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International Journal of Secondary Metabolite (IJSM) purposes the publication of articles related to secondary metabolites of plant and allied organisms (algae, fungi, and lichens). IJSM is open-access, peer-reviewed academic journal published electronically and quarterly. The journal is the goal to improve the research culture and help knowledge spread rapidly in the academic world by providing a common academic platform in the scope. The journal is published in English.

IJSM is published 4 issues per year (March, June, September, December), and accepting manuscripts related to secondary metabolites of plant and allied organisms (algae, fungi, and lichens). Research areas covered in the journal are phytochemistry, biochemistry, biotechnology, ethnopharmacology, biological and pharmacological activities (antimicrobial activity, antioxidant activity, antiulcer activity, anti-convulsant activity, anti-anxiety activity, antidiabetic activity, anti-gout activity, antiprotozoal activity, anti-inflammatory activity, antispasmodic activity, antiparasitic activity, anti-mutagenic activity, anticholinesterase activity, antidepressant activity, hepatoprotective activity, anti-anxiety activity, anti-convulsant activity, anti-spasmolytic activity, anticancer activity). IJSM welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Authors are required to frame their research questions and discuss their results in terms of major questions in plant biology. Contribution is open to researchers of all nationalities. The following types of articles will be considered:

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2. Research notes: These include articles such as preliminary notes on a study or manuscripts new records on secondary metabolites.

3. Reviews: Reviews of recent developments, improvements, discoveries, and ideas in various fields of plant and allied organisms (algae, fungi, and lichens) will be requested by the editor or advisory board.

4. Letters to the editor: These include opinions, comments relating to the publishing policy of the International Journal of Secondary Metabolite, news, and suggestions. Letters are not to exceed one journal page.

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Research Article

In vitro antimicrobial and antioxidant activities of *Sambucus williamsii* and *Sambucus pendula*

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Abstract: The present study aimed to compare the *in vitro* antimicrobial and antioxidant activities of *Sambucus williamsii* and *Sambucus pendula*. The antimicrobial activity of the two plants was evaluated using the disc diffusion and minimal inhibition concentration (MIC) method against three Gram-positive bacteria (*Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 9327 and *Listeria monocytogenes* ATCC 15313), four Gram-negative bacteria (*Escherichia coli* ATCC 15489, *Psendomonas aeruginosa* ATCC 27853, *Pseudomonas fluorescens* KCCM 41443 and *Salmonella typhimurium* KCCM 11862) and one yeast (*Saccharomyces cerevisiae* IFO 1950). The tested ether and ethyl acetate fractions of ethanol extract from *Sambucus williamsii* showed significant antimicrobial activity against *Bacillus cereus* and *Pseudomonas fluorescens*; conversely, the ether fraction of *Sambucus pendula* leaf showed no clear zone formation against any tested bacteria. In general, the fractions of the two *Sambucus* species exhibited a lower MIC against tested Gram-negative bacteria than the tested Gram-positive bacteria. The antioxidant activity was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay. The results showed that the half inhibitory concentration (IC_{50}) for the ethanol extract of *Sambucus williamsii* was the lower value, which means the greater antioxidant activity. On the other hand, the IC_{50} value of the hot water extract of *Sambucus pendula* was the lower value. The richness of the total polyphenol contents of the two *Sambucus* implies their potential as raw material sources for the pharmaceutical and cosmeceutical industries.

1. INTRODUCTION

Medicinal plants have been used for treating human diseases for thousands of years because they contain diverse organic compounds such as alkaloids, flavonoids, terpenoids, saponins, and phenolic compounds, which can exert physiological effect in the human body. A number of such compounds isolated from plants can be used to develop drugs to inhibit the growth of bacterial pathogens and quench reactive oxygen species (ROS) with low toxicity (Akhtar *et al*., 2018).

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The genus *Sambucus* L. (elderberry) belongs to the Viburnaceae family. Plants of the genus *Sambucus* are widely distributed, perennial woody shrubs. *Sambucus* consists of 5-30 species and 6-11 subspecies, depending on the taxonomic system. Five of these species are naturally found in Korea (Lee, 1980; Młynarczyk *et al*., 2018). The most common species, *Sambucus nigra* L., does not occurr in Korea. Previous *in vitro* studies have indicated that *Sambucus* berry extracts possess anticarcinogenic, immune-stimulating, antibacterial, and anti-inflammatory properties (Dulf *et al*., 2015). The stem of *Sambucus williamsii* is an important folk medicine used for wound-healing and treating bone fractures. This plant is naturally in the valleys or forest edges of Korea. The leaf and stem extracts of *Sambucus williamsii* exhibit a higher antioxidant activity than butylated hydroxytolune, a positive control, mainly owing to its total polyphenol content (Chae & Cho, 2012). *Sambucus pendula* is a woody shrub that only grows on Ulleung-do Island of Korea. However, this plant has not yet been used for medicinal purposes in Korea.

The present research aimed to study the possible antimicrobial and antioxidant activities of *Sambucus williamsii* and *Sambucus pendula*. The activities of the extracts from the two plants are interest for their potential use in food and pharmaceutical industries.

2. MATERIAL and METHODS

2.1. Plant Material

The leaf and stem of *Sambucus williamsii* and *Sambucus pendula* was collected from Mt. Baegunsan in Gwangyang-si, Jellanam-do Province, and Ulleung-do Island, Gyeongsangbuk-do, Korea, respectively, in July 2020. The two provenances were air-dried at room temperature for 2 weeks. The air-dried sample was pulverized using an electric mill. The plants were authenticated by one of the authors, Prof. K.W. Yun and voucher specimens were deposited in the Herbarium of Sunchon National University (SCNUH 20200023 and 20200024), Korea.

2.2. Antimicrobial Activity

2.2.1. *Test bacteria*

The tested bacteria included three gram-positive bacteria (*Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 9327, and *Listeria monocytogenes* ATCC 15313), four gram-negative bacteria (*Escherichia coli* ATCC 15489, *Psendomonas aeruginosa* ATCC 27853, *Pseudomonas fluorescens* KCCM 41443, and *Salmonella typhimurium* KCCM 11862), and one yeast strain (*Saccharomyces cerevisiae* IFO 1950). These strains were obtained from American Type Culture Collection and Korean Culture Center of Microorganisms.

2.2.2. *Preparation of extract*

Air-dried and powdered plant materials (100 g each) were soaked in 1,000 mL of ethanol at room temperature for 24 h and then filtered through Whatman No.2 paper. The crude ethanol extract was partitioned with 500 mL hexane, and the top layer was concentrated (comprising the hexane fraction). The remaining layer was successively fractionated with 500 mL of diethyl ether and ethyl acetate (forming the ether and ethyl acetate fractions, respectively). The remaining residue was the aqueousfraction. Each fraction was concentrated with a rotary evaporator (30 °C) to 30 mL and stored at 5 °C until tested.

2.2.3. *Determination of antimicrobial activity*

Each bacterial strain was grown in a nutrient broth at 30 °C for 18-24 h prior to testing and subcultured three times for another 18-24 h. The turbidity of bacterial cell suspensions was brought to 0.3 optimal density at 660 nm by adding sterile broth and was then used for the tests. We poured 0.1 mL of the bacterial cell suspensions uniformly onto nutrient broth agar plates. The paper disks containing the extracts were carefully placed on the seeded Petri dishes. The diameters of the resulting inhibition zones were measured in mm after the cultures were incubated at 38 °C for 24 h. At the end of the incubation period the antimicrobial activity was

evaluated by measuring the inhibition zone. The minimal inhibition concentration (MIC) was determined as the lowest concentration that produced an inhibition zone. MIC was measured for the ether and ethyl acetate fractions.

2.3. Antioxidant Activity

2.3.1. *Preparation of extract*

The powdered leaf and stem (100 g) was macerated in 1000 mL of ethanol and hot water for 6 h, respectively. The percolates were then filtered (ADVANTEC No.2 filter paper). The extracts were then concentrated *in vacuo* to 100 mL at 30°C and tested for the DPPH and ABTS radical scavenging activities.

2.3.2. *DPPH radical scavenging activity*

The DPPH radical scavenging activity of the extracts was evaluated using a modified version of the method described by Blois (Blois, 1958). Briefly, 160 µL of each extracts at various concentrations (100 µM as the final concentration) were added to 40 µL of DPPH solution (1.5×10^{-4} M). The solutions were gently mixed and allowed to stand at room temperature for 30 min, after which the optical density was measured at 520 nm using a microplate spectrophotometer (EL800; Bioteck, Vinooski, VT, USA). The DPPH radical scavenging activity of each extract was expressed in terms of IC_{50} values (the concentration required to inhibit DPPH radical formation by 50%). L-Ascorbic acid was used as a positive control.

2.3.3. *ABTS radical scavenging activity*

The experiment was performed using a modified ABTS decolouration assay (Re *et al*., 1999). ABTS radical cation was produced by reacting the ABTS stock solution (7 mM in water) and 2.45 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing the mixture to react at room temperature in the dark for 12 h. The solution was then diluted with ethanol to an absorbance of 0.70 ± (0.02) at 734 nm. The diluted ABTS (2.0 mL) was added to 50 µL of each extract and then the absorbance was measured on a microplate spectrophotometer reader (EL800; Bioteck, Vinooski, VT, USA) at 734 nm (Jaitak *et al*., 2010). The ABTS radical scavenging activity of each extract was expressed in terms of IC_{50} values (the concentration required to inhibit ABTS radical formation by 50%). L-Ascorbic acid was used as a positive control.

2.3.4. *Determination of total polyphenol content*

The total polyphenol content was determined using a modified version of the Folin-Denis method (Velioglu *et al*., 1998). Each 25 µL sample extract (1mg/mL) was mixed with 500 µL of Folin-Denis' reagent (diluted 10-fold with distilled water) was added. The mixtures were allowed to stand at room temperature for 5 min and then centrifuged at 1200 rpm for 10 min, following which the supernatant was collected. The clear supernatant (0.1 mL) of the samples was mixed with 0.75 mL of Folin-Denis' reagent. After 5 min, 500 µL of sodium bicarbonate (7.5 % in distilled water) was added and the solution was allowed to stand at 30 °C in darkness. Absorbance was measured at 765 nm using a microplate spectrophotometer (EL800; Bioteck). A standard curve prepared from gallic acid (100-1000 µg/mL) was used for quantification and the total polyphenol content was expressed as mg (of gallic acid)/g (dry weight).

2.3.5. *Determination of total flavonoid content*

The total flavonoid content was determined according to the method described by Moreno *et al*. (2000), with slight modifications. Each sample fraction (10 µL;1 mg/mL) was diluted with 80% aqueous ethanol (90 µL). An aliquot of 0.5 mL was added to test tube containing 2 µL of 10% aluminum nitrate, 2 µL of 1 M aqueous potassium acetate and 86 µL of 80% ethanol and the solution was allowed to stand at room temperature for 40 min. The absorbance was then measured at 415 nm using a UV-visible spectrophotometer (HP-8453; Agilent Technologies,

Santa Clara, CA, USA). A standard curve prepared from quercetin (100-1000 µg/mL) was used for quantification and the total flavonoid content was expressed as mg quercetin/g dry weight.

2.4. Statistical Analysis

Data with triplicate are analyzed using the SPSS software (version 24.0; IBM Corp., Armonk, NY, USA) and expressed as the mean ± SD. The statistical significance of the differences between means was determined using one-way analysis of variance. The level of significance was set at $p<0.05$.

3. RESULTS and DISCUSSION

3.1. Antimicrobial Activity

The antimicrobial activity of the ether and ethyl acetate fractions of the *Sambucus williamsii* extract was evaluated according to their MIC values against three gram-positive and four gram-negative bacteria and one yeast (Table 1). *Pseudomonas fluorescens* and *Escherichia coli* showed higher sensitivity and resistance to the two tested fractions of *Sambucus williamsii*. *Listeria monocytogenes*, a gram-positive bacterium, exhibited lower MIC value to the tested fractions. All the tested fractions of *Sambucus williamsii* affected *Bacillus cereus* and *Pseudomonas fluorescens* (MIC, 0.25-1.5 mg/mL). No inhibition against *Escherichia coli* was observed with any of the tested fractions of *Sambucus pendula* (Table 2). Stronger growth inhibitory effects on *Pseudomonas fluorescens* and *Salmonella typhimurium* were observed with the two fractions of the stem extract. The ethyl acetate fraction exhibited a higher antimicrobial activity than the ether fraction. In general, antimicrobial activity is associated with the outer layers of gram-positive and gram-negative bacteria. For gram-positive bacteria, antibacterial agents can easily destroy bacterial cell walls. Contrastingly, the outer membrane of gram-negative bacteria provides a hydrophilic surface to block external hydrophobic substances, resulting in stronger resistance to antibacterial compounds (Tian *et al*., 2018). In our experiments, both higher sensitivity and resistance to the tested fractions of the two plants were observed for the tested gram-negative bacteria. MIC against *Saccharomyces cerevisiae* was not detected for any tested fractions from the two plants. The antimicrobial activity of plant extracts forms the basis for many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine, and natural therapies (Hammer *et al*., 1999).

Table 1. MIC of the two fractions of the ethanol extract of leaf and stem from *Sambucus williamsii* against tested microorganisms.

MIC (mg/mL)	Samples			
	Ether fraction		Ethyl acetate fraction	
	Leaf	Stem	Leaf	Stem
Gram(+) bacteria				
Bacillus cereus	1.5	0.25	1.0	0.5
Bacillus subtilis	-	1.5	-	-
Listeria monocytogenes	-	0.25	0.25	0.1
Gram(-) bacteria				
Escherichia coli	1.0	-	-	-
Psendomonas aeruginosa	-	-	1.0	0.5
Psendomonas fluorecens	1.0	1.5	0.5	0.25
Salmonella typhimurium	1.5	-	0.25	0.25
Yeast				
Saccharomyces cerevisiae	-	-	-	-

- : No clear zone was formed.

Table 2. MIC of the two fractions of ethanol extract of leaf and stem from *Sambucus pendula* against tested microorganisms.

MIC (mg/mL)	Samples			
	Ether fraction		Ethyl acetate fraction	
	Leaf	Stem	Leaf	Stem
Gram(+) bacteria				
Bacillus cereus	-	0.25	1.5	0.25
Bacillus subtilis	-	-	-	-
Listeria monocytogenes	-	0.1	-	0.1
Gram(-) bacteria				
Escherichia coli	-	-	-	-
Psendomonas aeruginosa	-	0.25	1.5	0.25
Psendomonas fluorecens	-	0.1	1.5	0.1
Salmonella typhimurium	-	0.1	1.5	0.1
Yeast				
Saccharomyces cerevisiae	-	-	-	-

- : No clear zone was formed.

3.2. Antioxidant Activity

The antioxidant activity of extracts is related to the compounds capable of protecting a biological system against the potentially harmful effects of oxidative processes (Fernandez-Agullo *et al*., 2013). The results are expressed as IC_{50}, which defined as concentration of plant extract with 50% DPPH and ABTS free radical scavenging potential (Akhtar *et al*., 2018). The lower the IC_{50} value, the higher the antioxidant activity. Free radicals are highly reactive and toxic substances that cause several health problems including cancer, diabetes, aging, cardiovascular disorders and liver cirrhosis (Khuda *et al*., 2021). The DPPH free radical scavenging activities of the extracts of the two *Sambucus* are shown in Table 3. The IC_{50} values of the leaf ethanol extracts from the two *Sambucus* were lower than those of ascorbic acid, the positive control. This result was in agreement with that reported in a previous study on the DPPH scavenging activity of *Sambucus williamsii* (Chae & Cho, 2012). In particular, DPPH scavenging activity was the highest in the leaf ethanol extract of *Sambucus williamsii* (IC_{50}, 0.18 ± 0.07 mg/mL).

Table 3. DPPH free radical scavenging activity of solvent extracts of leaf and stem from *Sambucus williamsii* and *Sambucus pendula*.

Samples			DPPH free radical scavenging activity (IC_{50}, mg/mL)
Sambucus williamsii	Leaf	Ethanol	0.18 ± 0.07
		Hot water	1.60 ± 0.11
	Stem	Ethanol	0.23 ± 0.05
		Hot water	1.16 ± 0.09
Sambucus pendula	Leaf	Ethanol	0.21 ± 0.06
		Hot water	1.02 ± 0.07
	Stem	Ethanol	2.25 ± 0.09
		Hot water	1.05 ± 0.05
Ascorbic acid			0.27 ± 0.01

Values are expressed as mean±SD (n=3).

Table 4 shows ABTS radical scavenging activities of *Sambucus williamsii* and *Sambucus pendula* extracts. In general, the IC_{50} values of hot water extracts of the two plants were lower than those of ethanol extracts; in particular, the ABTS scavenging activity was found to be highest in the stem ethanol extract of *Sambucus williamsii* (IC_{50}, 0.71 ± 0.01 mg/mL).

Rungruang *et al.* (2021) confirmed that the phenolic compound is the important molecule that can reduce ABTS via hydrogen atom transfer or electron donation. The IC_{50} values of ABTS in all the tested extracts displayed a different pattern of those of DPPH. The DPPH scavenging activities of all the tested extracts were better than those for the ABTS. It was shown that methanolic extracts of *Sambucus ebulus* had a better phenolic profile and this was reflected in their antioxidant activity (Barak *et al*., 2020).

Table 4. ABTS radical scavenging activity of solvent extracts of leaf and stem from *Sambucus williamsii* and *Sambucus pendula*.

Samples			ABTS radical scavenging activity (IC_{50}, mg/mL)
Sambucus williamsii	Leaf	Ethanol	2.27 ± 0.08
		Hot water	1.63 ± 0.04
	Stem	Ethanol	0.71 ± 0.01
		Hot water	1.23 ± 0.13
Sambucus pendula	Leaf	Ethanol	2.11 ± 0.04
		Hot water	1.08 ± 0.07
	Stem	Ethanol	6.24 ±0.12
		Hot water	1.99 ± 0.02
Ascorbic acid			0.25 ± 0.01

Values are expressed as mean±SD (n=3).

Plant are known to have antimicrobial and antioxidant activities owing to the presence of polyphenols and flavonoids (Kumar *et al*., 2018). Our results showed that the total polyphenol content (TPC), expressed as Gallic acid equivalents (GAE) per g dry weight (DW) of a sample, ranged from 681.6 ± 10.4 to 1,031.0 ± 11.5 mg Gallate/g DW in the ethanol extract of the two *Sambucus* and from 925.3 ± 19.6 to 1596.9 ± 24.7 mg Gallate/g DW in the hot water extract. The highest DPPH radical scavenging activity of the leaf ethanol and hot water extracts of *Sambucus williamsii* is associated with high TPC. The stem ethanol extract of *Sambucus pendula*, which had the lowest TPC, exhibited the weakest DPPH and ABTS scavenging activities (Table 5). Milena *et al.* (2019) showed that the 50% ethanol extract of *Sambucus nigra* had the highest content of bioactive compounds and exhibited very strong antioxidant activity in all applied assays.

Table 5. Total polyphenol content of solvent extracts of leaf and stem from *Sambucus williamsii* and *Sambucus pendula*.

Samples			Total polyphenol content (mg Gallate/g DW)
Sambucus williamsii	Leaf	Ethanol	1031.0±11.5
		Hot water	1596.9±24.7
	Stem	Ethanol	833.5±12.1
		Hot water	1114.0±24.2
Sambucus pendula	Leaf	Ethanol	904.5±18.5
		Hot water	1314.0±56.2
	Stem	Ethanol	681.6±10.4
		Hot water	925.3±19.6

Values are expressed as mean±SD (n=3).

Table 6. The total flavonoids content of solvent extracts of leaf and stem from *Sambucus williamsii* and *Sambucus pendula*.

Samples			Total flavonoid content (mg Quercetin/g DW)
Sambucus williamsii	Leaf	Ethanol	388.7±5.8
		Hot water	388.7±90.2
	Stem	Ethanol	42.0±0.0
		Hot water	42.0±5.1
Sambucus pendula	Leaf	Ethanol	902.0±10.0
		Hot water	902.0±15.3
	Stem	Ethanol	22.0±0.0
		Hot water	22.0±5.8

Values are expressed as mean±SD (n=3).

Some well-known compounds, such as flavonoids, have many important biological activities, including the ability to inhibit enzymes and have antioxidant, anti-inflammatory, antitumor, antithrombogenic, antiosteoporotic, and antimicrobial activities (Akhtar *et al*., 2018; Ding *et al*., 2011). Total flavonoid content (TFC) was calculated as quercetin equivalents ($y=0.0002x + 0.0458$, $R^2=0.9942$). The ethanol and hot leaf water extracts of *Sambucus pendula* showed the highest TFC, with 902.0 mg Quercetin/g DW. In contrast, the stem ethanol and hot water extracts of *Sambucus pendula* contained 22.0 mg quercetin/g DW, showing the lowest TFC (Table 6). It has been reported that flavonoid compounds have a wide range of chemical and biological activities, and variations in their phytochemical contents depend on the season, geography, nutrient content of the environment, light density, photoperiod, and temperature (Omar *et al*., 2018).

4. CONCLUSION

This is the first study to compare the antimicrobial and antioxidant activities of *Sambucus williamsii*, and *Sambucus pendula* extracts. The ethyl acetate fractions of the ethanol extracts from the two species showed more potent antibacterial activity than the ether fractions. Our results also showed that ethanol extracts of *Sambucus williamsii* exhibited remarkable antioxidant activity, as determined by DPPH and ABTS radical scavenging assays. Total phenolic and flavonoid content showed no clear association with antioxidant activity. The results obtained will be useful for further research, such as the identification of specific compounds responsible for the antibacterial or antioxidant activities. The extracts from the two *Sambucus* belonging to the Viburnaceae family have potential applications in the pharmaceutical and cosmeceutical industries.

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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

Kyeong Won Yun: Conceptualization, methodology, and plant species identification. **Kyeong Won Yun** and **Kyoung-Sun Seo**: supervision, extraction, antimicrobial and antioxidant bioassays, data analysis, writing, original draft preparation, and editing.

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Research Article

Genetic structure and molecular analysis of the species of the genus *Artemisia* L. (Asteraceae) distributed in Azerbaijan

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Abstract: In this study, the phylogenetic relationships and genetic structure of 10 collected genotypes of *Artemisia szowitziana* (Besser) Grossh and *Artemisia fragrans* Willd. species from different regions of Azerbaijan were investigated using RAPD primers. A total of 94 amplicons were amplified using selected RAPD primers. Among them 3 band with OPA-02 primers and 12 band with OPW-17 primers were amplified. The highest polymorphism among the investigated RAPD markers was 100, 90.91 and 90%, which has been obtained by OPW-17, OPT-19, and OPT-20 primers, respectively. However, the calculated average value of polymorphism was as high as 72.79% based on 10 different RAPD markers. The average PIC (Polymorphism Information Content) value (0.864) has indicated the rich genetic diversity among the studied samples. The cluster analyses by using Jaccard similarity index and UPGMA method classified all *Artemisia* L. samples into 6 major groups. Principal Component Analysis (PCA) justified 74.22% of the total variance. Based on the results obtained, it has been observed that there is a wide range of diversity in the molecular level between the population of *Artemisia szowitziana* (1-2 and 4-6 samples) and *Artemisia fragrans* (3, 5, 7 and 8, 9, 10 samples) and between population of the *Artemisia* species in Azerbaijan.

1. INTRODUCTION

The genus *Artemisia* L. is one of the largest genera of the Asteraceae family. *Artemisia* has 40 or 42 species in Azerbaijan (Alesgerove & Ibadullayeva, 2011). The species are mostly spread in arid areas and dry valleys of the Azerbaijan region (Hajıyev & Malıkov,1999). While *Artemisia* is a diverse genus of plants with 500 species (Aalı *et al.*, 2014), *Artemisia* species are used medicinally throughout the world. Despite the harmful effects of some of its species (invading nurseries and farmlands; toxic and allergenic for humans), the *Artemisia* genus has a wide area of usage in different fields, including pharmaceutics, landscape architecture, and agriculture (Badr, *et al.*, 2011; Barney, *et al.*, 2003; Hayat, *et al.*, 2010).

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The generic name *'Artemisia'* is derived from 'Artemis', which refers to Diana, a Greek Goddess. The name of the Goddess 'Artemis' was given to the genus because one of the species *Artemisia vulgaris* L. was much used in medicine for women's disease in folk remedy (Takeda, 1971; Yasmin *et al.,* 2016). The plant grows wild in the temperate Himalayas. It is common in the Kashmir Valley and the Shimla and Nainital hills (Yasmin & Naeasegowda, 2016). 5 decamer primers generated 195 RAPD fragments, of which 155 fragments were polymorphic with 79.48% of polymorphism. Most of the RAPD markers studied showed different levels of genetic polymorphism. The pairwise similarity coefficient values of Nei and Li ranged from 0.50 to 0.79 for 5 species of these antimalarial medicinal plants.

Artemisia species, particularly *A. iwayomogi* Kitam., *A. capillaris* Thunb., *A. princeps* Pamp. and *A. argyi* H. Lev. & Vaniot, are important medicinal materials that are utilized in traditional Asian medicines (Murray & Thompson, 1980). The genetic variability among individuals of *Artemisia capillaris* from Terengganu and Kelantan, Malaysia was examined by using random amplified polymorphic DNA. A total of 335 (Terengganu) and 370 (Kelantan) RAPD fragments which were all polymorphic fragments (100%) with sizes ranging from 150 to 3000 bp were scored for Terengganu samples, while 124 polymorphic fragments (95%) with sizes ranging from 200 to 2500 bp were scored for Kelantan samples. Genetic distance for samples ranges from 0.0000 to 0.26000 (Terengganu) and from 0.1300 to 1.5300 (Kelantan). The similarity index ranges from 0.0000 to 0.7838 (Terengganu) and 0.1167 to 0.8758 (Kelantan) (Sayed *et al.,* 2009). RAPD markers were used to study the genetic variability in a wild medicinal plant species *Artemisia judaica,* which grows in desert areas in the south of Jordan. A total of 1073 bands were obtained, 165 of which were polymorphic. Similarly, values among the studied accessions ranged from 0.61 to 0.02 (Al-Rawashdeh, 2011). The diversity between *Artemisia monosperma* Delile and *A. judaica* species was analyzed based on the assessment of morphological, karyological, and molecular variations. The analysis of morphological variation and molecular polymorphism as revealed by RAPD confirmed the differentiation of *A. monosperma* and *A. judaica* L. as two distinct species and showed wider variations among *A. judaica* populations compared to the *A. monosperma* populations. The latter population is clearly distinguished from the other populations by RAPD profiling, supporting the recognition of some populations of *A. judaica* in South Sinai as a separate variety (Badra, *et al.,* 2012**)**. The RAPD method efficiently discriminated various *Artemisia* herbs. In particular, the non-specific primer 329 (5-GCG AAC CTC C-3), which shows polymorphism among *Artemisia* herbs, amplified 838 bp products, which are specific only to *A. princeps* and *A. argyi*. Based on the nucleotide sequence of the primer 329 product, a Fb (5-CAT CAA CCA TGG CTT ATC CT-3) and R7 (5-GCG AAC CTC CCC ATT CCA-3) primer-set was designed to amplify a 254 bp sized SCAR (sequence characterized amplified regions) marker, through which *A. princeps* and *A. argyi* can be efficiently discriminated from other *Artemisia* herbs, in particular, *A. capillaris* and *A. iwayomogi* (Lee, *et al.,* 2006). The aim is to perform a phylogenetic analysis of the 18S-26S rDNA, the internal transcribed spacer (ITS) nucleotide sequences of 19 *Artemisia* samples collected from the Ordu Province of Türkiye. This analysis revealed two unique haplotypes within our samples, including a rare one (Haplotype-I, represented by a single sample) and a common one (Haplotype-II, represented by 18 samples) (Koloren *et al.,* 2016). cDNA of subsp. *tridentata* and subsp. *vaseyana* of *A. tridentata* Nutt. species were normalized and sequenced. Assembly of the reads resulted in 20,357 contig consensus sequences in *A. tridentata* subsp. *tridentata* and 20,250 contig consensus sequences in *A. tridentata* subsp. *vaseyana*. A total of 20,952 SNPs and 119 polymorphic SSRs were detected between the two subspecies (Prabin, *et al.,* 2011).

Yenikend Reservoir was created in 2000 to produce electricity. Vegetation around the reservoir is constantly changing due to the rise and fall of the water level. From this point of view, the places near the reservoir are considered ecologically sensitive zones. Species of the

genus *Artemisia* are generally desert and semi-desert elements and have extensive intraspecific polymorphism. The adaptation characteristics of the species of the genus to different environmental conditions, especially around the water reservoir, were manifested in their development. Although the rise and fall of the water level in the reservoir destroys the surrounding vegetation, the regeneration and sustainability of the coenoses is evident with the dominance of wormwood species. To this end, phylogenetic relationships, genome polymorphism, and similarity indices of 10 samples of *A. fragrans* and *A. szowitsiana* species collected both from the reservoir area and other regions of Azerbaijan were studied comparatively.

2. MATERIAL AND METHODS

2.1. Plant Materials and DNA Isolation

In this study 10 different samples of *Artemisia szowitziana* (Besser) Grossh. (Figure 1) and *Artemisia fragrans* (Figure 2) were collected from the territories of Shamkir, Tovuz, Goranboy, Mingachevir and Lerik regions of Azerbaijan (Figure 3). Table 1 reflects the characteristic features of the collection area of the species studied, soil type, and composition of coenosis formed with the presence of plants and GPS data.

Figure 1. *Artemisia szowitziana*

Figure 2. *Artemisia fragrans*

Table 1. Information on the collection areas of the species studied.

No	Names of samples	Collector number	Areas where samples were collected and time of collection	Information about the area	The composition of phytocoenosis
1.	*A.szowitsiana*	1a	Shamkir district 10.06.2014	N 40.874210°; E 46.044440° 120 m a.s.l. dry gray-brown soils	*A.szowitsiana* + *A.fragrans* + *Euphorbia segueriana* + *Plantago major* + *Cichorium intybus*
2.	*A. szowitsiana*	2a	Shamkir district 03.10.2014	N 40.890620°; E 46.042456° 136 m a.s.l. dry gray-brown soils	*A.szowitsiana* + *A.fragrans* + *Euphorbia segueriana* + *Plantago major* + *Cichorium intybus*
3.	*A.fragrans*	1b	Shamkir district 03.10.2014	N 40.927860° ; E 46.192541° 120 m a.s.l. dry gray-brown soils	Wormwood semi-deserts (*Artemisia fragrans* +*Ephemeretum*)
4.	*A. szowitsiana*	3a	Shamkir district 12.04.2015	N 40.954657°; E 46.176473° 153 m a.s.l. roadside chestnut soils	*Artemisia fragrans* + *A. szowitsiana* + *Salsola dendroides*
5.	*A.fragrans*	2b	Shamkir district 09.07.2015	N 40.919154°; E 46.227273° 128 m a.s.l. water surrounding, gray-meadow soils	*Artemisia fragrans*
6.	*A.szowitsiana*	4a	Tovuz district 01.03.2016	N 40.999568°; E 45.610382° 558 m a.s.l. dry gray-brown soils	*Artemisia fragrans* + *A. szowitsiana* + *Salsola dendroides*
7.	*A.fragrans*	3b	Tovuz district 04.05.2016	N 40.975573°; E 45.618824° 478 m a.s.l. roadside chestnut soils	*Artemisia fragrans* + *Ephemeretum* + *Salsola dendroides*
8.	*A.fragrans*	4b	Mingachevir district 21.07.2016	N 40.919872°; E 46.490358° 116 m a.s.l. waterside gray-meadow, soils	*Artemisia fragrans*
9.	*A.fragrans*	5b	Goranboy rayonu 29.09.2016	N 38.777636° ; E 48.411884° 118 m a.s.l. roadside chestnut soils	*Artemisia fragrans* + *Ephemeretum*
10.	*A.fragrans*	6b	Lerik district 10.06.2017	N 38.777636° ; E 48.411884° 1232 m a.s.l. roadside meadow-brown, soils	*Artemisia fragrans*+*Alhagi pseudoalhagi*

The genomic DNA was extracted from the green leaves of plant samples using the CTAB protocol (Murray & Thompson, 1980). The extracted DNA was dissolved in sterile ionized water and then stored at -20° C. The concentration and purity of the genomic DNA for PCR were determined by spectrophotometry (Biotech, Epoch 5, USA). The DNA samples were then diluted to 20 ng/µl. To study the genetic diversity of *Artemisia szowitziana* and *Artemisia fragrans* Willd. at molecular level from different areas and various environmental conditions of Azerbaijan, 10 different RAPD primers were selected.

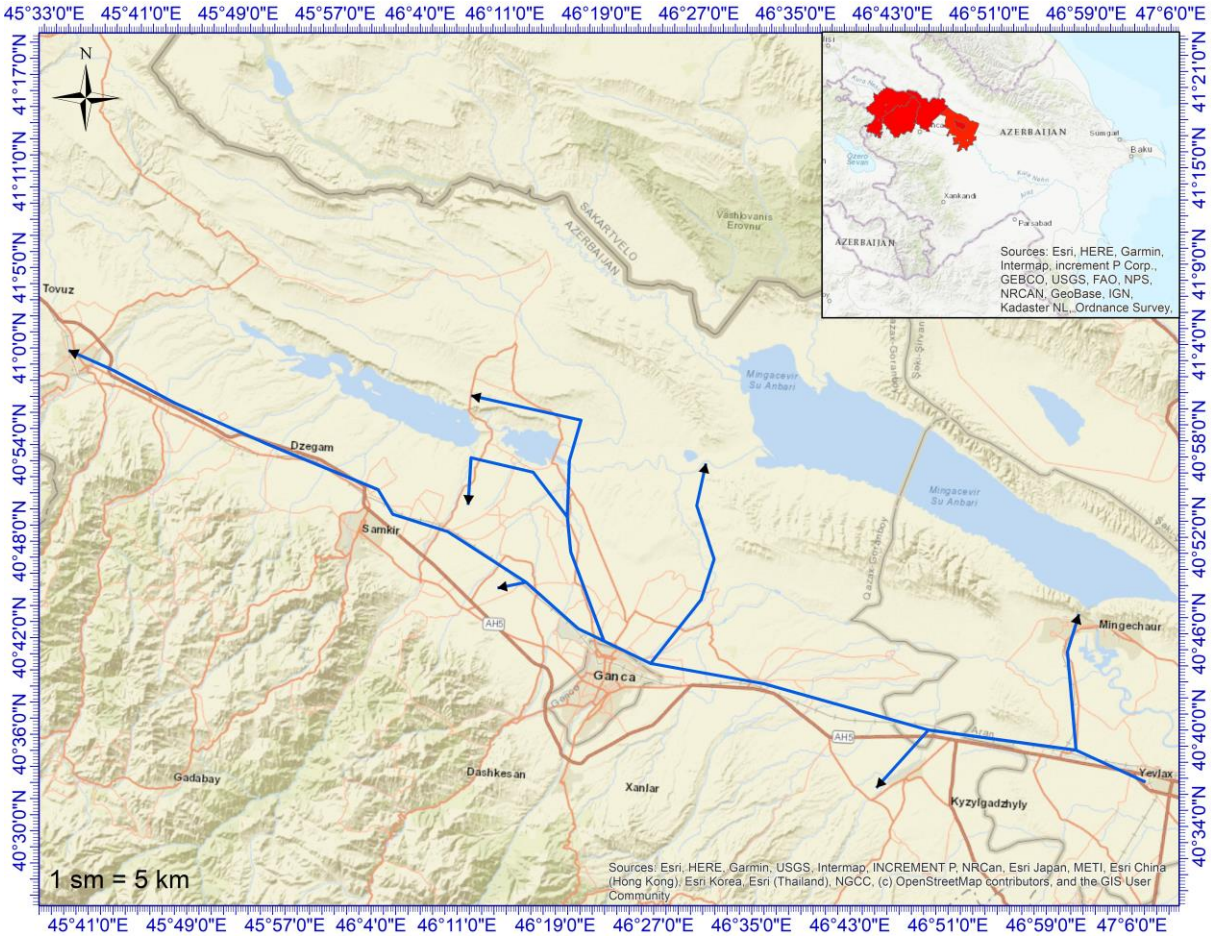

Figure 3. Dispersion map of *Artemisia* samples in different cities of Azerbaijan Republic.

2.2. PCR Amplification and Gel Electrophoresis

PCR was set up in a 25µl reaction volume containing 40 ng of genomic DNA, 1 U Taq DNA polymerase (Fermentas), 1XPCR buffer, 0.5 µM RAPD primer, and 0.2 mM of dNTP mix (Sigma). Amplification reactions were performed in a thermocycler (Applied Biosystems, 9700) using the following program: 95°C for 5 minutes followed by 30 cycles each of 94°C for 1 minute (denaturation), 36°C (varied with primer) for 1 minute (annealing), 72°C for 1 minute (extension), and finally at 72°C for 10 minutes. Amplified PCR products were separated by electrophoresis on 1% agarose gels at 100 V with 1XTAE (Tris acetate EDTA) buffer (pH-8.0). The gels were stained with ethidium bromide (0.5 mg/mL) and visualized in a gel documentation system («UVIPRO», UK). A 50 and 100-1000bp DNA ladders (Fermentas) were used as a molecular size standard. Figure 4 illustrates DNA fragments amplified with the primers studied.

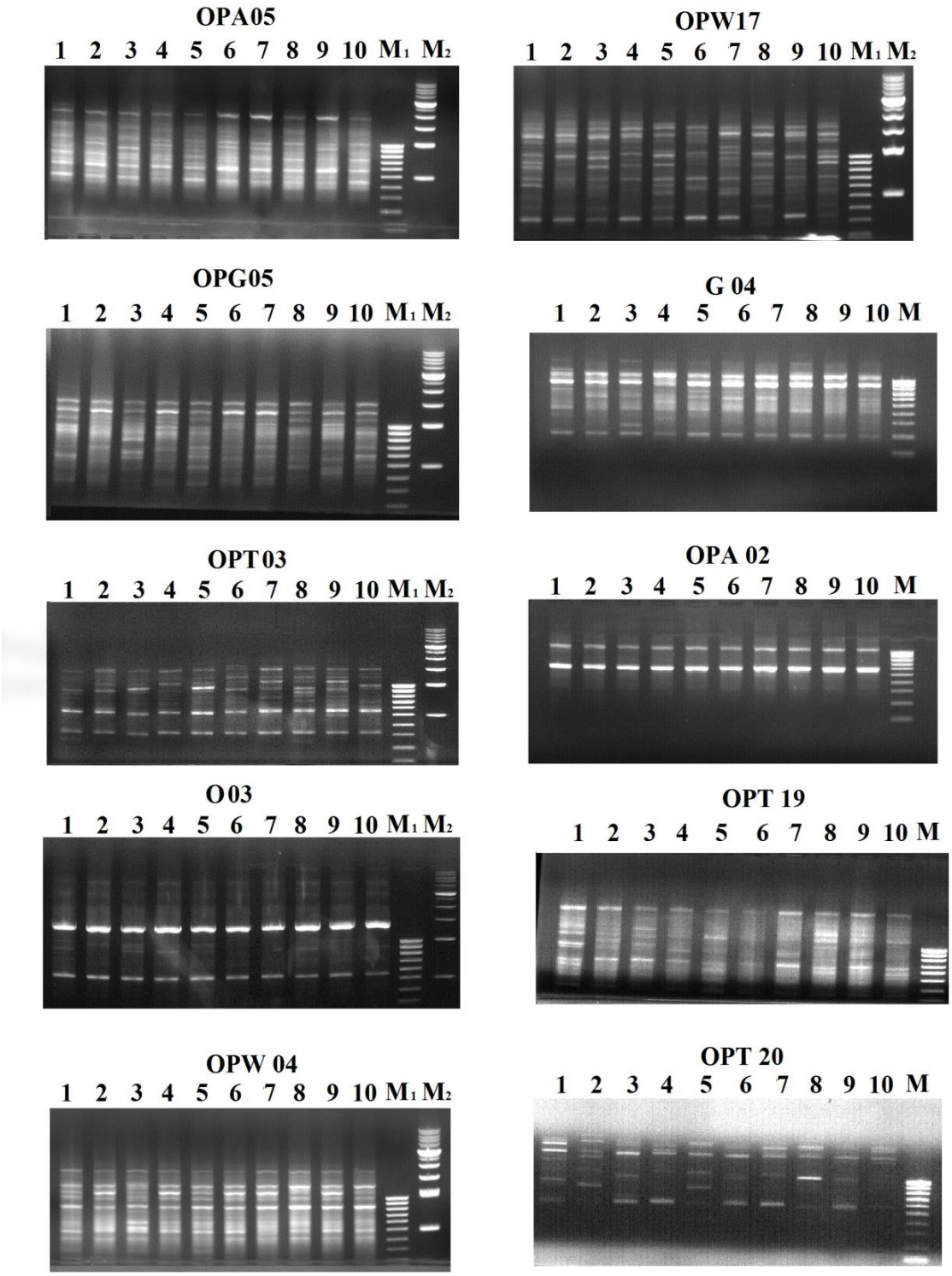

Figure 4. 1% agarose gel-analysis of DNA fragments amplified with 10 different RAPD primers on the chromosomal DNA extracted from the samples belonging to different populations of *Artemisia szowitziana* and *Artemisia fragrans* species distributed in Azerbaijan. M_1-DNA-marker (100-1000 b.p.); M_2-DNA marker (500-10000 b.p.).

1- *A. szowitziana* (dryland, Shamkir), **2-** *A. szowitziana* (dryland, Shamkir), **3-** *A. fragrans* (dryland, Shamkir), **4-** *A. szowitziana* (roadside, Shamkir), 5- *A. fragrans* (waterside, Shamkir), **6-** *A. szowitziana* (Tovuz),**7-** *A. fragrans* (Tovuz), **8-** *A. fragrans* (Mingachevir), **9-** *A. fragrans* (Goranboy), **10-** *A. fragrans* (Lerik).

2.3. Data Analysis

Banding patterns produced by ten RAPD markers were scored for the absence (0) and presence (1) of bands. Initially, by observing the banding patterns produced by different RAPD primers, a total number of bands, polymorphic bands, and percentage polymorphism were obtained. Further, the potential of these molecular markers for estimating genetic variability was assessed by measuring polymorphism information content (PIC). The PIC values were calculated using the formula PIC = 1- ∑pi 2, where pi is the frequency of its allele.

Based on the presence (1) or absence (0) of DNA bands synthesized with each of the primers, the cluster analysis was conducted, Jaccard's genetic similarity index between the studied genotypes of *Artemisia* was determined, and the dendrogram illustrating the cluster analysis with the UPGMA (unweighted pair group with arithmetic average) method was compiled (Figure 5).

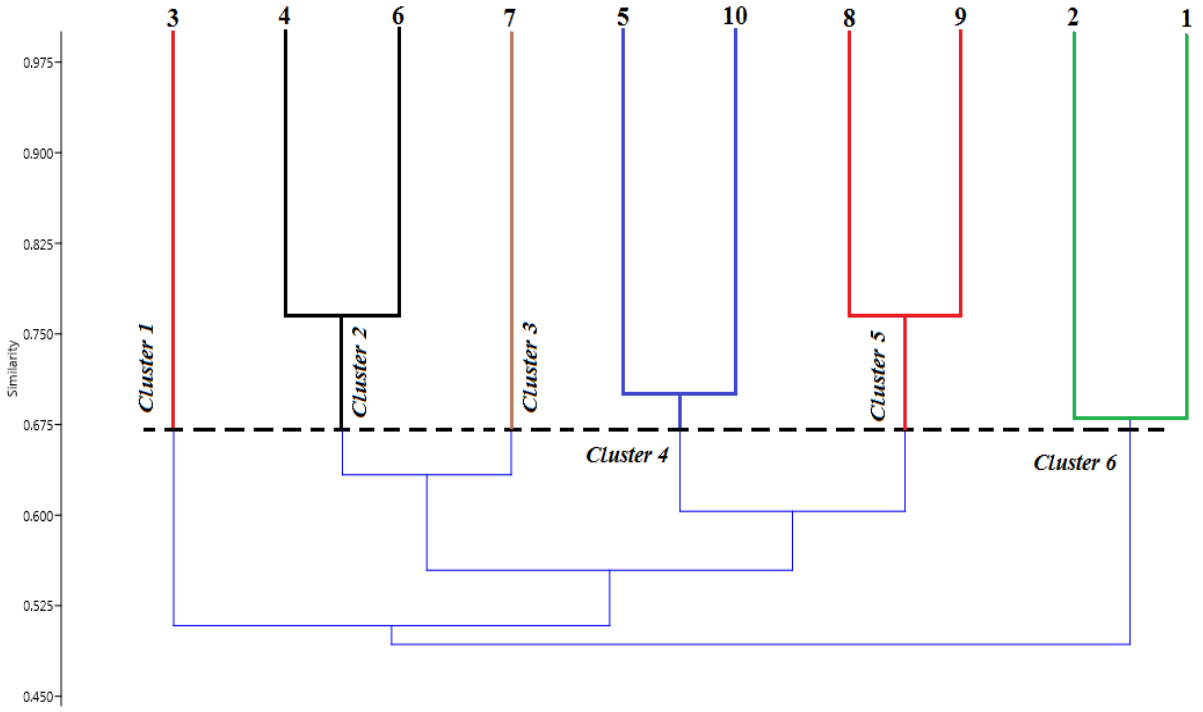

Figure 5. The dendrogram based on RAPD markers illustrating genetic relations between the samples belonging to different populations of *Artemisia szowitziana* and *Artemisia fragrans.*
1- *A. szowitziana* (dryland, Shamkir), **2-** *A. szowitziana* (dryland, Shamkir), **3-** *A. fragrans* (dryland, Shamkir), **4-** *A. szowitziana* (roadside, Shamkir), **5-** *A. fragrans* (waterside, Shamkir), **6-** *A. szowitziana* (Tovuz), **7-** *A. fragrans* (Tovuz), **8-** *A. fragrans* (Mingachevir), **9-** *A. fragrans* (Goranboy), **10-** *A. fragrans* (Lerik, Talysh).

3. RESULTS

RAPD-markers were used to study the genetic structure and phylogenetic relations of the samples belonging to *Artemisia* species characteristic of Azerbaijan at the genomic level. A total of 94 DNA spectra were amplified based on the selected RAPD-markers among the *Artemisia* samples. The number of amplicons varied from 3 to 12 which, in turn, reflects the different levels of polymorphism with molecular markers among the samples studied.

For each primer, the average number of amplified spectra was found to be 9.4. During the experiment, only 3 spectra were amplified with the OPA-02 primer, whereas 12 spectra were obtained with OPW-17. In addition, 11 spectra were amplified with the OPW-04 and OPT-20 primers.

Among the markers studied the highest level of polymorphism was registered for OPW-17, OPT-19 and OPT-20 primers (100, 90.91 and 90%, respectively) (Table 2). During the genetic diversity studies of the samples belonging to the *Artemisia annua* species based on RAPD and ISSR markers, Kumar *et al.*, (2011) found the level of polymorphism with these markers to be equal to 96.9% and 86.02%, respectively.

Table 2. List of the RAPD primers used in the present study.

№	Primers	5'→3' sequencing	№	Primers	5'→3' sequencing
1	G04	AGCGTGTCTG	6	OPT20	GACCAATGCC
2	O03	CTGTTGCTAC	7	OPW04	CAGAAGCGGA
3	OPA-02	TGCCGAGCTG	8	OPW17	CTCCTGGGTT
4	OPA-05	AGGGGTCTTG	9	OPT03	TCCACTCCTG
5	OPT19	GTCCGTATGG	10	OPG05	CTGAGACGGA

At the same time, Shafie *et al.*, (2009) reported polymorphism level to be equal to 67% with RAPD markers and to 63.16% with ISSR markers during the investigation of the phylogenetic relations of *Artemisia capillary* samples. In another study conducted by (Al-Rawashdeh, 2011), the low average polymorphism value (16.3%) was registered using RAPD markers during the genetic analysis of the samples belonging to *Artemisia judaica* species.

In our specific study, the lowest level of polymorphism was obtained with OPA-02 RAPD primer, while the average value of polymorphism calculated based on 10 different RAPD markers was found to be high and equal to 72.79%. Furthermore, in our study, high PIC (polymorphism information content) values – 0.95 and 0.9 – were observed with OPA-02 and OPT-19 markers, respectively. At the same time, PIC values were determined to be equal to 0.89 for O-03 and OPT-20 markers, and 0.88 with OPW-17, OPG-05 and OPT-03 RAPD markers. The average value of PIC equal to 0.864 obtained in the study of genetic diversity of the samples belonging to different populations of *Artemisia szowitziana* and *Artemisia fragrans* species characteristic of Azerbaijan serves as an indicator of high genetic diversity among the samples taken.

As can be observed, the cluster analysis has classified the *Artemisia* samples studied into 6 main groups. From the dendrogram it becomes evident that the first and the third clusters consist of only one sample, and in these clusters genotype 3 (cluster 1) and genotype 7 (cluster 3) are located. The location of these two samples in different clusters demonstrates that there is a substantial genetic distance between them and the other *Artemisia* samples.

During the analysis Jaccard's similarity index between genotypes 3 and 7 was determined to be equal to 0.43. The second cluster contains genotypes 4 and 6, and the genetic similarity index between these two samples is 0.76. Genotypes 5 and 10 comprise the fourth cluster, and the genetic distance index between them is 0.7. Clusters 5 and 6 contain two genotypes each as well. The fifth cluster combines genotypes 8 and 9 while the sixth cluster combines genotypes 1 and 2. In the fifth and the sixth clusters the genetic similarity index between the samples included is 0.76 and 0.68, respectively. In the study, the largest genetic distance was observed between genotypes 1 and 6. Badr et al. (2012) used cluster analysis to study the genetic diversity of Artemisia populations distributed in northern Saudi Arabia using morphological and molecular markers. They showed the classification of the samples studied into 5 clusters based on morphological traits and into 4 main groups on the basis of RAPD markers. Yasmin and Naeasegowda (2016) described the clustering of *Artemisia* samples into 4 main groups by using EST-SSR and 3 main groups using RAPD markers.

In addition to the cluster analysis, the principal components analysis is used in our study to analyze the genetic structure of *Artemisia* samples. Four components were obtained by PCA, explaining 74.22% of the total variation. The first, second, third, and fourth components accounted for 26.05, 18.78, 15.87, and 13.51%, respectively. A biplot (Figure 6) was constructed based on the two components. As can be observed, in contrast with the cluster analysis results, all the *Artemisia* samples included in the present study were divided into 4 main groups by PCA.

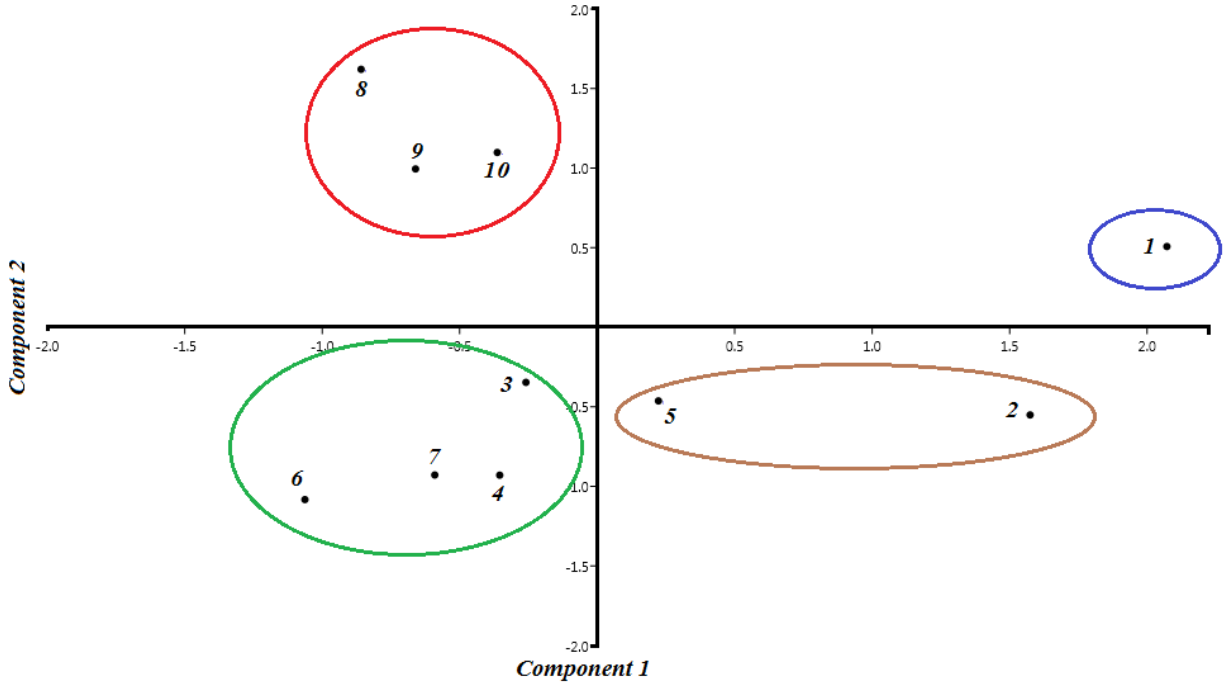

Figure 6. Biplot constructed on the basis of the two components.
1- *A. szowitziana* (dryland, Shamkir), **2-** *A. szowitziana* (dryland, Shamkir), **3-** *A. fragrans* (dryland, Shamkir), **4-** *A. szowitziana* (roadside, Shamkir), **5-** *A. fragrans* (waterside, Shamkir), **6-** *A. szowitziana* (Tovuz), **7-** *A. fragrans* (Tovuz), **8-** *A. fragrans* (Mingachevir), **9-** *A. fragrans* (Goranboy), **10-** *A. fragrans* (Lerik, Talysh).

RAPD markers are widely used genetic markers in the study of the genetic structure of different plants. The advantages of these markers are the ease of implementation, simplicity, and most importantly the absence of requirements for initial information on any part of the genome in the design of these markers. RAPD markers are universal and do not depend on the plant source. This advantage is highly important for medicinal plants, as the amount of DNA in the dried material of these plants is low, and their genome sequencing can be quite a difficult process (Shinde *et al.*, 2007). Sometimes sequence-based analysis fails to assess differences between species due to similarity in the DNA sequence of the regions amplified, but RAPD markers involve both coding and non-coding regions of the genome and are considered the most suitable ones for revealing diversity between species (Choo *et al.*, 2009).

This study shows that OPA-02, OPT-19, O-03, and OPT-20 primers, which show high levels of polymorphism compared to the other markers, can be used as suitable molecular markers for genetic diversity analysis. In addition, the cluster and principal component analysis implemented based on RAPD markers revealed high genetic diversity among samples of *Artemisia* distributed in Azerbaijan. The current study has confirmed the benefits of RAPD molecular analysis in genotype identification and assessment and differentiation of *Artemisia*

samples. Thus, the genetic data obtained in this experiment present valuable knowledge for breeders by providing new samples of medicinal plants in future breeding programs.

4. CONCLUSION

The genetic structure and polymorphism among *Artemisia* populations from different regions of the Republic of Azerbaijan were efficiently determined using molecular markers. The identification of *Artemisia* samples from Azerbaijan contributes to our knowledge of genetic relationships and the strategies required to protect natural populations and preserve genetic diversity.

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

Narmin Sadigova: Investigation, Resources, Original draft, Visualization. **Zarifa Suleymanova**: Investigation, Methodology, Visualization. **Javid Ojaghı:** Investigation, Methodology, Software, Visualization, Editing. **Sayyara Ibadullayeva:** Investigation, Resources, Designation of plant species. **Alamdar Mammadov:** Investigation, Methodology, Formal Analysis.

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Research Article

Phytochemical constituents of the roots of *Heliotropium verdcourtii* (Boraginaceae)

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Abstract: The medicinal value of medicinal plants lies in some bioactive constituents that produce a definite physiological action on the human body. *Heliotropium verdcourtii* is a deciduous shrub or small tree traditionally used in the treatment of various diseases including fever, dry cough, measles, convulsions, epilepsy, diarrhea, and other ailments. The chemical constituents of the roots of the plant were not investigated to date. The aim of the study was to investigate the phytochemicals present in the roots of *Heliotropium verdcourtii*. The freshly collected root of the plant was chopped and air dried under shade. The dried and finely grounded plant root was extracted through maceration with *n*-hexane, chloroform/methanol (v/v 1:1), and methanol successively. The extracts were subjected to qualitative phytochemical tests for screening the classes of secondary metabolites present in the plant. Compound isolation of the chloroform/methanol (v/v 1:1) extract was performed through silica gel chromatographic separation. The structures of all isolated compounds were determined by spectroscopic methods as well as comparison with previous reports in the literature. The yields of *n*-hexane, chloroform/methanol (v/v 1:1), and methanol extracts were 2.2 g (0.4%), 25 g (5.0%), and 19.8 g (4.0%), respectively. The qualitative phytochemical test of the extracts revealed the presence of flavonoids, terpenoids, phenolics, saponins, glycosides and alkaloids. Silica gel chromatographic separation afforded a mixture of three isomeric triterpenoids identified as α-amyrin, β-amyrin, and bauerenol. To the best of our knowledge these bioactive compounds were isolated from the root of this plant, for the first time.

1. INTRODUCTION

The use of herbs and medicinal plants for primary human health care is a universal phenomenon. Today, as much as 80% of the people in the world depend on traditional medicine as primary health care (Kimutai, 2017). There is therefore need to investigate such plants to understand their chemical constituents. The genus *Heliotropium* being a small tree or shrub comprises about 40 species and belongs to the family Boraginaceae (Weigend *et al.*, 2016). Many *Heliotropium* plants are mainly found being spread in tropical Asia, Africa, Australia,

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Europe, and Northern America (Gottschling & Hilger, 2004; Miller, 2003; Retief & Van Wyk, 2001). The bark, leaf juice, leaves, roots, seeds, stems, twigs and whole plant parts of *Heliotropium* are used as aphrodisiac, laxative, ethnoveterinary medicines, as traditional medicines, for ulcers and headaches, in treatment for schizophrenia, absorption of calcium, muscle protein, post-surgery recovery, sports injuries (Maroyi, 2021; Tidke *et al*., 2021).

Heliotropium verdcourtii is a deciduous shrub or small tree commonly found in the Savannah and secondary jungle of West Africa which includes Cameroon, Ghana, Gabon, Congo, and Nigeria (Ogundajo & Ashafa, 2017). The whole plant parts of *H. verdcourtii* are mainly used as aphrodisiac, laxative and ethnomedicines for gastro-intestinal problems, wounds, malaria, fever, typhoid, convulsions, epilepsy, toothache and respiratory infections (Jeruto *et al.*, 2011; Jeruto *et al.*, 2015; Li *et al.*, 2008; Maroyi, 2021; Oladunmoye & Kehinde, 2011). The presence of phenolic acids, lignans, flavonoids, nitrile glycosides, quinonoids, steroids, triterpenoids, and pyrrolizidine alkaloids was reported, in the genus *Heliotropium* (Jeruto *et al.*, 2011; Li *et al.*, 2010).

Phytochemistry of *H. verdcourtii* is characterized by anthraquinones, alkaloids, essential oils, flavonoids, fatty acids, glycosides, proanthocyanidins, phenols, pseudotannins, saponins, reducing sugars, tannins, steroids and terpenes (Ogundajo & Ashafa, 2017; Maroyi, 2021). Four triterpenoids namely, α-amyrin, β-amyrin, bauerenol, and a 12-13 epoxy ursane type pentacyclic triterpene were isolated from the leaves of the hexane extract (Chaluma *et al.*, 2018). Pharmacological activity tests of the extracts and the chemical constituents isolated from *H. verdcourtii* revealed antidiabetic, antibacterial, antihyperglycaemic and antioxidant activities (Maroyi, 2021).

H. verdcourtii is a plant highly distributed in Ethiopia, where it is locally named *Game* in Amharic, *Hulaga*, in Afan Oromo, and *Gidincho* in Sidama. It is a highly used as hedge plant in Ethiopia. Traditionally it is used for the treatment of various diseases like toothache, dysentery, tetanus, skin diseases and gastric ulcers (Chaluma *et al.*, 2018). The decoction of the leaves of *H. verdcourtii* is used to improve the quality and quantity of milk products of livestock in Ethiopia (Bezabih *et al.*, 2017). In Kenya an infusion and sap of the leaf is used to treat fever and as laxative agent, respectively. The root juice is used for healing wounds (Maundu and Tengnäs, 2005).

Despite the traditional use of the plant's roots against various life threatening diseases, there are few scientific reports dealing only with the phytochemical screening and biological activities of the root of *H. verdcourtii*. Recent reviews on the species, however, show that leaves of the plant are extensively studied, phytochemically (Maroyi, 2021). Hence this paper presents the results of the isolation and identification of chemical constituents from the roots extract of *H. verdcourtii* of Ethiopian origin.

2. MATERIAL and METHODS

2.1. Experimental

Grant thermostatic bath shaker (GLS-400) was used in the course of maceration of plant material. TLC spots were detected by a UV-2550 (SHIMADZU) UV-Vis spectrometer (Shimadzu, Kyoto, Japan). Column chromatography (CC) was performed with column size 3 cm × 30 cm packed with silica gel 60, size 0.063-0.200 mm (70-230 mesh ASTM). Thin layer chromatography (TLC) was performed on aluminum sheets, silica gel 60 F_{254}, and layer thickness 0.2 mm (Merck). NMR spectrum data was generated with 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR, TMS as internal standard and $CDCl_3$ as solvent with the chemical shifts reported in parts per million (ppm).

2.2. Collection and Preparation of Plant Materials

H. verdcourtii specimens (leaves, flowers, seeds, stems) were collected on October, 2021 from Rufo Waeno Kebele, Aleta Chuko Woreda, Sidama Region, Ethiopia, for authentication of the plant. The plant was authenticated by botanist Retta Regassa Department of Plant Science, Hawassa College of Teachers Training, Hawassa, Ethiopia and a specimen was stored in the Herbarium of Hawassa College of Teachers Training with voucher no: HHF/0021-24. In addition to the aforementioned morphological parts, the roots were also collected for phytochemical analysis. The plant material was prepared in such a way that the root of *H. verdcourtii* was washed with tap water to remove soil particles and other foreign materials, and air dried in a shade for three weeks. The air-dried root was pulverized into powder using electric grinder. The pulverized plant material was kept in sealed plastic container and put on a dry cup board until used for extraction.

2.3. Extraction

The pulverized root (500 g) of *H. verdcourtii* was soaked in *n*-hexane (1.5 L) in a 5 liter Erlenmeyer flask, at room temperature. The flask was shaken for 72 h on an orbital shaker. The solution was filtered and the filtrate was concentrated under vacuum at 36 to 38 °C. The marc was further extracted with $CHCl_3$:MeOH (*v/v* 1:1) and methanol successively, likewise. The extracts were put in refrigerator until used for further analysis.

2.4. Phytochemical Screening Tests

The *n*-hexane, $CHCl_3$:MeOH (*v/v* 1:1), and methanol extracts were subjected to qualitative phytochemical screening tests for the presence of the classes of secondary metabolites including alkaloids, terpenoids, flavonoids, phenolics, glycosides, and saponins, following standard procedures (Harborne, 1973; Parekh & Chands, 2008).

2.5. Compound Isolation and Structure Elucidation

Solvent selection for the chromatographic separation was performed by various proportions of solvent mixtures of *n*-hexane, chloroform, dichloromethane, ethyl acetate, and methanol. The *n*-hexane-ethyl acetate solvent system showed better TLC profile and it was chosen for silica gel column chromatographic separation. Silica gel slurry of *n*-hexane was used for packing the column in order to achieve list polarity to the mobile phase. The $CHCl_3$:MeOH (v/v 1:1) extract (15 g) was adsorbed on silica gel and was added to the column. Separation of the components through column chromatography was conducted with increasing polarity in *n*-hexane-ethyl acetate solvent systems of various proportions. 22 fractions of each 30 ml were collected and the components in each fraction were analyzed by TLC. The fractions (on TLC) were concentrated using rotary evaporator. The concentrated and dried compounds were put in vial and stored in a refrigerator until sent for spectral analysis. Fraction number 11 (*n*-hexane: EtOAc; 4:1) resulted in a white solid compound (Rf = 0.57) with minor impurity. This was further purified by washing with *n*-hexane and resulted in 250 mg of white solid compound. The structure elucidations of the isolated chemical constituents were determined by generating ^{1}H- and ^{13}C-NMR data and by comparing the experimental spectroscopic data with previous reports in the literature.

3. RESULTS and DISCUSSION

3.1. Extraction Yield

Phytochemical investigation of specimens of plant origin is needed to increase the amount of chemical constituents and to maintain their activities (Aziz *et al.*, 2003). Obtaining high extract yield is an important step in the course of secondary metabolite investigation and detection of biologically active compounds. Choice of appropriate extraction method is also essential for the tweaking of phytochemical constituents leaving out avoidable materials with the aid of the

solvents. Further selection of suitable extraction process and optimization of various parameters are very important for up scaling from bench scale to large scale phytochemical analysis. The most commonly used extraction techniques include conventional techniques such as maceration, percolation, infusion, decoction, hot continuous extraction etc. In this study cold maceration technique is used. The extraction yield of the plant material is presented in Table 1.

Table 1. Percent yield of the crude extracts.

Extract	Mass of extract (g)	Yield (%)
n-Hexane	2.2	0.4
$CHCl_3$:MeOH (v/v; 1:1)	25	5.0
Methanol	19.8	4.0

Extraction solvent choice needs to be based on the plant material matrix properties, chemical properties of the secondary metabolites, matrix-metabolite interaction, efficiency and desired properties (Ishida *et al.*, 2001; Hayouni *et al.*, 2007). The extractability of solvents depends on compound solubility in the solvent, the mass transfer and the strength of matrix interaction with heat and mass diffusion rate (Dhanani *et al.*, 2017). The extraction solvent choice also depends on what natural compounds or classes of natural compounds one is looking for. In this study, however, the focus was performing total phytochemical analysis of the plant roots. The high yield of the $CHCl_3$:MeOH (*v/v* 1:1) extract suggests that constituents in the plant specimen are moderately polar.

3.2. Phytochemical Screening

Plants of the genus *Heliotropium* are rich in bioactive constituents such as phenolic acids, lignans, flavonoids, nitrile glycosides, quinonoids, steroids, triterpenoids, and pyrrolizidine alkaloid (Jeruto *et al.*, 2011; Li *et al.*, 2008). The previous report on phytochemical screening of the leaf extracts of *H. verdcourtii* shows the presence of alkaloids, saponins, glycosides, terpenoids, anthraquinones, phenolics, and flavonoids (Ogundajo & Ashafa, 2017). In this study, the result of the qualitative phytochemical test of the extracts revealed the presence of flavonoids, terpenoids, phenolics, saponins, glycosides and alkaloids which is in agreement with previous works.

The classes of secondary metabolites found in the plant have the following biological activities. Flavonoids are known by antioxidative, free radical scavenging, coronary heart disease prevention, hepatoprotective, anti-inflammatory, anticancer and antiviral activities (Kumar & Pandey, 2013). Saponins are known for their biological activities such as antimicrobial, antifungal, anti-inflammatory, antiviral, antioxidant, anticancer, and immunomodulatory effects (Juang & Liang, 2020). Glycosides are known to possess remarkable therapeutic potential and pharmacological activities. Analgesic, anti-inflammatory, cardiotonic, antibacterial, antifungal, antiviral, and anticancer effects are some of the pharmacological activities (Soto-Blanco, 2022). Terpenoids have antimicrobial and antidiarrheal activities (Prashant *et al.*, 2011). Phenolic constituents exhibit antibiotic, antimicrobial, and antidiarrheal activities (Jacob and Burri, 1996; Prashant *et al.*, 2011). Alkaloids exhibit a wide range of activities. They are not only biosynthesized in nature against herbivores but also decrease bacterial or fungal influx (Adamski *et al.*, 2020). They are therefore constituents that have high prospective in medicine, plant defense, veterinary, or toxicology. The presence of such classes of secondary metabolites supports the ethnomedicinal use of the species.

3.3. Isolated Compounds and Their Structure Elucidation

Fractionation of the chloroform:methanol (*v/v* 1:1) extract resulted in a white solid compound with Rf = 0.57 (*n*-hexane-EtOAc 4:1). The ^{1}H-, ^{13}C-NMR, and DEPT-135 spectral data, however, revealed the white solid compound being a mixture of three compounds (isomers) where purification through silica gel column chromatography and recrystallization was not successful. The experimental spectroscopic data was compared with spectroscopic data reported in literature (Carothers *et al.*, 2018; Chaluma *et al.*, 2018; Liliana *et al.*, 2012; Mesfin, 2018; Raga *et al.*, 2013; Sathish *et al.*, 2017) for structure elucidation.

3.3.1. *Compound 1*

^{1}H-NMR spectrum: δ 5.12 (1H, t *J* = 6.62 Hz) is a proton attached to sp^2 hybridized carbon (H-12). δ 3.16 (1H, t *J* = 6.71 Hz) is a characteristic peak of a proton attached to a carbon atom bearing a hydroxyl group (H-3). δ 0.80 (H-25, s, H-30, d *J* = 6.70 Hz), 0.81 (H-24, s), 0.86 (H-29, d *J* = 6.54 Hz), 0.88 (H-26, s), 0.92 (H-23, s), 1.00 (H-28, s) and 1.06 (H-27, s) are eight aliphatic methyl signals. Moreover, the remaining proton signals for five methine and eighteen methylene protons were observed in the aliphatic region.

^{13}C-NMR spectrum: The signals at δ 38.0 (C-1), 27.3 (C-2), 18.4 (C-6), 32.9 (C-7), 23.3 (C-11), 28.8 (C-15), 26.6 (C-16), 31.3 (C-21), and 41.5 (C-22) are methylene (CH_2) carbons, 79.2 (C-3), 55.2 (C-5), 47.7 (C-9), 124.4 (C-12), 59.0 (C-18), 39.6 (C-19), 39.7 (C-20) are methine (CH) carbons and 28.2 (C-23), 15.6 (C-24), 15.6 (C-25), 16.8 (C-26), 23.3 (C-27), 28.1 (C-28), 17.4 (C-29), 21.3 (C-30) are methyl (CH_3) carbons. The absence of peaks on DEPT-135 spectrum at δ 38.8 (C-4), 41.2 (C-8), 36.9 (C-10), 139.6 (C-13), 42.0 (C-14) and 33.8 (C-17) confirms these peaks belong to quaternary carbon atoms. The two olefinic peaks at δ 124.4 and 139.7 were for C-12 and C-13 of which the latter is sp^2 hybridized quaternary carbon, respectively. The signal at δ 79.2 belongs to sp^3 hybridized oxygenated methine carbon (C-3).

3.3.2. *Compound 2*

The ^{1}H- and ^{13}C-NMR spectral data of this compound is similar to **compound 1** except some differences in the ^{13}C-NMR spectra. The ^{13}C-NMR spectra peaks at δ 145.3 and 121.7 belong to the olefinic carbons (C-13 and C-12) and δ 40.0 and 37.7 are C-20 and C-19 respectively. The difference in the chemical shift of the C-20 and C-19 is due to the shift of the methyl groups towards C-20 which resulted in an increase in C-20 and a decrease in C-19 chemical shift values. The chemical shift at C-3 also showed a slight shift and appeared at δ 79.0 which also distinguishes this compound from **compound 1**.

The experimental spectroscopic data of **compound 1** was in good agreement with reported data for (3*β*)-urs-12-en-3-ol commonly known as *α*-amyrin, viminalol, or *α*-amyrinol (Liliana *et al.*, 2012; Sathish *et al.*, 2017; Chaluma *et al.*, 2018). *α*-Amyrin (**1**) (1, Figure 1) is a triterpene which possesses a double bond between C-12 and C-13 where the hydrogen at the 3*β* position is replaced by a hydroxyl. It is a hydride derivative of pentacyclic triterpene known as ursane.

The spectroscopic data of **compound 2** agrees with previously reported data of *β*-amyrin (**2**) (2, Figure 1) (Chaluma *et al.*, 2018; Liliana *et al.*, 2012; Sathish *et al.*, 2017).

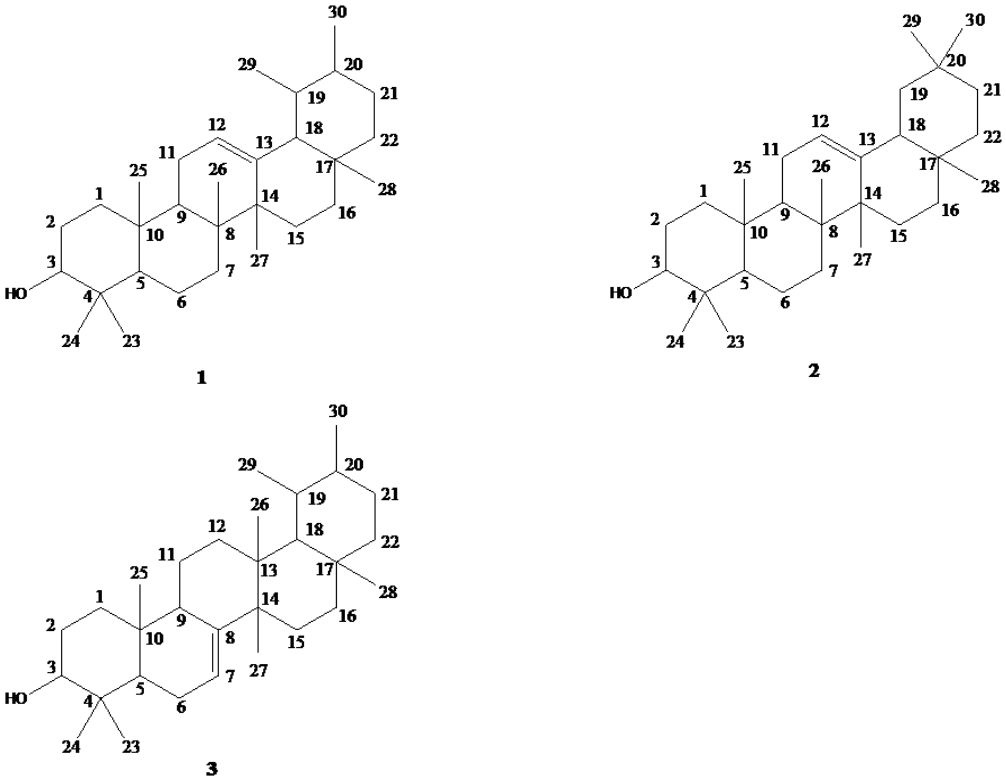

Figure 1. Structures of α-amyrin (**1**), β-amyrin (**2**), and bauerenol (**3**), respectively.

3.3.3. *Compound 3*

^{1}H-NMR spectrum: the chemical shift at 5.41 (1H, t J = 7.1 Hz) characterizes olefinic proton. The chemical shift at 3.24 (1H, t J = 6.70 Hz) is the characteristic peak of methine proton attached to a carbon atom bearing hydroxyl functional group. The signals at δ 2.19, 1.54, 1.27, and 1.14 belong to the methine (CH) protons. The signals at δ 2.16, 1.97, 1.64, 1.61, 1.60, 1.54, 1.50, 1.49, 1.48, 1.43, 1.19, 1.18, 1.14, and 1.09 are methylene (CH_2) protons. The signals at δ 1.05, 1.02, 0.97, 0.96, 0.95, 0.90, 0.84, and 0.75 are methyl protons.

^{13}C-NMR spectrum: The two peaks at δ 145.3 and 116.4 are olefinic carbons (C-8 and C-7). The peaks at δ 37.7 (C-16), 36.9 (C-1), 32.4 (C-12), 31.5 (C-22), 29.2 (C-21), 28.9 (C-15), 27.7 (C-2), 24.2 (C-6), 16.9 (C-11), are methylene (CH_2) carbons. The peaks at δ 77.2 (C-3), 54.9 (C-18), 50.4 (C-5), 48.2 (C-9), 35.3 (C-19), 32.0 (C-20) are methine (CH) carbons and the peaks at δ 39.9 (C-28), 27.6 (C-23), 25.7 (C-29), 23.7 (C-26), 22.7 (C-27), 22.5 (C-30), 14.7 (C-24), and 13.0 (C-25), are methyl (CH_3) carbons. The peaks at δ 41.5 (C-14), 38.8 (C-4), 37.7 (C-13), 35.3 (C-10) and 32.0 (C-17) are quaternary carbons.

DEPT-135 spectrum: the upward peaks at δ 116.4 (C-7), 77.2 (C-3), 54.9 (C-18), 50.4 (C-5), 48.2 (C-9), 35.3 (C-19), 32.0 (C-20) are methine (CH) carbons whereas the peaks at δ 39.9 (C-28), 27.6 (C-23), 25.7 (C-29), 23.7 (C-26), 22.7 (C-27), 22.5 (C-30), 14.7 (C-24), and 13.0 (C-25), are methyl (CH_3) carbons. The downward peaks at δ 37.7 (C-16), 36.9 (C-1), 32.4 (C-12), 31.5 (C-22), 29.2 (C-21), 28.9 (C-15), 27.7 (C-2), 24.2 (C-6), 16.9 (C-11), are methylene (CH_2) carbons. The absence of peaks at δ 145.3 (C-8), 41.5 (C-14), 38.8 (C-4), 37.7 (C-13), 35.3 (C-10) and 32.0 (C-17) implies they belong to quaternary carbons.

The above ^{1}H- and ^{13}C-NMR data were in good agreement with the reported data for bauerenol (**3**) (3, Figure 1) (Carothers *et al.*, 2018; Chaluma *et al.*, 2018; Mesfin, 2018; Raga *et al.*, 2013; Sathish *et al.*, 2017).

Ursane type triterpenes are widely distributed in the plant kingdom, as aglycones or in combined forms, and have several biological activities. *α*-amyrin is usually found in oleo-resin of the various species of *Bursera* or *Protium* of the Burseraceae family. It exhibits several biological activities *in vitro* and *in vivo* conditions against several health-related conditions, such as microbial, inflammation, cancer cells, and viral and fungal infections (Liliana *et al.*, 2012). Bauerenol, on the other hand, showed cytotoxic and apoptotic potential against human HepG2 cancer cells and it is also anti-*Trypanosoma brucei* agent (Carothers *et al.*, 2018). In addition to this bauerenol prevents migration, proliferation and invasion of retinoblastoma cells through induction of autophagy, apoptosis and cell cycle arrest (Chen *et al.*, 2022). *β*-Amyrin possesses anti-inflammatory, anti-fibrotic, and anti-apoptotic effects on dimethyl nitrosamine–induced hepatic fibrosis in male rats (Thirupathi *et al.*, 2017). The presence of *α*-amyrin might have caused the root of the species to have the traditional medicinal effects as wound healing (Maundu & Tengnäs, 2005).

4. CONCLUSION

In this study, phytochemical screening and compound isolation were carried out on the root of *H. verdcourtii*. The classes of secondary metabolite screening test of the *n*-hexane extract revealed the presence of terpenoids, flavonoids, and glycosides, alkaloids, saponins, and phenolics. The presence of these bioactive constituents is significant as they may account for the wide scope ethnomedicinal use of the species. Silica gel column chromatographic separation of the chloroform/methanol (*v/v* 1:1) extract has led to the isolation of the mixture of three biologically active ursane type pentacyclic triterpenes identified as *α*-amyrin, *β*-amyrin, and bauerenol. The presence of *α*-amyrin might be the cause of the root to have a wound healing property. This is the first report of the isolation of the aforementioned chemical constituents from the root of *H. verdcourtii*, of Ethiopian origin.

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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

Tegene Tesfaye Tole: Methodology, Supervision, Visualization, Formal Analysis, and Writing original draft. **Habtamu Hailu Feso**: Investigation, Resources, Visualization, Formal Analysis. **Legesse Adane**: Methodology, Supervision, and Validation.

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Research Article

Identification and assessment of biological activities of *Gymnanthemum amygdalinum* (Delile) Sch.Bip. ex Walp. collected from Bongabon, Nueva Ecija

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Abstract: The medicinal potential of plants encompasses a diverse array of compounds with therapeutic applications. These compounds have the potential to contribute to the development of innovative pharmaceuticals that enhance overall health. This study highlights the molecular identification, phytochemical analysis, teratogenic and cytotoxic effects of *Gymnanthemum amygdalinum* collected from Bongabon, Nueva Ecija. Morphological and molecular identification confirmed the identity of *G. amygdalinum* having 100 % similarity to their corresponding sequences. Also, *G. amygdalinum* exhibited secondary metabolites such as essential oils, phenols, sugars, anthraquinones, coumarins, anthrones, tannins, flavonoids, steroids, and alkaloids. The plant extract has teratogenic effects as mortality rate was observed at 1000 and 10,000 ppm, correlated with low hatchability rate at the same concentrations. These findings demonstrated the potential for anticancer, leading to further evaluation of cytotoxicity employing *Artemia salina* and hepatocellular carcinoma cell lines (HepG2). As a result, *G. amygdalinum* was found to be moderately toxic in brine shrimp lethality assay with a mortality rate of 10 ppm and higher. Similarly, it is moderately toxic in HepG2 at a median concentration of 1000 ppm and highly toxic at 4000 ppm. Collectively, *G. amygdalinum* extract exhibits teratogenic and cytotoxic effects and is suitable for further studies at the same or higher concentrations. Accordingly, it is recommended to proceed to the next phase of study for anticancer and antiproliferative. This study provides a scientific foundation for future research, supporting the researchers in uncovering the medicinal potentials of not only *G. amygdalinum* but other medicinal plants as well.

1. INTRODUCTION

The use of medicinal plants is an important aspect of traditional medicine in the Philippines, particularly among those who live in distant mountainous areas remote from cities. Its origins are found in the traditions among various ethnic Filipino communities. To date, herbal medicines have been recognized by researchers to be one of the foundations for discovering the potential medication of plants.

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Phytochemicals from plants are being studied for direct medicinal applications as well as prototype lead compounds for the development of newly manufactured synthetic or semisynthetic medicines (Chattopadhyay & Maurya, 2015). Locals in Bongabon, Nueva Ecija, commonly use medicinal plants instead of commercially produced medicines since these are more readily available and it is a traditional practice. When residents become ill or infected with a disease, they simply boil the leaves of those well-known medicinal plants found in their vicinity or from the nearby mountains. Furthermore, there is a particular plant in their area that is highly utilized for its medicinal value even though this plant has no local name and is still unidentified. In this regard, it is vital to identify this plant using a molecular approach to provide the local people residing in the area with accurate information regarding the identity of the plant they utilized. DNA barcoding is being used in an increasing number of studies to identify unknown organisms and determine their taxonomy. This plant molecular identification method involves obtaining one or more DNA sequences of one or more candidate genes and comparing them to a repository of genes associated with plant taxa (Simpson, 2019).

Locals, on the other hand, believe that due to its high efficacy as a traditional medicine in treating diseases, this plant could potentially be used to treat cancer. However, extensive research and several clinical trials are required before one can claim such potential of this plant. In connection to this, several bioassays, teratogenicity, and cytotoxicity were conducted to come up with the initial data on the anti-cancer potential of this plant. Teratogens are chemical, physical, or biological agents that can cause developmental defects. Some teratogens cause death, while others cause cell apoptosis. Teratogens are also capable of causing developmental abnormalities by altering gene expression patterns, inhibiting cell interactions, or preventing the morphogenetic movement of cells (Fenderson, 2009). The zebrafish is a good animal model that can be used to assess the teratogenic effects of some medicines because of its characteristics and traits that are similar to those of other vertebrate animals particularly humans (Dulay *et al*., 2012). Teratogens and teratogenic agents may be developed as anticancer drugs, therefore, anticancer and teratogenic effects are two closely related concepts (Blagosklonny, 2005). It has been proven through experimentation that anticancer plants have cytotoxic effects (Ghorani *et al*., 2018). Cytotoxicity test is one method of determining toxicity, which is an *in vitro* test that is mainly performed to screen potentially toxic compounds that affect basic cellular functions (Fotakis & Timbrell, 2006). Therefore, this study aims to molecularly identify the medicinal plant collected from Bongabon, Nueva Ecija, to determine its secondary metabolites and anti-cancer potential by evaluating its teratogenic and cytotoxic effect on zebrafish, brineshrimp nauplii, and hepatocellular carcinoma cell lines. In addition, this research can significantly contribute to the body of knowledge, mainly in the pharmaceutical and biomedical fields.

2. MATERIALS and METHODS

2.1. Sample Collection

The plant material was collected in Brgy. Calaanan, Bongabon, Nueva Ecija. Plant leaves were collected and used in the study since this is the part of the plant that was utilized by the local people. To prevent deterioration of plant components, leaves were immediately air dried after collection. Fresh leaves, on the other hand, were collected for molecular identification and placed in a 2 mL microtube containing Cetyl trimethylammonium bromide (CTAB) buffer.

2.2. Morphological Identification

The collected plants were photographed in their natural habitat. The main external plant structures and shapes were observed. A taxonomist from the Department of Biological Sciences, College of Science, Central Luzon State University confirmed and authenticated the identification of the plant specimen.

2.3. Nucleotide Sequencing

The genomic DNA of the plant samples was extracted from the dried leaf sample using the (CTAB) method of Murray and Thompson (1980) with minor modifications. About 100 mg of plant sample was grounded using mortar and pestle. Six hundred (600) µL of 2X CTAB buffer and 70 µL of 20 % Sodium dodecyl sulfate (SDS) were added to the ground sample and then thoroughly homogenized and incubated for 45 min in a dry bath (Labnet AccublockTM). Chloroform iso-amyl (19:1 v/v) was added and spun (Beckman Coulter) at 12,000 rpm for 30 min. After transferring the aqueous phase, 500 µL of ice-cold isopropanol was added, and incubated overnight. Following incubation, it was spun for 10 minutes at 10,000 rpm with 70 % ethanol.

The pellet DNA was dried for 3 hours of incubation at room temperature, and 100 µL 1X TE Buffer was added to completely dissolve the DNA pellet. To check the DNA quality, 2 µL of the DNA sample and 2 µL of loading dye were loaded in each well of a 1 % agarose gel and run in gel electrophoresis (Accuris My GelTM) for 30 min at 100V. The gel was then visualized in a UV trans-illuminator (UVitec Cambridge). DNA samples were purified, then aliquoted into 1:50 µL of nuclease-free water and used for PCR amplification.

The nucleotide ribosomal DNA region of the samples was amplified using the *rbcL* gene marker and the following components of GoTaq® Green Mastermix with 1 µL of 10 µM forward primer *rbcL* ATG TCA CCA CAA ACA GAG ACT AAA GC and 1 µL of 10 µM reverse primer *rbcL*724 GTA AAA TCA AGT CCA CCR CG. The PCR was performed using a thermal cycler (BioRad T100™) and the parameter was set to 95°C for 3 minutes (initial denaturation), followed by 35 cycles of 95°C for 30 seconds (denaturation), 51.1°C for 30 seconds (annealing), 72°C for 1 minute (extension), 72°C for 10 minutes (final extension). The PCR product was checked using gel electrophoresis (Accuris My Gel™) for 30 minutes at 100V; 2 µL from the PCR product was loaded in each well. The gel was visualized in the UV trans-illuminator Gel Documentation System (Uvitec Cambridge Gel Documentation System). Once amplified, DNA was quantified using the Spectrophometer (Multiskan™ Go™ ThermoFisher). When the expected size of amplified DNA fragments was confirmed, PCR amplicons were stored in the microtubes and sent to Apical Scientific Sdn Bhd in Malaysia for PCR purification and double pass DNA sequencing procedure using the forward and reverse primers. The BioEdit software was used for the visual presentation of chromatograms to check the sequence quality. The sequences of the samples were queried using BLAST (Basic Local Alignment Search Tool) of NCBI (National Center for Biotechnology Information) to compare with similar nucleotide sequences stored in the Genbank for proper identification.

2.4. Phytochemical Analysis

2.4.1. Preparation of extract

Ethanol extraction was used in the study. The air-dried leaves were ground using a mill grinder and sieved to remove large portions of the leaves. Ten grams of powdered leaves were soaked in ninety percent (90 %) ethanol and kept in a well-sealed flask for three days with constant agitation. After 3 days, the sample was filtered using filter paper (Whattman No. 2), then the filtrate was concentrated to dryness using a rotary evaporator (DLabTM).

2.4.2. Phytochemical screening

The screening was performed to detect the secondary metabolites of the crude extract. The extract was spotted on a 7 x 4 cm labeled TLC (thin layer chromatography). This was made in the developing chamber using an acetate-methanol (7:3) mixture. To test the separation of the different substances, the spots for specific metabolites were seen using TLC plates that were subjected to UV light and a hot plate. Vanillin-sulfuric acid reagents were used to detect the presence of phenols, steroids, triterpenes, and essential oils. Secondary metabolites such as

anthraquinones, coumarins, and anthrones were detected using methanolic potassium hydroxide. The potassium ferricyanide-ferric chloride reagent was used to identify the phenolic compounds and tannins. Finally, the presence of flavonoids was determined using a Dragendorff's reagent.

2.5. Teratogenicity Assessment

The teratogenic assessment used in this study was based on the published article by De Leon *et al*. (2020). The treatment concentrations were calculated using the standard dilution method, $C_1V_1 = C_2V_2$, with the extract being diluted with embryo water (Hank's solution). Each vial contained three milliliters of treatment concentration, along with four embryos in the segmentation phase and incubated at 26±°C. After 12, 24, 36, and 48 hours of incubation, teratogenic activity was observed under a compound microscope (Bell Photonics) at 40x magnification. Teratogenic (head and tail malformations, scoliosis, growth retardation, stunted tail, and limited movement) and lethal (coagulation, tail not detached, no somites, and no heartbeat) morphological endpoints were evaluated. The hatchability and mortality rate of the eggs were also assessed.

2.6. Brine Shrimp Lethality Assay

Brine shrimp lethality assay based on McLaughlin *et al*. (1998) as cited by De Leon *et al*. (2020) was used to evaluate the cytotoxicity of the *G. amygdalinum* leaf extract. The LC_{50} was evaluated according to the rating of Aldahi *et al*. (2015) stating that LC_{50} of <249 µg/mL is highly toxic, LC_{50} of 250-499 µg/mL is moderately toxic and LC_{50} of 500-1000 µg/mL is mildly toxic. Moreover, values above 1000 µg/mL are non-toxic according to the rating of (McLaughlin *et al*., 1998). Under laboratory conditions, all the treatments were laid out in a completely randomized design (CRD), at a 5 % level of significance, and one-way analysis of variance (ANOVA) was used to determine the least significant differences (LSD) between treatments.

2.7. Cytotoxicity Assay using Hepatocellular Carcinoma Cell lines

A total of 10 mg of leaf extract was weighed in 2 mL capacity microtubes, added with 250 µL of Dimethylsulfoxide (DMSO- ATCC® 4-X™), and mixed for 40 minutes, acquiring 40,000 parts per million (ppm) concentration. Each extract was diluted to 4000 ppm concentration from 40,000 ppm concentration using the Eagle's Minimum Essential Medium (EMEM) (ATCC® 302003™). Extracts were serially diluted in two-folds from 4000 ppm concentration, (highest concentration) until it reached 125 ppm (lowest concentration) used for the treatment. The positive control: 5-fluorouracil (Sigma-Aldrich, Germany) was also prepared with a similar concentration and diluted with EMEM.

Human hepatocellular carcinoma [HepG2] (ATCC® HB-8065™) cells were cultured in T25 flasks using Eagle's Minimum Essential Medium (ATCC® 302003™), supplemented with 10% Fetal Bovine Serum (FBS) (HyClone™ Sera SH30071.03) and 1% Penicillin-Streptomycin Solution (ATCC® 302300™). Cells were grown and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells at 80 % confluence were used for cell seeding.

The cytotoxicity activity of the extract was tested against [HepG2] (ATCC® HB-8065™) using Promega CytoTox 96® Non-Radioactive Cytotoxicity Assay (LDH). A 5000 cells/100 µL media were seeded in 96 well plates, it was then incubated for 4 hours in a CO_2 incubator allowing cells to attach. Once attached, cells were treated in triplicates with the prepared extract of leaves and the controls. After 16-18 hours of incubation, controls were treated with lysis solution, following the manufacturer's protocol with minor modifications, and incubated for 45 mins. The incubated plates were spun down using a refrigerated centrifuge (Centurion Scientific Limited, United Kingdom) at 500 rcf, 25°C for 5 mins. A total of 30 µL of supernatant from the plates was transferred into a new plate and 30 µL of Promega CytoTox 96® Non-

Radioactive Cytotoxicity Assay (LDH) reagent was added and incubated again for 30 minutes. The plates were then read using a Multiskan Go™ (Thermo Fisher Scientific, USA) microplate reader with 20-second low shaking settings at 490 nm absorbance. Following a logarithmic model of *in vitro* cytotoxicity versus compound concentration, as established by multiple standard industry assays, compounds with percent cytotoxicity near 50 % were immediately flagged indicating that the least active concentration would be toxic to the patient. The value of 10 % cytotoxicity is arbitrarily chosen as the threshold for flagging compounds for testing discretion. This level of cytotoxicity implies that a subsequent increase in compound concentration to increase bioactivity may potentially lead to cytotoxicity of 50 %. Samples were tested in duplicates in two (2) independent trials. Samples with a highly cytotoxic profile were given an extended margin of 5 % only if the other trial exhibited a moderately cytotoxic, mildly cytotoxic, or non-cytotoxic profile (Table 1). The decision to pursue (Discontinue or Proceed) downstream experiments and orthogonal assays is based on the concurrence of both hepatic and nephric cytotoxic classifications of the samples.

$$\% \text{ Cytotoxicity} = \frac{(Experimental - Blank\ 3) - (Negative\ control - Blank\ 1)}{(Positive\ control\ 1 - Blank\ 2) - (Negative\ Control - Blank\ 1)}$$

The experimental data were all analyzed with GraphPad Prism Version 8.0.2 and the mean and standard deviation were presented. Nonlinear Regression was used to examine the LC_{50} for Dose Response Inhibition. For statistical analysis, an unpaired t-test was used, and a P-value of 0.05 was considered statistically significant.

Table 1. Criteria for classification of cytotoxicity.

Classification*	Cytotoxicity (%)
Non-cytotoxic	< 0 %
Mildly cytotoxic	0-1 %
Moderately cytotoxic	1-10 %
Highly cytotoxic	>10 %

*Based on NIMBB-DMBEL, UPD (Bataclan *et al.*, 2019).

3. RESULTS

3.1. Morphological Identification

Plant morphology plays a vital role in plant identification as it provides a visual and structural basis for differentiating between different plant species. As identified, it is a large shrub 2-3 meters long. The leaf is characterized as elliptical with entire margins, a reticulate venation pattern, and an alternate phyllotaxy arrangement. Alara and Abdurahman (2021) stated that this plant can reach a height of 1-6 m above sea level and has elliptical-shaped petiolate leaves that are 6 mm in diameter and 20 cm long. More so, an expert plant taxonomist from the Department of Biological Sciences, College of Science, Central Luzon State University has confirmed and authenticated the identity of the collected plant as *Gymnanthemum amygdalinum*.

3.2. Nucleotide Sequencing

To further confirm the identity of the collected medicinal plant from Bongabon, Nueva Ecija, molecular identification was conducted using the *rbcL* gene marker. By providing the DNA sequences of the collected medicinal plant, the National Center for Biotechnology Information-BLAST analysis confirms the identity of *G. amygdalinum*, with 100 % similarity to their respective GenBank sequences (Table 2). The identity was determined by the maximum percent similarity of plant samples obtained from GenBank as well as the comparison of Genbank sequences to the actual photograph and morphological characteristics of the plant. Considering

the 100% similarity and 100% query cover, which indicate sequence diversity between the collected plant and the *G. amygdalinum* species in GenBank, the *rbcL* gene used for identifying *G. amygdalinum* proves to be a potent molecular marker.

Table 2. Molecular Identification of *G. amygdalinum.*

Plant	BLAST Identity	% Identity	Query Cover	Accession Number
	*Gymnanthemum amygdalinum*	100 %	100 %	MN627973.1

3.3. Phytochemical Analysis

Detecting phytochemicals in plants can reveal their potential health benefits. Different phytochemicals present in *G. amygdalinum* extract were analyzed to understand its nutritional and medicinal properties and how it can be used for various purposes. The extract was evaluated for the presence of 14 secondary metabolites (Table 3), out of these 10 secondary metabolites was found present in *G. amygdalinum* extract which includes alkaloids, anthraquinones, anthrones, coumarins, essential oils, flavonoids, phenols, steroids, sugars, and tannins.

Table 3. Phytochemical composition of *G. amygdalinum.*

Plant constituents	Results
Alkaloids	+
Amino acids	-
Anthraquinones	+
Anthrones	+
Coumarins	+
Essential oils	+
Fatty acids	-
Flavonoids	+
Phenols	+
Steroids	+
Sterols	-
Sugars	+
Tannins	+
Triterpenes	-

*present (+), absent (-)

3.4. Teratogenicity Assessment

The lethal effect of various concentrations of *G. amygdalinum* ethanol extract on zebrafish embryos was assessed, and the mean percentage mortality of the embryos after 12, 24, 36, and 48 hours of exposure is presented in Table 4. At 12 hours post-treatment application, mortality was observed as 33.33 % in the 10,000 ppm and 22.20 % in the 1000 ppm which increased to 55.60 % and 33 %, respectively, after 24 hours. Moreover, at 48-hour exposure, the mortality increased at 77 % at 10000 ppm and 44.40 % at 1000 ppm. No mortality was recorded at lower concentrations of 100 ppm, 10 ppm, 1 ppm, and control throughout the observation period. As a result, the mortality of zebrafish embryos was proportionate to the concentrations of plant

extract and to the time the embryos were exposed. The percentage of mortality increased along with an increase in concentration and exposure length.

This finding indicates that *G. amygdalinum* ethanol extract has teratogenic effects on embryos which correlates to the percent hatchability at lower concentrations after 48 hours of exposure. The percent hatchability of zebrafish embryos after 48 hours of exposure to various concentrations is shown in Table 5. Hatchability is a major indicator of a successful development process. As a result, a disrupted hatching process could imply distinctive developmental problems. All embryos from concentrations ranging from 0 ppm to 100 ppm were fully formed and had hatched. However, at higher concentrations, a delay in embryo hatching was observed having a 55.60 % hatchability at 1000 ppm and 22.20 % at 10000 ppm which are the same concentrations that have exhibited mortality rates. Furthermore, the lethal effect of no heartbeat was observed at concentrations 1000 and 10,000 ppm (Table 6), while other toxicological endpoints such as coagulation and not detached tail were not observed at all concentrations. Thus, the teratogenicity assay confirmed the lethal effect of *G. amygdalinum* on zebrafish embryos. Figure 1 shows the morphological development of embryos exposed to various concentrations of *G. amygdalinum* extract. At higher concentrations (1000 ppm and 10,000 ppm), stunted growth of the embryos was observed compared to normal development at lower conce The lethal effect of various concentrations of *G. amygdalinum* ethanol extract on zebrafish embryos was assessed, and the mean percentage mortality of the embryos after 12, 24, 36, and 48 hours of exposure is presented in Table 4. At 12 hours post-treatment application, mortality was observed as 33.33 % in the 10,000 ppm and 22.20 % in the 1000 ppm which increased to 55.60 % and 33 %, respectively, after 24 hours. Moreover, at 48-hour exposure, the mortality increased to 77 % at 10000 ppm and 44.40 % at 1000 ppm. No mortality was recorded at lower concentrations of 100 ppm, 10 ppm, 1 ppm, and control throughout the observation period. As a result, the mortality of zebrafish embryos was proportionate to the concentrations of plant extract and to the time the embryos were exposed. The percentage of mortality increased along with an increase in concentration and exposure length.

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3.5. Cytotoxicity Assay using *Brine shrimp*

Given that *G. amygdalinum* extract was revealed to be teratogenic to the zebrafish embryo in the teratogenicity assay, this is a positive indicator that the plant extract might also be cytotoxic. To examine the cytotoxicity, an *in vivo* cytotoxicity assay was performed using brine shrimp (*Artemia salina*). The brine shrimp lethality assay is an efficient approach for preliminary toxicity research and testing which employs brine shrimps instead of mice and rats as *in vivo*

animal models. Table 7 shows the mortality rate of brine shrimp nauplii after 24 hours of exposure to various concentrations of *G. amygdalinum* extract. The highest mortality rate was observed at 10,000 ppm with 100 % while the lowest was observed at 10 ppm with 6.67 % mortality. The analysis of variance showed that, after 24 hours of extract exposure, there was no significant difference in all treatment concentrations at a 5 % level of significance. Based on the data, the median lethal concentration (LC_{50}) of the extract was obtained using probit regression analysis. LC_{50} value is the most important measure in determining the toxicity of a plant extract. The computed LC_{50} value is 263.03 which is considered to be moderately toxic according to the rating of Aldahi *et al*. (2015) stating that LC_{50} of <249 µg/mL is highly toxic, LC_{50} of 250-499 µg/mL is moderately toxic, and LC_{50} of 500-1000 µg/mL is mildly toxic.

Table 4. Mortality rate at 12, 24, 48 hpta of embryo at different concentrations.

Concentration (ppm)	% Mortality		
	12 Hours	24 Hours	48 Hours
0	0.00^{b}	0.00^{c}	0.00^{c}
1	0.00^{b}	0.00^{c}	0.00^{c}
10	0.00^{b}	0.00^{c}	0.00^{c}
100	0.00^{b}	0.00^{c}	0.00^{c}
1000	22.20^{a}	33.00^{b}	44.40^{c}
10000	33.33^{a}	55.6^{a}	77.80^{c}

*Means with the same letter superscript are not significantly different from each other at a 5% level of significance.

Table 5. Percent hatchability after 48 hpta exposure at different concentrations

Concentration (ppm)	% Hatchability
	48 Hours
0	100.00 ^{a}
1	100.00 ^{a}
10	100.00 ^{a}
100	100.00 ^{a}
1000	55.60 ^{b}
10000	22.20^{c}

*Means with the same letter superscript are not significantly different from each other at a 5% level of significance.

Table 6. Lethal effects of various concentrations at 12, 24, and 48 hours of exposure.

Toxicological Endpoints	Time of exposure	Concentration (ppm)					
		0	1	10	100	1000	10000
Coagulation	12	–	–	–	–	–	–
	24	–	–	–	–	–	–
	48	–	–	–	–	–	–
No Heartbeat	12	–	–	–	–	+	+
	24	–	–	–	–	+	+
	28	–	–	–	–	+	+
Tail not detached	12	–	–	–	–	–	–
	24	–	–	–	–	–	–
	28	–	–	–	–	–	–

*observed (+), not observed (-)

Table 7. Mortality rate of Brine shrimp nauplii after 24 hours of exposure to different concentrations.

Concentration (ppm)	% Mortality
	24 Hours
0	0.00[d]
1	0.00[d]
10	6.67 [d]
100	23.33[c]
1000	63.33[b]
10000	100.00[a]

∗Means with the same letter superscript are not significantly different from each other at a 5% level of significance.

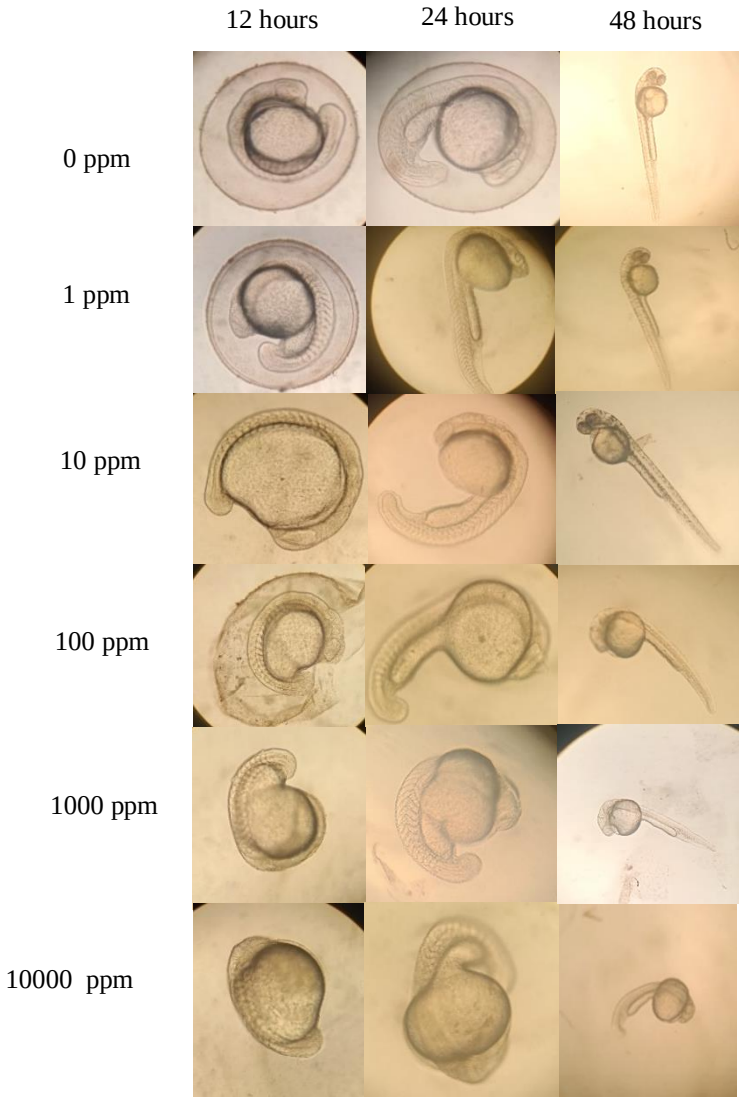

Figure 1. Morphological development of embryos exposed to different concentrations.

3.6. Cytotoxicity Assay Using Hepatocarcinoma Cell Lines

With regards to the findings that *G. amygdalinum* extract exhibits cytotoxic effects in an *in vivo* assay using brine shrimp, an *in vitro* assay was carried out using human hepatocarcinoma cell lines. These cell lines are efficient in *in vitro*, and they also maintain the genomic and transcriptome landscapes of primary HepG2. The cell-based cytotoxicity of *G. amygdalinum*

extracts was analyzed using five concentrations, with 1000 ppm being used as the median concentration, since teratogenic and *in vivo* cytotoxic effects were observed at this concentration. Starting at 4000 ppm and decreasing by half until reaching 250 ppm was found to be the lowest concentration.

Table 8 shows that after two separate trials, four (4) concentrations were found to be cytotoxic. The percent cytotoxicity was 1.82 for 500 ppm, 8.11 % for 1000 ppm, 17.21 % for 2000 ppm, and 21.26 % for 4000 ppm. Cytotoxicity levels of 500 ppm and 1000 ppm were categorized as moderately toxic, while concentrations of 2000 ppm and 4000 ppm were categorized as highly toxic. Figure 2 illustrates a dose-dependent trend in which the cytotoxicity increases as the concentration increases. The results indicate that a lower concentration of 250 ppm was not acceptable for further analysis as its toxicity level was found non-cytotoxic. However, 500 ppm, 1000 ppm, 2000 ppm, and 4000 ppm can be used in the next phase for anti-cancer, anti-proliferative, and apoptotic studies since they have the potential to suppress various cancer cell actions and are advantageous for thorough studies. Also, different cell lines could well be employed for further research using the same concentrations.

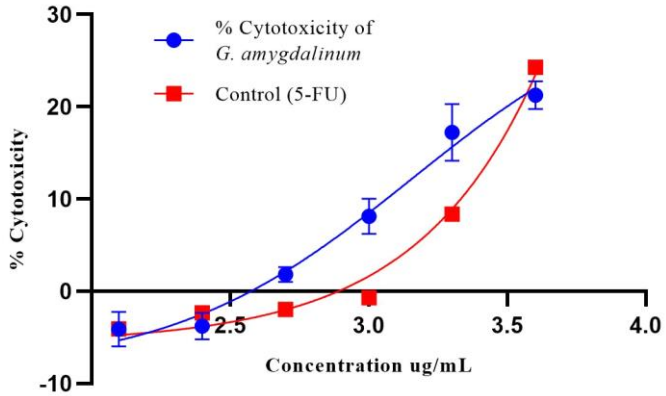

Figure 2. Cytotoxicity index plots for HepG2 cell lines (1 ppm =1 µg/mL).

Table 8. Calculated percent cytotoxicity in HepG2 cell lines.

Concentration (ppm)	% Cytotoxicity of the plant	Toxicity Level
4000	21.2595	Highly cytotoxic
2000	17.2105	Highly cytotoxic
1000	8.1175	Moderate cytotoxic
500	1.824	Moderate cytotoxic
250	-3.7765	Non-cytotoxic

4. DISCUSSION

All over the world, medicinal plants are used as alternative or complementary medications. For centuries, medicinal plants have been used in medicine and folk medicines to prevent and cure diseases by utilizing various indigenous biological systems (Elnour *et al*. 2023). In Bongabon, Nueva Ecija, the local people implement their accumulated traditional knowledge, thus they are very fascinated with utilizing medicinal plants rather than pharmaceutical products as it provides a less expensive option for treating various diseases. Studies on these medicinal plants include accurate identification, pharmacological, and toxicological evaluations are of the utmost importance for the development of new drugs.

Proper identification of plant species is therefore important to further studies into the various advantages they provide. In the present study, a medicinal plant that was widely utilized by the

locals in Bongabon, Nueva Ecija was morphologically and molecularly identified as *Gymnanthemum amygdalinum* with 100 % similarity to their corresponding GenBank sequences. The *rbcL* gene marker, which was utilized to identify *G. amygdalinum*, is a powerful molecular marker as Tnah *et al*. (2019) and Eraga *et al*. (2020) have both successfully employed this molecular marker to identify *G. amygdalinum*. In addition to this, there have been reported plants that belong to the same family of Asteraceae that confirmed its identity by also using *rbcL* gene in molecular identification such as *Cirsium arvense* (Cao *et al*., 2022a), *Argyranthemum frutescens* (Zhao *et al*., 2022), *Mikania glomerata* and *Mikania laevigata* (Bastos *et al*., 2011).

The naturally occurring bioactive compounds known as phytochemicals are derived from various plant parts and are primarily responsible for all plants' biological activities. It is used to describe plant chemicals that are not nutrients but may have health benefits by lowering the risk of developing chronic diseases (Cao *et al*., 2022b). Table 3 shows the presence of essential oils, phenols, sugars, anthraquinones, coumarins, anthrones, tannins, flavonoids, steroids, and alkaloids in *G. amygdalinum* extract. *G. amygdalinum* belongs to the family of Asteraceae, according to Soković *et al*., (2019), certain plants belonging to the family Asteraceae are rich in phytochemicals. The majority of phytochemicals proved to have useful attributes, including anti-arthritic, antibacterial, antimalarial, antidiabetic, and others. It can strengthen the immune system, reduce the rate at which cancer cells grow, and protect against DNA damage, which can result in cancer and other illnesses (Kumar *et al*., 2023). In this study, the presence of important phytochemicals such as flavonoids was found. Flavonoids have a broad range of anticancer properties, making them suitable candidates for further research into the development of novel cancer chemopreventive medicines, as flavonoid-rich foods could induce advantageous changes in the gut microbiota, lowering the risk of developing cancer and normalizing vital cellular functions (Kopustinskiene *et al*., 2020).

Furthermore, renowned for having a bitter flavor, the *G. amygdalinum* is commonly named a bitter leaf. The bitterness of the *G. amygdalinum* prevents the locals of Bongabon from eating the leaves as vegetables. However, according to Engel (2007), bitterness generally denotes toxicity, it is possible that bitterness in plants could be a useful indicator of therapeutic potential. In teratogenicity assay, to determine direct-acting teratogens and conduct preliminary evaluations of embryotoxic substances (Weigt *et al*., 2011), zebrafish was used as it has increasingly been recognized as an efficient animal model in determining teratogenic effects. The embryos were considered dead if no visible heartbeat or observed as coagulated during the experiment. The number of deaths caused by a specific condition is known as mortality. In this study, mortality was observed at greater concentrations of 1000 ppm and 10,000 ppm (Table 4), which is correlated with the low hatchability rate at the same concentrations (Table 5). On the other hand, the lethal effects of no heartbeat were also observed at higher concentrations.

Teratogenicity assay confirmed the lethal effect of *G. amygdalinum* on zebrafish embryos. Additionally, there were no abnormalities in the morphological development of embryos exposed to various concentrations of Gamygdalinum extract, however, there was stunted growth of embryos at higher concentrations (1000 ppm and 10,000 ppm) compared to the normal development at lower concentrations. Thus, *G. amygdalinum* extract is teratogenic to the embryos. The mortality of zebrafish embryos was found to be related to the concentrations of *G. amygdalinum* extract used and the duration that the embryos were exposed. As the concentration and duration of exposure increased, the percent mortality correspondingly increased. This finding indicates that *G. amygdalinum* extract has teratogenic effects on embryos. In the study of teratogenicity of a rhizome plant using zebra fish, the toxicity effects were also discovered to be dose-dependent at dosage above 62.50 µg/mL, while, at 125.0 µg/mL, mortality of embryos was observed (Alafiatayo *et al*., 2019) Similar results were seen in the *Carica papaya* extract (De Castro *et al*., 2015) and *Moringa oleifera* (David *et al*., 2016)

as embryo-toxic in which teratogenicity and mortality were both concentration-dependent. Likewise, in the study of Jose *et al.* (2016), the toxic effects of *Garcinia mangostana* extract on developing zebrafish embryos were discovered to depend on the period of exposure, concentrations, and parts of the plant. The lyophilized water extract from the plant leaves was more toxic than the extract from stem bark.

Considering that *G. amygdalinum* extract was revealed to be teratogenic to the embryo in the teratogenicity assay. Determination of whether the plant extract possesses an impact on cell proliferation or exhibits direct cytotoxic effects, is of utmost importance. Drug screening typically uses cell cytotoxicity and proliferation assays (Adan, *et al*., 2016), thus, an *in vivo* cytotoxicity assay was performed using brine shrimp (*Artemia salina*). The highest mortality rate of 100 % was observed at 10,000 ppm while the lowest was observed at 10 ppm with 6.67 % mortality (Table 7). Results showed that, after 24 hours of extract exposure, there was no significant difference in all treatment concentrations at a 5 % level of significance. The computed LC_{50} value is 263.03 which is considered to be moderately toxic according to the rating of Aldahi *et al*. (2015) stating that LC_{50} of 250-499 µg/mL is moderately toxic. The assay results show that as the extract concentrations increased, the mortality of brine shrimp nauplii correspondingly increased, indicating that the mortality rate is considered high. Correspondingly, other reported studies have confirmed the moderate toxicity of *V. amygdalina* (Dosumu *et al*., 2017; Ijeh & Onyechi, 2010; Omede *et al*., 2018) while other species of *Vernonia* genus like *V. anthelmintica* shows mild cytotoxic activity (Patnaik & Bhatnagar, 2015).

To support the findings that *G. amygdalinum* extract shows cytotoxic effects in an *in vivo* assay employing brine shrimp, an *in vitro* assay was performed using human hepatocarcinoma cell lines (HepG2). Results show that the toxicity level was non-cytotoxic at lower concentrations (250 ppm) while the toxicity level was found to be moderate to highly cytotoxic at 500 ppm, 1000 ppm, 2000 ppm, and 4000 ppm. Therefore, it has the potential to be used in the next stage of cancer research. Congruent to the results, endemic plants from the same family of *G. amygdalinum*, such as *S. musilii* whole and *A. monosperma* leaves, demonstrated a capable anticancer effect when assessed with HepG2 (Khan *et al*., 2022). On the other hand, the study of Wong *et al*. (2013) on *G. amygdalinum* inhibits cancer growth in MCF-7 and MDA-MB-231 cells. The effect was mediated by the inhibition of breast cancer cell proliferation. When combined with doxorubicin, it showed synergism, implying that it can supplement current chemotherapeutic treatment.

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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. **Ethics Committee Number**: Central Luzon State University, 2023-511.

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Research Article

Content of saponin, tannin, and flavonoid in the leaves and fruits of Iranian populations from *Rhamnus persica* Boiss. (Rhamnaceae)

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Abstract: To study the phytochemical properties in the leave and fruit of Iranian populations of *Rhamnus persica* Boiss, eighteen different populations from six geographical regions of Kermanshah province were studied. The results showed that the highest and lowest leaf saponin was present in Aliabad (6.40 mg/g) in Eslamabad-e Gharb and Chaharzebar in Mahidasht (3.29 mg/g), respectively. In fruit saponin, the highest content was in the population of Arkavazi in Eslamabad-e Gharb (4.33 mg/g) and the lowest being in Mahidasht population (Chaharzebar) and Sarpol-e Zahab (Galin) each with 2.20 mg/g. About the leaf tannin, the highest and lowest values were available in Barf-Abad population in Eslamabad-e Gharb (4.57 mg/g) and Chaharzebar in Mahidasht (1.83 mg/g), respectively. In addition, in the case of fruit tannin, the highest amount in Meleh-Sorkh population belonged to Homeil (2.74 mg/g) and the lowest being in of Changizeh population (Sonqor) in 2.28 mg/g. Moreover, the highest and lowest leaf flavonoids were obtained in two populations including Gordi in Eslamabad-e Gharb (6.14 mg/g) and Changizeh from Sonqor (4.09 mg/g), respectively. Regarding fruit flavonoids, the highest and lowest amounts were observed in populations of Meleh-Sorkh from Homeil (3.45 mg/g) and Banganjab in Mahidasht (2.13 mg/g). These findings confirmed the effect of geographical location on changing the phytochemical characteristics of *Rhamnus* populations in Kermanshah province from Iran.

1. INTRODUCTION

Study of different medicinal plants in each country is the first step to enter the global trade market of these important plants. Various biochemical properties such as saponin levels can be studied in various medicinal plants and even used as a marker to identify, differentiate, and classify of these plants (Paterson, 2019). Saponins are steroidal and triterpenoid glycosides that exhibit various biological activities. Extensive presence in plants and potential for medicinal usages has led to the extraction and identification of them in various plant species. Although,

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these efforts are important for expanding knowledge about saponin structures that are naturally produced by plants, biosynthesis, and their distribution in have recently been considered (Ahmed *et al*., 2008; Faizal & Geelen, 2013).

Tannins are different compounds that are usually hard and astringent, as well as the ability to adhere. They contain proteins and cause their deposition. Due to this property, they have been used for centuries to turn animal skins into leather (in tanning). Tannins are divided into several general categories, which include hydrolyzable (gallotanes and agitates), dense, and false tannins (complex) (Omidbeygi, 2008). Flavonoids include anthocyanin pigments and isoflavonoids. The main skeleton for the flavonoid group is flavan, flavonos, flavonols, and anthocyanins that are the most important groups of them. The most prominent flavonoids are also anthocyanins and anthocyanins (Lila, 2004).

Rhamnus persica Boiss. (Figure 1) is one of the valuable medicinal plants in the flora of Iran and due to the presence of some bioflavonoids, vitamins (especially vitamin C), valuable minerals, and organic acids are very important in the pharmaceutical industry around the world (Sattarian *et al.*, 2017). The Family Rhamnaceae includes about 100 to 125 species that are distributed in tropical and subtropical regions of the northern hemisphere and *Rhamnus* is the largest genus in this family (Kurylo *et al.*, 2007). The fruits in this genus are round with four-leafed ovaries and the seeds have yellow grooves (Mubberley, 1987; Soufiyan & Dinarvand, 2007). Eight terrestrial species of genus *Rhamnus* are known in Iran and are widely found in the Zagros, Iran-Turanian, and Caspian regions (Soufiyan & Dinarvand, 2007).

The vast plateau of Iran, while being a special geographical unit on the planet, has a variety of climates and environments. Therefore, various plant species are distributed in the country so that the rich flora of Iran includes more than 7500 plant species. The effect of climatic factors on different plants is variable and the role of these factors on the growth and development and effective compounds in medicinal plants should always be investigated with appropriate research. Although, the quantity of secondary metabolites is affected by the set of plant gene bank; but, their content, concentration, and accumulation are significantly affected by the environmental conditions (Omidbeygi, 2008).

So far, there is no study on the phytochemical properties of *R. persica* in Iran including the measurement of its saponin, tannins, and flavonoids. The present study was performed by using eighteen ecotypes of this medicinal plant and its purpose was to determine the content of different compounds in the leaves and the fruits of this valuable medicinal plant for finding a superior population for its later usages. The authors of this article hope to introduce suitable populations of *R. persica* with higher chemical compounds for their usage in Iran and in trade of medicinal plants. This study is very important; because, it is new and can be considered by researchers from different aspects.

Figure 1. *Rhamnus persica*.

2. MATERIAL and METHODS

2.1. Collecting of Samples

In this study, eighteen different populations (ecotypes) of *R. persica* were studied in Kermanshah province from west of Iran which location of them is shown in Table 1. The leaves and fruits of the plant were collected from its natural habitats and samples were identified according to the identification key in the herbarium of Razi University, Kermanshah. Then, were maintained in the refrigerator under a temperature between 4°C to 8 °C.

Table 1. Geographical characteristics of plant collection areas from *R. persica*.

Name	Latitude	Longitude	Height from the see level (m)
Gordi (Homeil)	33 50 21	49 51 22	1353
Meleh-Sorkh (Homeil)	33 57 20	46 55 05	1538
Kandahar (Homeil)	34 53 23	46 52 00	1601
Barf-Abad (Eslamabad-e Gharb)	34 04 41	46 31 18	1409
Ali-Abad (Eslamabad-e Gharb)	34 07 20	46 26 21	1376
Arkavazi (Eslamabad-e Gharb)	34 07 49	46 28 19	1402
Navdar (Guilan-e Gharb)	34 03 22	46 59 03	1026
Anari (Guilan-e Gharb)	34 07 57	46 53 02	895
Vizhenan (Guilan-e Gharb)	34 05 34	45 43 32	946
Nasar (Sarpol-e Zahab)	34 19 01	45 51 36	992
Galin (Sarpol-e Zahab)	34 15 49	45 56 36	796
Sorkheh-Dizeh (Sarpol-e Zahab)	34 24 57	46 01 59	1128
Ali-Yar (Mahidasht)	34 10 42	46 49 36	1511
Chaharzebar (Mahidasht)	34 14 00	46 41 29	1561
Banganjab (Mahidasht)	34 17 48	46 40 34	1450
Parsineh (Sonqor)	34 41 50	47 26 43	1819
Soltan Taher (Sonqor)	34 40 22	47 42 45	1804
Cahngizeh (Sonqor)	34 50 16	47 05 05	2023

2.2. Preparation of Methanolic Extract from *R. persica*

For extraction, mature leaves and fruits of *R. persica* were dried in the shade condition at room temperature and then pulverized. Extraction was performed for 48h by percolation method with methanol 80%. At the end, the extract was concentrated by a rotary apparatus and dried by oven at 60 °C. Finally, the purified extract was stored at 4 °C until its usage (Pormorad *et al.*,2016).

2.3. Saponin Assay

To extract the saponin, purified extract of alcoholic extract was used by diffusion method. The extract from the solvent was separated by vacuum distillation to identify the saponin and determine its amount, pour 1 g of methanolic extract into a test tube, some distilled water were added, and then shaken violently for 5 min. After standing for 30 min, the amount of saponin was measured from the height of the foam created in it (Salehi *et al.*, 1992).

2.4. Flavonoid Assay

To prepare flavonoid extract, method of soaking in methanol 70% with a ratio of 3 to 1 (3 part of methanol and 1 from plant powder) for 24h was used. After this time, the extract was filtered and spilled in a 100 mL jug with a methanol 70%. Then, 2 mL of methanolic extract of the plant with 1.5 mL of methanol, 0.1 mL of aluminum chloride (methanol10%), 0.1 mL of potassium acetate (1M), and 2.8 mL of distilled water was combined. Finally, the solutions were placed under the room temperature for 30 min and absorbance of each reaction compound was measured at 415 nm by spectrophotometer (Ranjith, 2009). The standard curve was prepared

with methanolic solutions at concentrations of 250 to 1000 µg/mL and the flavonoid concentration of the extract was calculated from the following formula.

$P = (C \times 100/1000 \times M) \times 100$

P = Percentage of flavonoids concentration

C = Flavonoid concentration calculated based on standard curve (mg/mL)

M = Sample weight (g)

2.5. Tannin Assay

In order to measure tannin, 1 mL of methanol containing 20 mg of polyvinyl pyrrolidine was added to 1 mL of methanolic extract, put in a vortex for 10 sec and then maintained in ice for 30 min. The samples were centrifuged for 6 min at 6000 rpm. Then, 1.5 mL of the centrifuged solution was poured into microtubes and 10 mg of polyvinyl pyrrolidine was added to it and the extract was re-extracted according to the above method. After centrifugation, 1 mL of the extract was separated and the tannins were measured according to the phenol measurement method. According to this method, tannin compounds were precipitated with polyvinyl pyrrolidine and their amount was calculated from the difference of adsorption rate (Ranjith, 2009).

2.6. Statistical Analysis

Statistical analysis of the data was done in the form of randomized complete block design (RCBD) with three replications and comparison of means was performed by Duncan's multiple range test ($p \leq 0.01$) by using SPSS software, version 22.

3. RESULTS

The results of ANOVA from saponin, tannin, and flavonoids content in the leaves and fruits of *R. persica* are shown in Table 2. Accordingly, different populations have these important compounds in their structure which showed a significant difference at the level of 1%. Also, the results of the mean comparison from saponin, tannin, and flavonoids in the leaves and fruits are also shown in Table 3. Accordingly, in the case of leaf saponin, the highest value was in Ali-Abad population (6.40 mg/g) in Eslamabad-e Gharb and the lowest being Chaharzebar (3.29 mg/g) belong to Mahidasht. By comparing different populations, it was found that the differences between the populations of Sonqor (Soltan Taher) and, Sarpol-e Zahab (Nesar), difference between the populations of Guilan-e Gharb (Anari and Vizhenan), difference between the populations of Eslamabad-e Gharb (Barf-Abad) and Guilan-e Gharb (Navdar), difference among the populations of Sarpol-e Zahab (Galin and Sorkheh-Dizeh) and Mahidasht (Ali-Yar) and finally the difference among the populations of Homeil (Gordi), Mahidasht (Banganjab) and Sonqor (Parsineh and Cahngizeh) were not significant. Regarding the fruit saponin, the highest content was determined in the population of Eslamabad-e Gharb (Arkavazi) at 4.33 mg/g and the lowest being in the population of Mahidasht (Chaharzebar) and Sarpol-e Zahab (Galin) at 2.20 mg/g. Also, differences among the populations of Homeil (Gordi), Sarpol-e Zahab (Galin), Mahidasht (Banganjab), and Sonqor (Changizeh), differences among the populations of Homeil (Meleh-Sorkh), Eslamabad-e Gharb (Arkavazi), Guilan-e Gharb (Navdar), and Sonqor (Parsineh), difference between the populations of Sarpol-e Zahab (Nesar) and Mahidasht (Chaharzebar), and finally the differences among the populations of Guilan-e Gharb (Anari and Vizhenan) and Mahidasht (Ali-Yar) were not significant.

About the leaf tannins, the highest and lowest amounts were presented in Meleh-Sorkh (4.57 mg/g) populations in Eslamabad-e Gharb and Chaharzebar (1.83 mg/g) populations in Mahidasht, respectively. By comparing of different populations, it was found that the differences between the populations of Guilan-e Gharb (Navdar) and Sonqor (Soltan Taher), the difference between the populations of Sarpol-e Zahab (Nesar) and Sonqor (Parsineh), and

finally the difference between the populations of Galin and Sorkheh-Dizeh in Sarpol-e Zahab were not significant. In the case of fruit tannin, the highest amount in Meleh-Sorkh population (2.74 mg/g) belonged to Homeil and the lowest being in the population of Sonqor (Changizeh) was determined to be 2.28 mg/g. In this case, the differences between the populations of Sonqor (Soltan Taher) and Sarpol-e Zahab (Nesar) and finally the differences between the populations of Guilan-e Gharb (Navdar) and Mahidasht (Chaharzebr) were not significant.

Table 2. ANOVA of phytochemical properties from *R. persica*.

Sources of changes	Leaf Saponin	Fruit Saponin	Leaf Tannin	Fruit Tannin	Leaf Flavonoid
Treatment (populations)	23.834	0.846	1.483	0.069	0.285
Block (repeat)	0.117	0.141	0.117	0.0001	0.005
Error	0.216	0.1	0.002	0.002	0.0002

Values of treatment, block, and error.

The highest and lowest leaf flavonoids were obtained in Gordi population (6.14 mg/g) in Homeil and Changizeh population (4.09 mg/g) belonging to Sonqor, respectively. In this case, the difference between the populations of Navdar and Anari in Guilan-e Gharb, the difference between the populations of Guilan-e Gharb (Vizhenan) and Mahidasht (Banganjab), the difference among the populations of Homeil (Meleh-Sorkh), Sarpol Zahab (Galin), and Sonqor (Changizeh) were not significant. Regarding fruit flavonoids, the highest and lowest values were determined in the population of Meleh-Sorkh (3.45 mg/g) belonging to Homeil and Bangangab (2.13 mg/g), respectively. In this case, the difference between the populations of Gordi and Meleh-Sorkh in Homeil, the difference among the populations of Sarpol Zahab (Sorkheh-Dizeh), Guilan-e Gharb (Navdar) and Mahidasht (Ali-Yar and Chaharzebar), the difference among the populations of Guilan-e Gharb (Viznan) and Sarpol-e-Zahab (Nesar and Galin) and Mahidast (Ali-Yar), and finally the differences between the populations of Homeil (Kandahar) and Sonqor (Changizeh) were not significant.

Table 3. Mean comparison from leaf and fruit saponin, tannin, and flavonoids from *R. persica*.

Population	Leaf Saponin (mg/g)	Fruit Saponin (mg/g)	Leaf Tannin (mg/g)	Fruit Tannin (mg/g)	Leaf Flavonoid (mg/g)	Fruit Flavonoid (mg/g)
Gordi (Homeil)	3.38 ^{ji}	2.37 ^{f}	2.67 ^{fg}	2.68 ^{b}	6.14 ^{b}	3.44 ^{a}
Meleh-Sorkh (Homeil)	3.50 ^{lhi}	2.43 ^{ef}	3.63 ^{cd}	2.74 ^{a}	6.08 ^{c}	3.45 ^{a}
Kandahar (Homeil)	3.82 ^{fg}	2.73 ^{cdef}	3.67 ^{C}	2.63 ^{e}	6.04 ^{d}	3.34 ^{bc}
Barf-Abad (Eslamabad-e Gharb)	4.30 ^{e}	3.30 ^{bc}	4.57 ^{a}	2.65 ^{c}	4.40 ^{j}	3.12 ^{fg}
Ali-Abad (Eslamabad-e Gharb)	6.40 ^{a}	4.23 ^{a}	2.52 ^{h}	2.46 ^{k}	4.95 ^{e}	3.24 ^{de}
Arkavazi (Eslamabad-e Gharb)	3.93 ^{f}	4.33 ^{ef}	2.72 ^{f}	2.34 ^{n}	4.87 ^{f}	2.58 ^{j}
Navdar (Guilan-e Gharb)	4.33 ^{e}	2.50 ^{ef}	2.92 ^{e}	2.59 ^{f}	5.34 ^{a}	2.91 ^{h}
Anari (Guilan-e Gharb)	5.13 ^{d}	3.00 ^{bcde}	2.63 ^{g}	2.64 ^{d}	5.35 ^{a}	3.06 ^{g}
Vizhenan (Guilan-e Gharb)	5.07 ^{d}	3.00 ^{bcde}	2.32 ^{ji}	2.50 ^{j}	4.73 ^{g}	2.75 ^{i}
Nasar (Sarpol-e Zahab)	5.40 ^{c}	3.23 ^{bcd}	2.17 ^{k}	2.51 ^{i}	4.39 ^{j}	2.74 ^{i}
Galin (Sarpol-e Zahab)	3.77 ^{fgh}	2.20 ^{f}	2.25 ^{jk}	2.53 ^{h}	5.08 ^{c}	2.70 ^{i}
Sorkheh-Dizeh (Sarpol-e Zahab)	3.33 ^{fgh}	2.67 ^{def}	2.24 ^{jk}	2.54 ^{g}	4.85 ^{f}	2.89 ^{h}
Ali-Yar (Mahidasht)	3.70 ^{fgh}	3.03 ^{bcde}	2.39 ^{i}	2.63 ^{e}	5.35 ^{a}	2.88 ^{h}
Chaharzebar (Mahidasht)	3.29 ^{j}	2.20 ^{bcd}	1.83 ^{l}	2.59 ^{f}	4.51 ^{i}	2.92 ^{h}
Banganjab (Mahidasht)	3.38 ^{ij}	2.30 ^{f}	2.60 ^{g}	2.43 ^{l}	4.74 ^{g}	2.13 ^{fg}
Parsineh (Sonqor)	3.37 ^{ij}	2.53 ^{ef}	2.22 ^{k}	2.34 ^{m}	4.65 ^{h}	3.24 ^{de}
Soltan Taher (Sonqor)	5.60 ^{c}	3.40 ^{b}	2.85 ^{e}	2.52 ^{i}	4.88 ^{f}	3.16 ^{ef}
Cahngizeh (Sonqor)	3.38 ^{ij}	2.30 ^{f}	3.56 ^{d}	2.28 ^{o}	4.09 ^{c}	3.32 ^{bc}

Different letters indicate a significant difference in Duncan's multiple range test ($p \leq 0.01$).

4. DISCUSSION and CONCLUSION

Saponins have many medicinal properties; but, unfortunately few studies have been done on them in various plants, especially in *R. persica* and other plants in the family Rhamnaceae, some of which are discussed below. Generally, saponins were defined as a group of naturally occurring plant glycosides characterized by formation of strong foam in aqueous solutions. To date, saponins have been reported in more than 100 plant families, among which at least 150 types of them have significant anti-cancer properties. There are more than eleven premium classes of saponins including dammaranes, tirucallanes, lupanes, hopanes, oleananes, taraxasteranes, ursanes, cycloartanes, lanostanes, cucurbitanes and steroids. Due to their great structural diversity, saponins always show good antitumor effects through a variety of antitumor pathways. In addition, there is a large amount of saponin that has not yet been identified or studied by chemists (Ando *et al.,* 2008; Acharya *et al.,* 2010).

Certain saponins with strong antitumor effects have also been identified. Ginsenosides, which belong to dammaranes, prevent tumor cells from invading and metastasizing by inhibiting it angiogenesis and suppressing in vascular endothelial cells and then preventing their adhesion. Dioscinis are other steroid saponins whose aglycone diosgenin has been extensively studied for its antitumor effect by stopping cell cycle and apoptosis. Other important molecules discussed include oleanane saponins such as avicins, platycodons, saikosaponins, and soysaponins along with tubeimosides (Man *et al.*,2010).

Kimura *et al.,* (1981) in their studies on the medicinal plant *Hovenia dulcis* Thunb obtained three new saponins including C2(1a), D(2a), and G(3a) from its leaves, which are very valuable and further studies are needed to determine the effects of these newly identified compounds. Tadesse *et al.,* (2012) in their studies on the analysis of phytochemical properties in the root extract of the medicinal plant *Helinus mystacinus* (Aiton) E.Mey. ex Steud. from the family Rhamnaceae stated that the essential oil extract of this plant contains saponins, terpenoids, and glycosides and its methanolic extract also contained alkaloids, saponins, tannins, terpenoids, and glucosides. On the other hand, the oil extract of the plant did not contain any of these tested compounds. Based on phytochemical screening experiments, chloroform extract was subjected to column chromatography and showed two compounds called Betulinic Acid-1 and Benzoic Acid-2 in its structure. In the present study, the leaf and fruit extracts of *R. persica* were studied and only methanolic extraction was performed, which was one of the important reasons for the differences in the results of two studies. However, plant species were also different in them.

Seri Seriab *et al.,* (2020) in their studies on the medicinal plant *Ziziphus mauritiana* Lam from the family Rhamnaceae which is traditionally used in treatment of various diseases found that the main constituents of the methanolic extract from the leaves in growing plant was O-malonyl-ziziphus saponin compound with nine known compounds consisted of two saponins, six flavonoids, and a calcon derivative. The structure of these compounds was determined by analyzing D1, D2-NMR2 spectroscopy data as well as mass spectrometry. Part of *Zizyphus* saponin I (2) along with the other compounds was isolated from the leaves of this plant for the first time. Although in the present study, chemical separation and identification of saponins was not performed; but, this field of research can open many windows of studies on different genera of this family.

There are no direct studies on the study of phytochemical properties, such as the measurement of tannins and flavonoids in *R. persica*; but, there are studies on related species, some of which are mentioned. Pawlowska *et al.*,(2009) investigated flavonoids in *Zizyphus jujuba* and *Zizyphus spina-christi* fruit and concluded that the six major compounds were purified by Sephadex LH-20 column chromatography followed by using HPLC spectroscopy and NMRs. A glycoside C, 5'-di-C-β-d-glucosylphloretin was also detected in *Z. spina-christi*. Quantitative analysis of all compounds reported higher flavonoids content in *Z. jujuba*. Ahmed

et al., (2019) in their study on evaluation of antioxidant activity and phytochemical screening from leaves, skin, stem, and fruit of *Alphitonia philippinensis* Braid from Brunei Darussalam found that most of antioxidant activity in leaf extract can be higher total phenolic content and total flavonoids and flavonols are correlated. According to antioxidant content and results of the assay, the leaf extract had the highest antioxidant properties followed by fruit, shell, and stem. Also, Kohansal-Vajargah *et al.*, (2019) in their study on evaluation and comparison of morphological and phytochemical characteristics from 14 ecotypes of *Z. jujuba* found that flavonoid function was positively and significantly correlated with fruit length and width with length to width ratio. Phenol yield also had a positive and significant relationship with fruit length and width and tannin yield showed a positive and significant correlation with phenol. The results ofmorphological traits including leaf length and width and length to width ratio, fruit length, and width and length to width ratio and 1000-seed weight showed germination type of this medicinal plant in Iran. In terms of morphological traits, ecotype had the maximum effective substance and no statistically significant difference was observed in chemicals. Based on cluster analysis, 14 ecotypes were divided into two separate groups and evaluation of morphological and phytochemical traits showed high diversity among ecotypes. In another study, Murtala *et al.*, (2019) examined the phytochemical screening and physicochemical properties of *Ziziphus abyssinica* when found that phytochemical screening for presence of alkaloids, flavonoids, saponins, phenols, tannins, carbohydrates, glycosides, glycosides, showed ions and triterpenes in aqueous and methanolic extracts. However, steroids were not available. Quantitative chemical analysis showed that flavonoids (0.134 mg/g) were the highest chemical in the leaves while the lowest were saponins (0.6 mg/g).

Characteristics of the studied geographical areas and their effect on the amount of saponin, tannin, and flavonoids in the leaves and fruits of *R. persica* cannot be said with certainty. Based on means comparison, it can be said that similar areas in terms of the climate has almost the same amount of these valuable compounds; but, this position needs further research. Another subject that need more work is to accurately identify saponins types in leaves and fruits and even comparison of them to each other. Unfortunately, as mentioned earlier, studies on this valuable plant are very limited. The authors of this article hope that future researchers will take further steps in this area and explore more characteristics of this valuable 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 authors.

Authorship Contribution Statement

Peymen Asadi: Investigation and Resources. **Masoumeh Farasat**: Visualization and Software. **Mehrnoush Tadayoni**: Formal Analysis and Writing original draft. **Sina Attar Roshan**: Methodology. **Neda Hassanzadeh**: Supervision and Validation

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Research Article

Optimization of factors affecting *Agrobacterium*-mediated hairy root induction in *Vitex negundo* L. (Lamiaceae)

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Abstract: *Vitex negundo* L. is an aromatic, woody, blooming shrub in the Lamiaceae family which can grow into a small tree. Traditionally, *V. negundo* root has been used to cure diabetes, colic, boils, leprosy, and rheumatism. Keeping the importance of its roots in mind, an attempt has been taken for development of a protocol for efficient hairy root proliferation system. The *Agrobacterium rhizogenes* strains (MTCC 532 and MTCC 2364) were used for hairy root induction. For *A. rhizogenes* infection, both *in vitro* and *in vivo* leaves as well as internodes were used as explant. *In vitro* leaves and internodal explants were obtained by the inoculation of matured nodal segments on the optimum medium [MS + 2.0 mg/L N^6-Benzylaminopurine (BAP)] with *c.a.* 91.6% shoot regeneration and an average of 8.1 shoots per explants. *In vitro* leaf showed best hairy root induction followed by *in vitro* internode on ½ MS medium augmented with acetosyringone. Highest transformation efficiency was achieved using MTCC 2364 strain, while no transformation was observed in MTCC 532 strain. Different factors affecting transformation including co-cultivation period, infection time and optical density (O.D.) value were standardized. The highest efficacy, 88.8% hairy root induction was observed in *in vitro* leaves infected by MTCC 2364 for 60 minutes infection time with an O.D. value of 0.29 maintained over a 44-48 hours of co-cultivation period. The prescribed protocol may be used as a reference for development of industrial scale hairy root production for bioactive compound located in root of *V. negundo*.

1. INTRODUCTION

Vitex negundo L. (Lamiaceae) is an aromatic, woody and blooming shrub found mainly in India, Ceylon and China. The plant is also found at an altitude of 1500 m in the outer Himalayas (Usha *et al*., 2007). The term "*Vitex*" is originated from the Latin word 'vieo', meaning "to weave together", because of the flexible quality of the twigs and stems of the plant (Ahuja *et al*., 2015). *V. negundo* is usually an agroforestry plant and also used as fence. Besides, the plant is a traditional medicinal plant that has also been adopted in modern medicine. From root to fruit,

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the plant contains a variety of secondary metabolites that enhance the plant's ability to provide matchless medical benefits (Singh *et al*., 2016). Leaf is aromatic, used as tonic, and a vermifuge. The secondary metabolites present in the root juice (i.e. Dibutyl malate, Ajmalicine, Bruceantin, Beta carotene, Stearoyldelicone etc.) are expectorant, used as tonic for diuretic property and also used to treat colic, worms, rheumatism, dyspepsia, leprosy and boils (Gade *et al*., 2023; Ghani *et al*., 1998). Fruit is helpful for cerebrospinal, anxiety and emmenagogue therapies; dried fruit is a vermifuge, and blossoms are employed in cold and astringent treatments (Nadkarni *et al*., 2002). Scrofulous ulcers and sinuses are treated with floral oil of this plant. *V. negundo* has also been found as an antimicrobial (Dewade *et al*., 2010) and anti-inflammatory drugs (Chawla *et al*., 1992). Powdered seeds are used to cure spermatorrhoea and can be utilized as an aphrodisiac when mixed with milk and dried *Zingiber officinale* (Yunos *et al*., 2005).

Due to its immense medicinal property the plant plays an important role in pharmaceutical industry. Further secondary metabolite profiling of the *V. negundo* roots depicted about the presence of vitexin, isovitexin, negundin-A, negundin-B, acetyl oleanolic acid, sitosterol, alkaloids, phenols, flavonoids, glycosidic irridoids, tannins and terpene (Ladda & Magdum, 2012). Biotechnological intervention is useful for development of various efficient strategies to explore the therapeutic potential of *V. negundo.* Production of aerial parts like leaf, fruit, seed and flower has been already achieved by a number of workers (Vishwanathan & Basavaraju, 2010; Rana & Rana, 2014; Kamal *et al*., 2022) through *in vitro* regeneration of shoot using different explants like node and internode. But for large scale *in vitro* root production in *V. negundo* has not yet been reported in spite of being the source of most of the active compounds of pharmaceutical importance. *A. rhizogenes* interacts with plant cells through a defined segment of its large root inducing plasmid DNA (pRi T-DNA), which is conjugationally transferred into the plant genome and expressed. This results in the development of a tumors "hairy root" disease, distinguished by localized adventitious root proliferation at the infection site, and hiding two auxin biosynthetic genes (iaaM, iaaH). Under *in-vitro* culture, these genetically modified roots exhibit a rapid rate of growth along with abundant lateral branching, negative geotropism, cytogenetic stability, and sustained growth and biosynthetic potential in the medium without the need for growth regulator input (Sahu *et al*., 2015). In this context, *in vitro* root cultures in particular hairy root production could be the most appropriate alternative method which will be useful for secondary metabolites production in order to fulfill the requirement of drug designing and manufacturing. The advantages of production of secondary metabolites through hairy root culture also includefast growth, low doubling time, ease of maintenance of hairy roots, and their ability to synthesize a wide range of chemical compounds which could be a continuous source for the production of valuable secondary metabolites. To successfully induce and establish hairy root cultures of *V. negundo* using *A. rhizogenes*, various factors impacting genetic transformation must be meticulously evaluated and standardized. These include explant source and type, culture matrix, bacterial strains, bacterial cell density (by O.D. “Optical Density”), acetosyringone pre-culture, method of infection, infection time and duration of co-cultivation. Therefore, for the first time, efforts were made to optimize different factors to maximize the efficiency of the transformation and to develop a reproducible protocol for hairy root production in *V. negundo* which could serve as a feasible and sustainable source of pharmaceutically important phytochemicals.

2. MATERIAL and METHODS

2.1. *In vitro* Shoot Proliferation for *Agrobacterium rhizogenes* Infection

The *Vitex negundo* species was collected from Athagarh, Cuttack, Odisha, India and established in the experimental garden of Department of Botany, Ravenshaw University, Cuttack, Odisha (Figure 1 a). The *V. negundo* nodal explants were surface sterilized by washing with running tap water for 30 minutes, then treating with 2% Teepol (Reckitt Benckiser Ltd., India) for 10

minutes followed by 2% Bavistin (Antracol fungicide, Bayer, Crystal Crop Protection Ltd., India) treatment for 10 minutes. Finally, the explants were treated with 0.1% $HgCl_2$ (Merck, India) for 7 minutes as the last step of surface sterilization. For multiple shoot proliferation, the nodal explants were inoculated on different strengths of Murashige and Skoog's (1962) (MS) medium i.e., MS, ½ MS, ¼ MS, ⅛ MS either alone or, MS medium augmented with various concentrations (0.5 to 3.0 mg/L) of Benzylaminopurine (BAP; Hi Media, India). The well-developed *in vitro* shoots (Figure 1 b) were served as a source of *in vitro* explants (Leaf and Internode) for *A. rhizogenes* infection.

2.2. Maintenance of Bacterial Culture

The *A. rhizogenes* cultures MTCC 532 and MTCC 2364 (CSIR-IMTECH, MTCC, Chandigarh) were revived in nutrient broth (1.3 g/100 mL; Hi media, India) with a pH of 7.4±0.2. For bacterial growth, the temperature was set at 26±1°C for 24 to 48 hours inside incubator (Shaker and Incubator, N. Biotek, NB 205 QF). The O.D (Optical Density) of both bacterial cultures, MTCC 2364 and MTCC 532, maintained in between 0.02 and 0.8. after 24 to 48 hours of incubation were used for hairy root induction. After revival of both the strain in nutrient broth, the cultures were stored and maintained on Nutrient agar medium (1.3 g/100 mL nutrient broth and 1.8 g/100 mL bacteriological grade agar (Himedia, India) inside a refrigerator at 4-5°C for future use (Figure 1 e and f).

2.3. *Agrobacterium* Infection and Hairy Root Induction

2.3.1. *Transformation and hairy root induction in internodes*

For transformation and hairy root induction experiment, both *in vitro* and *in vivo* internodes were used. *In vitro* shoots from a 45 to 60-days-old culture were cut into small pieces (1.0 - 2.0 cm) and the excised *in vitro* internodes were punctured at one end. The punctured side was dipped in the bacterial suspension for different infection time 15-75 minutes. After the infection, the internodes were soaked with sterile tissue paper and inoculated on ½ MS and half MS + acetosyringone (19.6 mg/mL; Hi-Media; Figure 1 c) media by the other side of the piercing. These ½ MS media were gelled with 0.6% agar and pH was adjusted to 5.8±0.01. The co-cultivation time was optimized as 44-48 hours inside an incubator at 26°C. After 44-48 hours of co-cultivation, the internodes were transferred into a new flask with ½ MS + acetosyringone medium and kept in a culture room at 24°C under dark condition for hairy root induction. *Agrobacterium* infection in the *in vivo* internode was carried out, after surface sterilization, in the same way as *in vitro* internodes. The procedure of surface sterilization of the *in vivo* internode explant was carried out following the same procedure mentioned earlier for mature nodal explants.

2.3.2. *Transformation and hairy root induction in leaves*

Forty-five to sixty days old axenic shoot cultures were taken as the source for *in vitro* leaves. The leaves were cut into small segments (0.5 cm width ×1 cm length) and a sterile needle was used to pierce the mid rib and entire surface of the leaves. The pierced leaves were dipped in the bacterial suspension for 15-75 minutes. The leaves were dried with sterile tissue paper before being inoculated on ½ MS medium (Hi-Media, India; pH 5.8±0.01) and ½ MS + acetosyringone (Stock :196 mg/ 10 mL i.e., 0.1 M, Hi-Media, India; pH 5.8±0.01; Figure 1 d) media gelled with 0.6% agar. The co-cultivation time was optimized between 44-48 hours at 26°C inside an incubator. After 44-48 hours of co-cultivation, the leaves were transferred to fresh flasks containing ½ MS + acetosyringone (0.1 M) medium and kept in dark inside a culture room at 24°C. The *in vivo* leaves, after following the surface sterilization procedure were subjected to *A. rhizogenes* infection in the same way as *in vitro* leaves.

2.4. Statistical Analysis

All values are presented as the mean with three biological replicates. Mean values within column with different superscript alphabets are significantly different. Data were analyzed by analysis of variance (ANOVA) using Duncan's multiple range test ($p < 0.05$).

3. FINDINGS

3.1. *In vitro* Shoot Proliferation from *in vivo* Nodal Explants

Out of different strengths of MS basal media tried for *in vitro* shoot multiplication from *in vivo* nodal explants, the full-strength MS was found better in terms of shoot proliferation percentage, shoot number and shoot length whereas all these parameters were enhanced after the addition of BAP in different concentrations into the full-strength MS. The shoot regeneration from the nodal segments was observed after 7 days of inoculation of explants. Highest percentage of shoot regeneration (91.6) with highest number (8.1) of shoots with 8.33 cm average shoot length were recorded on MS medium augmented with BAP (2.0 mg/L) after 40-50 days of inoculation (Table 1).

Table 1. *In vitro* shoot proliferation from *in vivo* nodal explant of *Vitex negundo.*

Sl. No.	Media	Percentage of shoot proliferation	Mean shoot number	Mean shoot length (in cm)
1	⅛ MS	50.0^{f}	1.0^{ef}	0.38^{efg}
2	¼ MS	50.0^{f}	1.3^{e}	0.41^{ef}
3	½ MS	58.3^{de}	1.3^{e}	0.63^{def}
4	MS	62.5^{d}	1.5^{de}	0.88^{de}
5	MS + BAP (0.5mg/L)	62.5^{d}	1.6^{d}	1.51^{d}
6	MS + BAP (1.0mg/L)	66.6^{cd}	2.3^{cd}	2.16^{cd}
7	MS + BAP (1.5mg/L)	70.8^{c}	2.5^{c}	3.16^{c}
8	MS + BAP (2.0mg/L)	91.6^{a}	8.1^{a}	8.33^{a}
9	MS + BAP (3.0mg/L)	79.1^{b}	3.4^{b}	7.13^{ab}

Data pooled from 02 explants per flask, 04 flasks per replication and the experiments were repeated 03 times (2×4×3 =24). Each value is the mean from 3 replications. Mean values showed by different letters are significantly different at $p < 0.05$ (DMRT).

3.2. Induction of Hairy Roots

For *Agrobacterium* transformation, we tested four different explant types, including *in vivo* (leaf and internode) and *in vitro* (leaf and internode). Further, two different media types, including ½ MS without acetosyringone and ½ MS with acetosyringone, were employed for the inoculation after *Agrobacterium* infection. There was no rooting tendency in the two sets of control group i.e., *in-vivo* and *in-vitro* (internodes and leaves) explants of ½ MS and ½ MS with BAP medium, which were not infected by *A. rhizogenes*. However, only the *in vitro* (leaf and internode) inoculated on the ½ MS with acetosyringone medium showed positive response for hairy root induction.

3.2.1. *Hairy root induction from internode*

The *A. rhizogenes* strains MTCC 2364 and MTCC 532 were subjected for hairy root induction from both *in vivo* (mature) internode and *in vitro* internode, among which *in vitro* internode showed hairy root induction with MTCC 2364 strain. Different infection time (15, 30, 45, 60 and 75 minutes), O.D. values (0.042, 0.375 and 0.572) with co-cultivation time (44-48 hours) were examined for hairy root induction and the results were depicted in Table 2. The initiation of hairy root was observed after 15 days of infection whereas on the 30th day of observation i.e., with 30 minutes infection time 2.27 root numbers (1.66 cm), with 45 minutes infection time 1.5

root numbers (1.25 cm), 0.66 root numbers (0.83 cm) root length and with 60 minutes infection time 2.90 root numbers (2.92 cm) were observed. The rest of the explants of *in vitro* internode did not show any hairy root induction rather eventually all these explants became brown and died after few days of infection (Table 2). Overall, at O.D. value 0.042 with 60 minutes infection time and 44-48 hours co-cultivation period exhibited best results for hairy root induction (Table 2; Figure 1 g). Another strain MTCC 532 was not found effective with any type of explants tested for hairy root induction. It is noteworthy to explain that during co-cultivation period the explant inoculated on ½ MS medium fortified with acetosyringone only got success in hairy root transformation but explant inoculated on ½ MS without any addition of acetosyringone failed to respond.

Table 2. Factors affect hairy root induction in *in vitro* internode infected by MTCC 2364.

O.D.	Infection Time (in minutes)	Co-cultivation Period (in hours)	Average Hairy root induced (%)	Number of induced Hairy root (average)	Length of Hairy root (average)
0.042	15	44-48	0	0	0
	30		0	0	0
	45		22.21^{bc}	1.5^{abc}	1.25^{bc}
	60		66.65^{a}	2.90^{a}	2.92^{a}
	75		0	0	0
0.375	15	44-48	0	0	0
	30		38.88^{b}	2.27^{ab}	1.66^{b}
	45		0	0	0
	60		0	0	0
	75		0	0	0
0.572	15	44-48	0	0	0
	30		0	0	0
	45		11.10^{d}	0.66^{bc}	0.83^{c}
	60		0	0	0
	75		0	0	0

Data pooled from 03 explants per flask, 02 flasks per replication and the experiment repeated 03 times (3*2*3=18). Each value is the mean from 3 replications. Mean values showed by different letters are significantly different at $p < 0.05$ (DMRT).

3.2.2. *Hairy root induction from leaves*

The *A. rhizogenes* strains MTCC 2364 and MTCC 532 were also used for infection of both matured *in vivo* leaves and *in vitro* leaves, among which *in vitro* leaves showed hairy root induction with MTCC 2364 strain. The other strain MTCC 532 was not found effective for hairy root induction in both *in vivo* and *in vitro* leaf. Like the case of internode, during co-cultivation period the leaf explant inoculated on ½ MS medium fortified with acetosyringone only resulted in production of hairy roots but ½ MS without supplemented with acetosyringone could not be able to develop a single hairy root from either type of leaves. Different infection time (15, 30, 45, 60 and 75 minutes) at different O.D. (0.086, 0.290 and 0.375) with co-cultivation period of 44-48 hours were examined for hairy root induction (Table 3). The initiation of hairy root was observed from midrib and side cut of infected *in vitro* leaves after 15 days of infection (Figure 1 h). Overall, at O.D. value 0.290 with 45 minutes infection time and 44-48 hours co-cultivation period using MTCC 2364 strain exhibited best results (approx. 89%, 3.1 hairy roots with a length of 2.94 cm) for hairy root induction (Table 2). The *in vivo* leaves failed to show any hairy root induction, rather they eventually became brown in colour and became necrotic (Table 3).

Table 3. Factors influence hairy root induction in *in vitro* leaves infected by MTCC 2364.

O.D.	Infection Time (in minutes)	Co-cultivation Period (in hours)	Average Hairy root induced (%)	Number of induced Hairy root (average)	Length of Hairy root (average)
0.086	15	44-48	0	0	0
	30		61.10^{c}	2.1^{bc}	1.97^{bc}
	45		77.75^{abc}	2.61^{ab}	2.66^{abc}
	60		49.99^{d}	1.83^{d}	1.77^{d}
	75		0	0	0
0.290	15	44-48	0	0	0
	30		0	0	0
	45		83.32^{ab}	2.55^{abc}	2.78^{ab}
	60		88.86^{a}	3.1^{a}	2.94^{a}
	75		22.21^{g}	1.83^{d}	1.55^{de}
0.375	15	44-48	16.66^{gh}	0.83^{g}	0.62^{f}
	30		38.88^{ef}	1.36^{ef}	1.97^{bc}
	45		0	0	0
	60		0	0	0
	75		0	0	0

Data pooled from 03 explants per flask, 02 flasks per replication and the experiment repeated 03 times (3*2*3=18). Each value is the mean from 3 replications. Mean values showed by different letters are significantly different at $p < 0.05$ (DMRT).

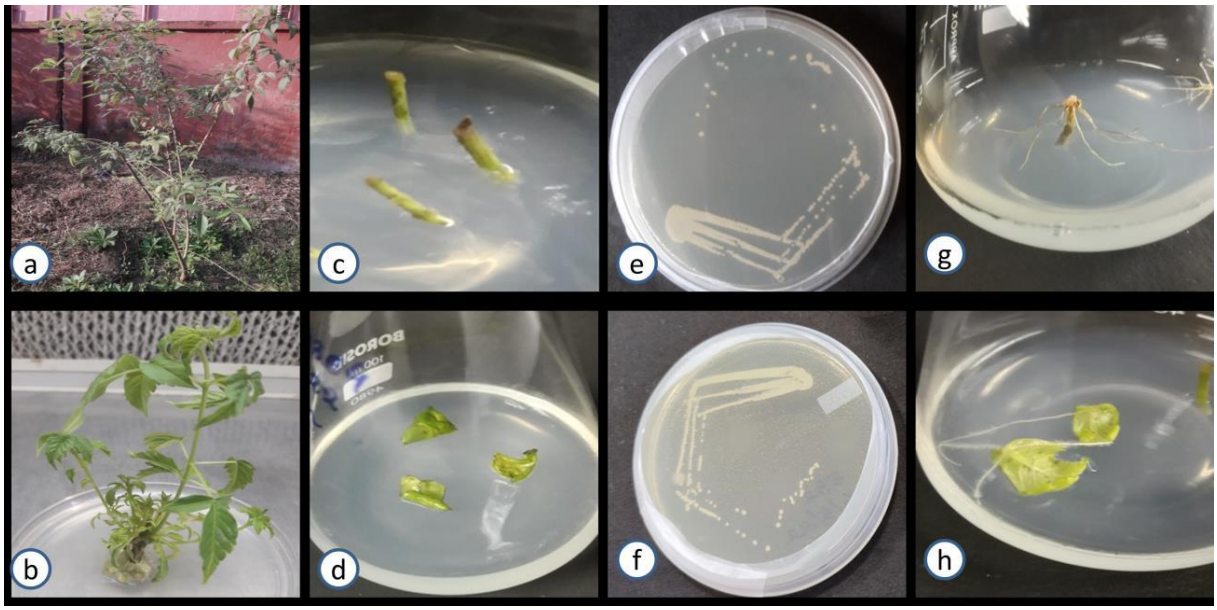

Figure 1. a: *In-vivo Vitex negundo* plant in the garden, **b:** *In-vitro* shoot culture in MS with BAP (2 mg/L) media, **c:** *In-vitro* internode infected by MTCC 2364 before hairy root induction, **d:** *In-vitro* leaves infected by MTCC 2364 before hairy root induction, **e:** MTCC 2364 grow in petriplate, **f:** MTCC 532 grow in petriplate, **g:** Hairy root induction from *in-vitro* internodes infected by MTCC 2364, **h:** Hairy root induction from *in-vitro* leaves infected by MTCC 2364.

4. DISCUSSION and CONCLUSION

Out of the two strains namely, MTCC 2364 and MTCC 532 employed in this hairy root induction experiment, the MTCC 2364 strain of *A. rhizogenes* was found effective for hairy roots induction, while the MTCC 532 strain was found ineffective in our experiment. Similar to this study, other researchers Muthiah *et al*. (2016) (*Bacopa monnieri*), Bathoju *et al*. (2017) (*Chlorophytum borivilianum*), and Bhagat *et al*. (2019) (*Rauwolfia serpentina*) also used these two types of strains for hairy root induction. However, in contrast to our study, in all these experiments, although MTCC 2364 was found to be effective for hairy root induction but the

strain MTCC 532 was more effective in term of percentage of hairy root induction, biomass, number and length of hairy root. On the other hand, MTCC 532 also works effectively for hairy root cultures of *Plumbago rosea*, *Rubia tinctorum*, *Arachis hypogaea*, and *Withania somnifera* (Brijwal & Tamta, 2015).

Different types of explants, *in vivo* leaf, internode, *in vitro* leaf and internode were used in this experiment, out of which, only *in vitro* leaf and *in vitro* internode explants responded to the induction of hairy roots. Of the two *in vitro* explants, the *in vitro* leaf performed better than the *in vitro* internode. Jesudass *et al*. (2020) (*Cucumis anguria*) also reported about the susceptibility of *in vitro* explant towards the infection of *Agrobacterium* strain which agrees with this result. Contrary to our result, Bhagat *et al*. (2019) got success in hairy root transformation in *R. serpentina in vivo* leaf explants. Swain *et al*. (2010) also proposed that stem internodes are significantly more sensitive than leaf explants, irrespective of the *Agrobacterium* strains used. But many of the researchers like Sajjalaguddam *et al*. (2016) (*Abrus precatorius*) and Bathoju *et al*. (2017) (*Chlorophytum borivilianum*) used *in vitro* explants like germinated seedling and shoot base respectively for hairy root induction experiment.

The O.D. value plays a crucial role for transformation and hairy root induction. Multiple investigations have reported that the *A. rhizogenes* and their densities have an impact on the rate of hairy root induction during transformation (Kumar *et al*., 2006; Shahabzadeh *et al*., 2014). All strains considerably enhanced the transformation frequency after identifying the best inducing O.D. (Majumdar *et al*., 2011). In our investigation, the best O.D. value at $O.D_{600}$ of the MTCC 2364 is 0.29 and 0.042 for the hairy root induction in *in vitro* leaf and internode respectively. Like our experiment different workers reported about different O.D. values of *A. rhizogenes* for optimum result for transformation. In *R. serpentina*, both MTCC 532 and MTCC 2364 showed 31% and 24% hairy root induction respectively at 0.6 optical densities of *A. rhizogenes* culture (Bhagat *et al*., 2019). According to the findings of Mahendran *et al*. (2022), different strains had different optimal induction concentrations. Further, lower bacterial concentrations resulted in lower transformation frequencies, whereas bacterial densities above the O.D. value 1.5 resulted in lower transformation rates due to severe necrosis. A higher cell density increased the T-DNA adherence to host cells, which resulted in browning of explants and a decrease in the frequency of hairy root induction.

In this experiment, we have tried some variable time (i.e., 40-44, 44-48, 48-52, 52-56 hours) of co-cultivation period whereas, the effective co-cultivation duration was standardised as 44-48 hours for hairy root induction. In corroboration to our result, Giri *et al*. (2001) as well as Brijwal and Tamta, (2015) also suggested about the 48 hours of co-cultivation duration as beneficial for hairy root induction for *Artemisia annua* and *Berberis aristate* respectively. Further, 48 hours of co-cultivation time was suggested by Srinivasan *et al*. (2023) for hairy root development in *Aerva javanica* by five different bacterial strains (ATCC 15834, R1000, LBA 9204, MTCC 2364, and MTCC 532).

Different infection times like 15, 30, 45, 60, and 75 minutes were studied and found that 60 minutes of infection time gave the best results for our explants; however, increasing the infection time up to 75 minutes in both *in vitro* leaf and *in vitro* internode decreased the rate of hairy root induction. The finding of Orlikowska *et al*. (1995) in the explants of safflower cv. and Kumar *et al*. (2023) in Plumbago zeylanica 'Centennial' reported a similar trend like our experiment. In *Carthamus tinctorius*, 30 minutes infection time was found suitable for higher transformation efficiency however, increasing the infection time up to 45 min caused a drop in effectiveness, which may be related to the explants' hypersensitive reaction to bacteria.

Gelvin (2000) explained that acetosyringone enhances the frequency of transformation of infected explants by activating *vir* genes. According to Brijwal & Tamta (2015), acetosyringone

in co-cultivation media not only enhanced explant transformation but also reduced the time needed for hairy root induction in contrast with acetosyringone free medium. They reported that in *Berberis aristata*, acetosyringone supplemented medium showed 72.22% transformation frequency whereas, acetosyringone free medium showed 61.11%. In this present hairy root experiment, ½ MS supplemented with acetosyringone (AS; 19.6 mg/mL) media effectively induces hairy roots whereas no hairy roots were observed from any type of the explants inoculated on ½ MS without addition of acetosyringone. Bhagat *et al*. (2019) and Moola *et al*. (2022) also suggested about the addition of acetosyringone in 125 µM for *Rauwolfia serpentina* and 100 µM for *Celastrus paniculatus* respectively for hairy root induction. However, Godwin *et al*. (1991) found that differences in transformation efficiency at various acetosyringone concentrations are significantly influenced by plant genotype and bacterial strains.

A protocol for hairy root transformation of *V. negundo* using *A. rhizogenes* strains MTCC 2364 was established. The *in vitro* leaf was found as most suitable explant for hairy root induction. However, this is a preliminary work and further research is required for molecular conformation analysis of transformed root, hairy root biomass enhancement, parameters optimization for production and enhancement of bioactive secondary metabolites of particular interest as well as designing of bioreactor for up-scaling of hairy roots of *V. negundo*. As *V. negundo* is a significant medicinal plant, hairy root cultures might be considered as a useful system for large-scale production of important secondary metabolites in general and those are located in the root in particular.

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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

Bhaswatimayee Mahakur: Experimental designing, data recording and writing-original draft. **Arpita Moharana**: Helps in writing and data analysis. **Sanjay K. Madkami**: Helps in DMRT data analysis. **Soumendra K. Naik**: Reviewing and supervision. **Durga P. Barik**: Supervision and Validation of data.

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Research Article

Antimicrobial, antioxidant and essential oil studies on *Veratrum album* L. (Melanthiaceae)

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Abstract: In this study, essential oil components of the *Veratrum album* L. and the antimicrobial and antioxidant properties of these components were determined. The chemical composition of the essential oils of dried aerial parts of *V. album* was analyzed using GC and GC-MS. Antimicrobial activity was determined with the disk diffusion method. Total antioxidant status (TAS), total oxidant status (TOS) and 2.2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity were detected for the antioxidant activity of the plant. According to the analysis results, the major essential oil components of the *V. album* were determined as hexacosane (39.5%), myristic (tetradecanoic) acid (22.8%), heptane (6.5%), anethole (4.9%) and 1,8- cineole (4.8%). The findings showed that the methanol extracts of the stem and leaf parts of the plant inhibited the growth of pathogenic microorganisms at different rates (14±0.1 - 34±0.3 mm). The TAS values of methanol extracts of stem and leaf parts of *V. album* were calculated as 3.75±0.07 and 3.91±0.01 mmol, while TOS values were calculated as 6.14±0.13 and 6.54±0.05 µmol. The scavenging activity of the DPPH radical increased depending on increasing concentrations of the plant extract.

1. INTRODUCTION

Plants have continued to play a dominant role in the protection of human health and to be an important alternative treatment method to alleviate the symptoms of diseases since ancient times due to various active substances they contain. Today, with the renewed interest in traditional medicine and the need and demand for more herbal medicines, the importance of studies with medicinal plants has increased even more. This revival of interest in plant-derived medicines stems from the current widespread belief that "green medicine" is safe and more reliable than expensive synthetic drugs, many of which have negative side effects (Nair & Chanda 2007). This has accelerated the search for new antimicrobial agents from various sources, such as medicinal plants (Cordell, 2000). Synthetic drugs are not only expensive and inadequate for the treatment of diseases, but also often have adulteration and side effects.

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Therefore, there is a need to investigate new infection control strategies (Sieradzki *et al.*, 1999; Dabur *et al.*, 2007).

Although the genus *Veratrum* is included in the Liliaceae family in the Flora of Türkiye (Edmondson, 1984), it has been included in the Melanthiaceae family in recent years with systematic and molecular studies (Reveal & Chase 2011; Seberg *et al.*, 2012). The genus *Veratrum*, the largest genus of the Melanthieae tribe is represented by 45 species globally and only one species in Türkiye. Zomlefer *et al.*, (2001; 2003) divided the genus *Veratrum* into two sections; *Veratrum* and *Fuscoveratrum; Fuscoveratrum* has two subsections: *Pseudoanticlea* and *Asiaveratrum*. The phylogenetic study of flower colors by Liao *et al.*, (2007) also supports this grouping. Zomlefer *et al.*, (2001; 2003) also examined the tepal shapes of the species, the nectariums in the tepals and the characteristics of the seeds in detail and evaluated them with the results of the molecular study. The most common *Veratrum* species in the world; *V. album* and *V. nigrum* are distributed in Central, Southern and Southeastern Europe. Species of the genus have a wide distribution range, mostly from temperate North America to Arctic Eurasia. The origin and largest center of diversity of the genus *Veratrum* is East Asia. About 20 of the *Veratrum* species grow naturally in China. Thus, it is very well known and widely used in traditional medicine in China. Medicines prepared from the dry stems and rhizomes of *Veratrum* species are known as "Li-lu" in Chinese sources (Atalay, 2013).

In ancient Chinese medical sources, Li-lu is mentioned as an herb that treats ailments, such as high blood pressure, inflammation, coagulation and spasm (Hollman, 2003; Wen *et al.*, 2005; Tang *et al.*, 2010). Among Indians and pre-industrial Europeans, *Veratrum* species have been used in the treatment of various diseases, including cough, sore throat, tonsillitis, mental illnesses, epilepsy, jaundice, scabies, bacterial infections, snake bites, venereal diseases and injuries (Li *et al.*, 2006; Li *et al.*, 2007; Tanaka *et al.*, 2011). *Veratrum* species are known by local names, such as “hellebore”, “American litter”, “false litter” and “white litter” (Li *et al.*, 2007). The pharmacological activities of *Veratrum* species have attracted attention for over 300 years. Extracts obtained from *Veratrum* species have been used as an insecticide against some harmful insect species until the 1950s (Jacobson, 1958). Thus, phytochemical studies have been conducted on *Veratrum* species since the 1930s, and over 100 alkaloid-type metabolites with different pharmacological properties have been identified (Rahman *et al.*, 1992). In addition to alkaloids, *Veratrum* species have also been reported to contain flavonoids and stilbenoids (Dai *et al.*, 2009; Hanawa *et al.*, 1992; Zhou *et al.*, 1999; 2001; Huang *et al.*, 2008). Among these, the metabolites with the highest biological activity were steroidal alkaloids (Ivanova *et al.*, 2011; Rahman *et al.*, 1992; Sener *et al.*, 1996; Zhou *et al.*, 1999; Zhu *et al.*, 2011). Exposure to alkaloids of the plant *Veratrum* causes similar toxic effects in animals and humans. The symptoms observed with exposure to *Veratrum* alkaloids include hypotension, vomiting (for species with this reflex), salivation, weakness, irregular pulse and slow breathing (Krayer & Acheson, 1946; Mulligan & Munro, 1987; Swiss & Bauer, 1951).

In line with these scientific studies in the literature, the present study aimed to investigate the antimicrobial and antioxidant activities and the mechanism of action of the extracts obtained from the stem and leaves of *Veratrum album*, about which we have little information on its use in folk medicine, despite being widely distributed in Türkiye. In addition, essential oil characterization of the extracts was performed.

2. MATERIAL and METHODS

2.1. Collection of the plant material

The plant material used in the present research was collected from the Kabaca plateau, Okuzyatagi locality in Artvin province (Lat: 41°7’58.019” N, Lon: 41°31’31.899” E, Alt: 2313 m). In addition, the general view of the plant is shown in Figure 1. It was collected from its

natural habitat during its vegetative period in June 2021, and dried in the shade. For this purpose, the plant specimens collected from nature were brought to the laboratory environment in cloth bags with frequent ventilation. Then, they were laid on blotting paper in a sun-free environment and allowed to dry in the shade. Most of the dried samples were used for essential oil studies, while a small amount of samples were used for antimicrobial and antioxidant studies. A group of plant specimens, which were turned into herbarium material, are kept in the Herbarium (FUH) of the Faculty of Science, Fırat University.

Figure 1. General appearance of *Veratrum album*.

2.2. Isolation of the Essential Oils

Air-dried aerial parts (stem and leaves) of the plant materials (250 g) were subjected to hydrodistillation using a Clevenger-type apparatus for five hours to yield essential oils. The obtained essential oil was analyzed using GC and GC-MS. A Shimadzu GC-MS (QP2020 model) with an FID detector was used. The system was equipped with a RXI-5MS (30m x 0.25 mm x 0.25 µm) capillary column through which helium was flowing as carrier gas. The column oven temperature program was as follows, the temperature was set to 40ºC and held for 2 min then heated to the final temperature of 240ºC at a ramp rate of 3ºC/min. The injection volume was selected as 1 µL in the split mode (This process was repeated two times). The column and analysis conditions were the same as in GC-MS, expressed above. The percentage composition of the essential oils was computed from GC-FID peak areas without correction factors. The MS results were compared with the Wiley-Nist W9N11 libraries in the device memory (Table 1).

2.3. Determination of Antimicrobial Effect

2.3.1. Test microorganisms

In this study, *Escherichia coli* ATCC25322, *Staphylococcus aureus* ATCC25923, *Klebsiella pneumoniae* ATCC 700603, *Bacillus megaterium* DSM32, *Candida albicans* FMC17, *Candida glabrata* ATCC 66032, *Trichophyton* sp. and *Epidermophyton* sp. microorganisms were used as test microorganisms. All microorganism cultures were obtained from Firat University, Faculty of Science, Department of Biology, Microbiology Laboratory culture collection.

2.3.2. Preparation of microorganism cultures and testing for antimicrobial effect

The antimicrobial activity of the plant extracts in methanol was determined according to the disk diffusion method (Collins & Lyne 2004). Bacteria strains (*E. coli*, *S aureus*, *K. pneumoniae*, *B. megaterium*) were incubated in Nutrient Broth (Difco) for 24 hours at 35 ± 1°C, yeast strains (*C. albicans* and *C. glabrata*) were incubated in Malt Extract Broth (Difco) for 48 hours at 25 ± 1°C and dermatophyte fungi (*Trichophyton* sp. and *Epidermophyton* sp.) were inoculated in Glucose Sabouroud Buyyon (Difco) and incubated at 25 ± 1°C for 48 hours. The bacteria, yeast and fungi cultures prepared in broth were inoculated into Mueller Hinton Agar, Sabouraud Dextrose Agar and Potato Dextrose Agar, respectively, at a rate of 1% (10^6 bacteria ml, 10^4 yeast ml, 10^4 fungi ml). After shaking well, 25 ml of the cultures were placed in sterile petri dishes of 9 cm diameter. Homogeneous distribution of the medium was achieved. Antimicrobial discs (Oxoid) of 6 mm diameter, each impregnated with 100 µL (1000 µg) extracts, were lightly placed on the solidified agar medium. After keeping the petri dishes prepared in this way at 4°C for 1.5-2 hours, the plates inoculated with bacteria were incubated at 37 ± 0.1°C for 24 hours, and the plates inoculated with yeast at 25 ± 0.1°C for 72 hours. As controls, different standard discs were used for bacteria (Streptomycin sulfate 10 µg/disc) and yeasts (Nystatin 30 µg/disc). Dimethyl sulfoxide (DMSO) was used for negative control. The zones of inhibition were measured in mm.

2.4. Determination of Antioxidant Activity

2.4.1. Total antioxidant assay (TAS) and total oxidant assay (TOS)

Total antioxidant (TAS) and total oxidant status (TOS) of the plant extracts were determined with Rel Assay kits (Rel Assay Kit Diagnostics, Türkiye). The TAS value was expressed as mmol Trolox equiv./L and Trolox was used as the calibrator (Erel, 2004). The TOS value was expressed as µmol H_2O_2 equiv./L and hydrogen peroxide was used as the calibrator (Erel, 2005).

2.4.2. 2.2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity

The antioxidant activity of the methanol extract of the plant extracts was determined according to the 2.2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity method (Cuendet *et al.*, 1997). The solution was prepared in methanol at a concentration of 25 mg/ml of the extract obtained. The prepared solution was diluted four times and the calibration curve of DPPH was obtained. For this purpose, 40 µL of the prepared solution was taken and added with 160 µL of DPPH solution. After thorough mixing, the vial was closed and kept in the dark for 30 minutes. The same procedures were repeated for all concentrations, and butylated hydroxyanisole (BHA) and methanol was used as a control. Afterwards, the absorbance of each mixture was read at 570 nm in the spectrophotometer. The percentage inhibition values were calculated according to the following equation:

$$I\ (\%) = (A_{control} - A_{sample}/A_{control}) \times 100$$

2.5. Statistical Analyses

Data were presented as mean ± standard deviation (SD) based on three replicates, and the significant difference ($p<0.05$) was determined by independent t-test, one-way and two-way analysis of variance with Duncan's test using SPSS v25.0.

3. RESULTS

The composition of the aerial parts essential oils of *V. album* was analyzed and a total of 21 compounds were identified. According to the results of the analysis, hexacosane (39.5%), myristic (tetradecanoic) acid (22.8%), heptane (6.5%), anethole (4.9%) and 1,8- cineole (4.8%) were the major components. The hydrodistillation of the aerial parts of *V. album* yielded 0.12 % of light yellowish oil. Table 1 lists the components identified from *V. album* corresponding to 87.5 % of the total essential oil and their retention index and percentage composition.

Table 1. Constituents of the essential oils from *V. album*.

No	RT	RI	Area	Name	Aerial parts %
1	5.1	930	48718	α-thujene	1.2
2	5.6	935	14757	α-pinene	0.1
3	5.7	960	58489	β-pinene	1.7
4	5.8	1017	52298	p-cymene	0.9
5	**5.9**	**1033**	**65509**	**1,8-cineole**	**4.8**
6	**6.0**	**1208**	**534856**	**Anethole**	**4.9**
7	62.9	1228	175344	Furan, 2-pentyl-	2.7
8	9.4	1280	55310	2-Heptanone	1.2
9	**9.9**	**1286**	**73828**	**Heptanal**	**6.5**
10	5.9	1404	25972	α-Ionone	2.3
11	8.6	1480	112848	1-Hexanol	0.6
12	12.2	1565	45719	Hydroperoxide, 1-ethylbutyl	0.4
13	12.4	1620	96863	Undecanone	1.0
14	61.9	1680	30506	2,3-Octanedione	0.6
15	10.3	2000	68987	Linoleic acid ethyl ester	0.4
16	12.6	2020	29708	Benzaldehyde	0.2
17	**55.5**	**2476**	**2281061**	**Hexacosane**	**39.5**
18	13.6	2508	43030	Dodecanoic acid	0.9
19	**58.6**	**2680**	**698636**	**Myristic acid**	**22.8**
20	63.4	2740	827808	Pentacosane	1.2
21	64.0	2798	182118	Heptacosane	1.7
				Total	87.5

** RI: retention indices; RT: retention time

The antimicrobial effects of methanol extracts of stem and leaf parts of *V. album* are seen in Table 2. In the results obtained, the antimicrobial effects of the stem and leaf parts of *V. album* against *E. coli*, *S. aureus*, *K. pneumoniae*, *B. megaterium*, *C. albicans*, *C. glabrata*, *Trichophyton* sp., *Epidermophyton* sp. were determined as 29 ±0.5 mm, 20±0.1 mm, 25±0.1 mm, 23±0.2 mm, 25±0.3 mm, 27±0.5 mm, 22±0.1 mm and 15±0.5 mm, respectively. Inhibition zones of the leaf part against *E. coli*, *S. aureus*, *K. pneumoniae*, *B. megaterium*, *C. albicans*, *C. glabrata*, *Trichophyton* sp., *Epidermophyton* sp. were detected as 34±0.3 mm, 25±0.5 mm, 24±0.6 mm, 23±0.0 mm, 19.7±0.6 mm, 25±0.2 mm, 21±0.5 mm and 14±0.1 mm, respectively (Table 2).

Antimicrobial effects of Streptomycin sulfate used as a control against *E. coli*, *S. aureus*, *K. pneumoniae* and *B. megaterium* ranged from 19±0.1 to 30±0.4 mm. The antimicrobial effects of Nystatin against *C. albicans*, *C. glabrata*, *Trichophyton* sp., *Epidermophyton* sp. were determined in the range of 20±0.2 - 25±0.7 mm (Table 2). The effects of extracts prepared from stem and leaf parts on some bacteria, yeast and dermatophyte species were found to be statistically significant ($p<0.05$). Extracts prepared from leaf parts showed a higher effect on *E. coli* and *S. aureus* when compared to extracts prepared from stem parts, while antimicrobial effects on other pathogenic microorganisms were found to be low. In addition, it was observed that the extracts obtained from both stem and leaf parts were more effective on *K. pneumoniae* and *C. glabrata* when compared to the control group (Table 2).

Table 2. Antimicrobial effect of *V. album.*

Microorganism	Stem	Leaf	Control	*F*	*p*
Escherichia coli	29.0±0.5 ^{fx}	34.0±0.3 ^{gy}	30.0±0.4 ^{ez}	*126.0*	*0.0*
Staphylococcus aureus	20.0±1.0 ^{bx}	25.0±0.5 ^{fy}	20.0±0.3 ^{bx}	*55.9*	*0.0*
Klebsiella pneumoniae	25.0±1.0 ^{dx}	24.0±0.6 ^{ex}	19.0±0.1 ^{ay}	*67.8*	*0.0*
Bacillus megaterium	23.0±0.2 ^{cx}	23.0±0.0 ^{dx}	25.0±0.0 ^{dy}	*300.0*	*0.0*
Candida albicans	25.0±0.3 ^{dx}	19.7±0.6 ^{by}	25.0±0.4 ^{dx}	*150.3*	*0.0*
Candida glabrata	27.0±0.5 ^{ex}	25.0±0.2 ^{fy}	20.0±0.2 ^{bz}	*354.5*	*0.0*
Trichophyton sp.	22.0±1.0 ^{cx}	21.0±0.5 ^{cx}	22.0±0.1 ^{cx}	*2.3*	*0.1*
Epidermophyton sp.	15.0±0.5 ^{ax}	14.0±1.0 ^{ax}	25.0±0.7 ^{dy}	*191.3*	*0.0*
F-value	117.9	336.8	341.0		
p-value	0.00	0.00	0.00		

Streptomycin sulfate (10 mg/disc) and Nystatin *(30 mg/disc) were used as standard antibiotic discs. The diameter of the paper discs was 6 mm.

The letters $^{a-g}$ indicate the comparisons in each column, and $^{x-z}$ the comparisons between the rows. Values with the same letters are not different from each other. Each value is expressed as the mean ± SD of three replicates (n=3, $p<0.05$)

TAS and TOS values of leaf parts were found to be higher than stem parts. In the results obtained, the TAS value of the stem and leaf parts of *V. album* was very high, and the TOS value was between normal values (Table 3).

Table 3. TAS and TOS values of parts of *V. album.*

	TAS (mmol Trolox equiv./L)	TOS (μmol H_2O_2 equiv./L)
Stem	3.8±0.7	6.2±0.1
Leaf	3.9±0.1	6.5±0.5
p-value	0.023	0.008

The inhibition percentages of DPPH radical scavenging activity of different concentrations of methanol extracts of stem and leaf parts of *V. album* were determined (Table 4). It was observed that the DPPH radical scavenging activity of stem parts of *V. album* was above 50% at 1000 µg/mL concentration (58.7±0.2) but below 50% at other concentrations. While the DPPH radical scavenging effect of BHA used as positive control was determined as 83.7±0.3%, the DPPH radical scavenging effect of methanol used as negative control was determined as 1.7±0.7%. The findings showed that the percentage of inhibition of the scavenging activity of DPPH radical in all concentrations of the leaf part of the same species was over 50%.

When the obtained results were compared with the controls, it was determined that the closest antioxidant effect to the control was in the methanol extract of the leaf part of *V. album* at 1000 µg concentration (75.5±0.4) (Table 4).

Table 4. Percent inhibition of the DPPH radical of stem and leaf parts of *V. album.*

Concentration	Stem	Leaf
1000 µg/mL	58.7±0.2^{a}	75.5±0.4^{x}
500 µg/mL	38.0±0.8^{b}	64.5±0.4^{y}
250 µg/mL	19.2±0.3^{c}	53.1±0.3^{z}
125 µg/mL	9.6±0.0^{d}	47.3±0.4^{t}
BHA (control I)	83.7±0.3^{e}	
MetOH (control II)	1.7±0.2^{f}	
F-value	20624.6	22928.8
p-value	0.00	0.00

Each value is expressed as the mean ± *SD* of three replicates (n=3, $p<0.05$)

The letters $^{a-f}$ indicate the comparisons in each column, and $^{x-t}$ the comparisons between the rows.

DPPH effects of different concentrations (125-1000 µg/mL) prepared from stem and leaf parts were found to be statistically significant (p<0.05). In addition, it was observed that the mean DPPH values of stem and leaf were statistically different for each concentration value (p<0.05). Depend on increasing concentrations, DPPH values of stem and leaf parts were found to be low when compared to control I (BHA). In terms of DPPH values, it was observed that the values obtained from the leaf were higher than the stem parts (Table 4).

4. DISCUSSION and CONCLUSION

The essential oil components of *V. album* are shown in Table 1. To our best knowledge, the essential oil components of this species were determined for the first time in this study. The essential oils isolated were a complex mixture of monoterpenes, sesquiterpenes and hydrocarbon. On the other hand, it was determined that hydrocarbons made up the higher contribution in *V. album* essential oil. Tabanca *et al.*, (2018) reported that when the active fractions of *V. lobelianum extracts* were further analyzed by GC-MS, ethyl palmitate and ethyl linoleate were identified. The mass spectra and linear retention indices (LRI) values that they determined were comparable with purchased authentic compounds of ethyl palmitate and ethyl linoleate (Tabanca *et al.*, 2018). In our study, the ethyl linoleate (Linoleic acid ethyl ester) content was 0.4%.

The reason for this variability in the composition of essential oils can be attributed to the differences in the geographical regions from which the species are collected and methodological conditions used. The extract of this plant is used in the treatment of various diseases in different countries (URL-1; URL-2). However, the essential oil content of the sample grown in the environment and conditions of our country was revealed for the first time by us in this study. Our aim is to determine the essential oil content of the sample grown in Türkiye in the first place. The environment and seasonal conditions in which the plant grows will of course affect the essential oil content. In addition, the essential oil contained in the plant can differ qualitatively and quantitatively in different phenological periods (vegetative, flowering and fruiting) of the plant. However, in order to standardize this content, it is necessary to determine the essential oil composition of the plant and to determine whether it contains valuable components. Based on the results obtained from this study, we can say that the *V. album* plant has very valuable components and the plant in question can be grown and produced consistently in a greenhouse environment created by imitating its natural habitat. Therefore, this study constitutes a basis for more comprehensive studies.

In another study conducted by Lin *et al.*, (2003) volatile oil of *Hemerocallis flava*, which for close *V. album* species, was obtained by simultaneous distillation–solvent extraction. Afterwards, the essential oil was analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) and 51 components were identified, constituting approximately 92% of the oil. The main components of the essential oil were 3-furanmethanol (47.9%), 2-furancarboxaldehyde (10.4%) and Furan, 2-pentyl (2.7%). When comparing the essential oil composition of the genus *Veratrum* with Hemerocallis genus studies, some similarity was found to be evident (Lin *et al.*, 2003).

It has been determined that the rhizome parts of *V. album* show antiviral effect against HSV at a concentration of 250 µg/mL, and against SINV virus at a concentration of 500 µg/mL (Hudson *et al.*, 2000). It has been reported that the ethanol extract of the same species inhibited the growth of *Mycobacterium tuberculosis* at concentrations lower than 100 µg/mL (Tosun *et al.*, 2005). The antimicrobial effects of *Veratrum* alkaloids against *P. ovale*, *T. mentagrophytes* and *S. cerevisiae* have been tested and found that only jerveratrum alkaloids showed antimicrobial activity (Wolters, 1970) Alkaloids obtained from the *V. album* have antioxidant effects (Atalay *et al.*, 2019).

In this study, hexacosane found at a high rate is a substance with antimicrobial properties. According to a study in which the inhibition zones of isolated terpenoids have also been recorded, the results indicated that hexacosane was more effective against *E. coli* and hexacosanoic acid had a greater activity against *A. flavus* (Singh & Singh 2003). Another study investigating the antimicrobial effect of hexacosane has reported that it was a highly effective compound with inhibition zone of 29, 27, 26 and 25 cm against *Klebsiella pneumoniae, Salmonella typhi, Mithecithinne staphaureus* and *Proteus vulgaris*, respectively (Rukaiyat *et al.*, 2015). These results are consistent with the findings obtained in the present study.

Veratrum is one of the most critical genera that are rich in a pharmaceutical alkaloids worldwide. This study showed that the essential oil obtained from *V. album* might have a potential to be used in subsequent pharmacological and biological screening tests. In addition, it is thought that such studies will be useful in comparing essential oil compositions and providing basic data for taxonomic and essential oil evaluation studies of the genus, and contribute scientific agriculture and product diversity as well as various industries including medicine, cosmetics, landscaping, flavor and food.

In the present study, the leaf parts of the *V. album* showed a better antimicrobial effect against bacteria, while the stem parts showed a better antimicrobial effect against yeast and dermatophyte fungi. The antioxidant activity of the leaf parts of the plant was better. Especially the plant's leaf parts extract showed a better effect at increased concentrations. We think that the antimicrobial and antioxidant effects of the plant are due to the alkaloids in its structure. In particular the antimicrobial and antioxidant effects of alkaloids called jervine have been reported in previous studies (Wolters, 1970; Atalay *et al.*, 2019). However, biological studies on the species are limited in the literature, and the results of this study provide valuable insights into the literature.

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

Pelin Yılmaz Sancar: Investigation, Resources, Visualization, Formal Analysis and Writing-original draft. **Şule İnci:** Antimicrobial and Antioxidant studies, Writing-original draft. **Azize Demirpolat:** Essential oil studies, Writing-original draft. **Sevda Kırbağ:** Antimicrobial-Antioxidant studies, Statistical analysis, Supervision, Validation and Writing-original draft. **Şemsettin Civelek:** Supervision, Validation and Writing-original draft.

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Research Article

Investigation of Sterol structures and biological activities in Cochineal and *Hibiscus sabdariffa* extracts

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Abstract: In the future, it is necessary to discover natural resources with no or less toxicity and side effects instead of synthetic drugs. Therefore, it is crucial to recognize, isolate, measure, and reveal the biological activities of substances in the structure of natural resources. In this study, the two extracts prepared from the plant (*Hibiscus sabdariffa)* and the insect (*Dactylopius coccus)* were examined. Sampling of the released substances was performed using Gas-Chromatography-Mass Spectrometry (GC-MS). In addition, viability, apoptosis, and oxidative stress of the derivatized samples were determined. Due to the biological differences between the samples, the chemical structures observed in the GC-MS analysis were not the same. It was observed that stigmasterol and its derivatives were found in the pomegranate sample, whereas naphthol and its derivatives were more abundant in the Cochineal insect sample. The cell viability increased with increasing concentrations of stigmasterol, which is present in large amounts in the structure of the pomegranate flower. However, the cell viability decreased with the Cochineal insect sample. Apoptosis and oxidative stress test results were also found to be different and contrasted for both samples. Therefore, the present study presents a new, natural, and different source that can be used as an antiviral and anticancer agent.

1. INTRODUCTION

Natural substances come from three different sources, namely plants, animals, and minerals. Dyes obtained from plants are particularly environmentally friendly and biodegradable (Geetha, 2013; Saxena, 2014; Kumar, 2020). Phytochemicals, natural, biologically active chemical derivatives found in plants, can be divided into three basic categories: The first category is terpenoids, which contain aliphatic alkenes and have cyclic structures. The second category includes flavonoids, which mostly contain an aromatic benzene ring and its derivatives. The third category comprises alkaloids which share structural similarities with flavonoids (Belay, 2014; Ogunyemi, 2020). Another natural source of matter is insects, as there are many organic

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substances in the structure of insects. Similar to flavonoids in plants, insects, such as kermes, cochineal, and lac insects, contain dyes in vibrant colors, particularly red (Singh, 2017). Especially in the textile field in Europe, the red color of the American Cochineal (*Dactylopius coccus*) is proposed to be superior to other insect reds and is preferred. The dye obtained is known as carmine in the form of carminic acid and is preferred due to its relatively lower cost compared to the kermes insect dye (Gonzalez, 2002; Deveoglu, 2020; Baaka, 2022). Various methods have been tested to dissolve the dye and to observe the dye content and the performance of the solvent (Borges, 2012; Dapson, 2007; Gabrielli, 2018; Nallar, 2021; Serrano, 2011; Yousaf, 2018).

Furthermore, studies have been conducted to determine the antioxidant activity of carminic acid (CA). CA exhibits a crucial antiradical activity like that of known antioxidants such as ascorbic acid quercetin and Trolox (Gonzalez, 2010). Excessive fructose intake poses a risk in kidney patients. In this case, according to the results of a study investigating the positive effect of carminic acid, CA significantly decreases inflammation and oxidative stress response in the kidneys of mice through regulating Nuclear factor kappa B (NF-κB) and Nuclear factor erythroid2-related factor 2 (Nrf-2) signaling pathways. Carminic acid supplementation protects against inflammation and oxidative stress by inducing Nrf-2 signaling (Li, 2021). A study was conducted to increase the role of natural hydroxyanthraquinones in oxidative stress in comparison with the cytotoxic effect of carminic acid (Nemeikate-Ceniene, 2002). Another investigation revealed that carminic acid has free radical scavenging activity, providing a food additive (Guo, 2010). Apoptosis, or programmed cell death, is a well-known phenomenon in many cellular systems and determines cell biological activity. Apoptosis is characterized by several changes in the cell, including the degradation of nuclear chromatin into internucleosomal fragments, and cell membrane degradation, presumably by the activation of an endogenous endonuclease (Obeng, 2020).

Colorectal cancer, a type of colon cancer formed by the growth of tissues in these parts, is more common in developing countries, and the death rate is the fourth among other cancer types (Marmol, 2017). Some compounds used in cancer treatment have been obtained from some naturally occurring organisms because they have been used in traditional treatments for many years (Esmeeta, 2022). Emodin (6-methyl-1,3,8-trihydroxyanthraquinone), isolated from the *Rheum palmatum* plant, is an active ingredient and has been widely used in traditional treatments in China (Chun-Guang, 2010; Srinivas, 2003). It is one of the best-known remedies in Chinese herbal medicine and is used for the soothing treatment of constipation, gastrointestinal bleeding, and ulcers.

Therefore, this study aimed to investigate the biological activities by determining and comparing the chemical contents of cochineal and roselle extracts obtained from two different natural sources.

2. MATERIAL and METHODS

Dried cochineal insects were obtained from a Natural Dye company in İstanbul. Other chemicals and solutions used were of analytical grade and locally manufactured.

2.1. Sample 1 Preparation

Dried Roselle (*Hibiscus sabdariffa*) obtained from a market in the Talas region of Kayseri province was pounded in a mortar. A 5 g sample was added to 100 mL of distilled water (dH_2O), and it was boiled for 30-35 minutes. It was kept covered at room temperature and then filtered the next day. The water in the filtrate was poured into an Eppendorf tube to evaporate, and the remaining solid was used for derivatization.

2.2. Sample 2 Preparation

The cochineal was bought from a company that sells natural dyes in İstanbul and dried in an oven at 60 degrees (NUVE 500) for 6 hours. After grinding it in a hand mill, 5 g was placed into a beaker with precision weighing. 100 mL of dH_2O was added, boiled for 30 minutes, and kept covered overnight. The next day, it was filtered with Whatman paper. The water in the filtrate was poured into an Eppendorf tube to evaporate and the remaining solid was used for derivatization.

2.3. Derivatization of Samples

0.5 g was taken from each of the solids separated from Sample 1 and Sample 2. After dissolving it with methyl alcohol, derivatization with *N*, *O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was applied for 4 hours at 100°C. After the derivatization process, the sample was filtered and was ready for injection and the analysis was started.

2.4. Cell Culture

DLD-1 colorectal carcinoma cells were used for this study. The cells were cultured in Dulbeccos's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin, 1% L-glutamine, and in a humidified 37°C 5% CO_2 incubator. When the cells reached 85-90% confluence, they were detached from the flasks by using a trypsin-EDTA solution. For adequate cell numbers, the cells were cultured in the same conditions.

2.5. MTT Assay

For the MTT assay, $3x10^5$ cells were seeded in 96 well plates for test cell proliferation. After 24 hours, both Cochineal extracts were added to the cells as 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 µL. After 48 hours 0.5 mg/mL of the last concentration of MTT reagent was added to the wells and 3 hours later all the well contents were aspirated and 100 µL of Dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The purple color was formed by dissolving the formazan crystals and was read by using an ELISA reader at 560nm (Promega, Glomax, USA).

2.6. Apoptosis and ROS Activity Assays

For Apoptosis and ROS Activity Assays, $4.5x10^5$ cells were seeded in the 6 well plates. After 24 hours, both Cochineal extracts were added to the cells as 200 µL and 25 µL (correlated with 12.5µL and 1.56µL). Annexin V and Oxidative Stress assays (Luminex, USA) were performed according to the manufacturer's instructions. The Annexin V kit presents the percentage of live cells: early, late, and total apoptotic cells. The Oxidative stress assay presents ROS (+) and ROS (-) cell percentages.

2.7. Immunocytochemistry

For the immunocytochemistry (ICC) staining, 5 x105 cells were seeded on the sterile 1 cm^2 glass microscope slides in 24 well plates, and after 24 hours both Cochineal extracts were added to the cells at 12.5 and 1.56 µL. After 48 hours cells were fixed by 4% paraformaldehyde for 12 min. The cells were then rinsed 3x5 times and mounted with propidium iodide (PI) and 4',6-diamidio-2-phenylindole (DAPI) containing mounting medium for 15 minutes. The cells were washed again and were imagined by using a fluorescence microscope (Nikon, Ti-Eclipse).

2. 8. GC-MS Analysis

GC-MS analysis of the sample 1 and sample 2 extracts was performed on a SHIMADZU QP2010 ULTRA GC System fitted with a Rtx-5MS capillary column (30 m 0.25 mm inner diameter, 0.25 µm film thickness, max. temperature, 350 °C) coupled to a SHIMADZU GC-MS. Pure, ultra-high helium (99.99%) was used at a sustained flow rate of 1.0 mL/ min. Ion source temperatures and transfer line injection were all 290 °C. The ionizing energy was 70 eV. The electron multiplier voltage was obtained from an auto-tuning. The oven temperature was

programmed from 60°C (hold for 2 minutes) to 280°C at a rate of 3°C/min. The samples were diluted with a convenient solution (1/100, v/v) and filtered. The particle-free diluted extracts (1 µL) were aspirated into a syringe and injected into the injector at a split ratio of 50:1. All data were obtained from the full-scan mass spectra within the scan range of 40-850 amu. The percentage composition of the sample extracts was expressed as a percentage by peak area. The characterization and identification of the chemical compounds in various sample extracts were based on the GC retention time. The mass spectra were computer-matched with those of standards available in mass spectrum libraries.

2.9. Statistical Analysis

T-test was used for statistical analysis. *p* values less than 0.05 were considered statistically significant.

3. RESULTS

3.1. Cell Culture

Cells were grown in culture plates without any contamination and by keeping their epithelial-like cell morphology.

3.2. MTT Assay

Cell viability decreased when both samples were compared with the control. However, in Sample 1, cell proliferation and viability increased in the right proportion with sample concentration. In Sample 2, the viability and proliferation decreased as the sample increased. In other words, two samples showed adverse effects on cells in terms of viability and proliferation. Sample 1 showed the highest concentration and Sample 2 the lowest concentration values closest to the control, and unlike all other data, there was no statistically significant difference as they gave a value close to the control when compared to the control ($p > 0.05$), which is shown in Figures 1a and 1b.

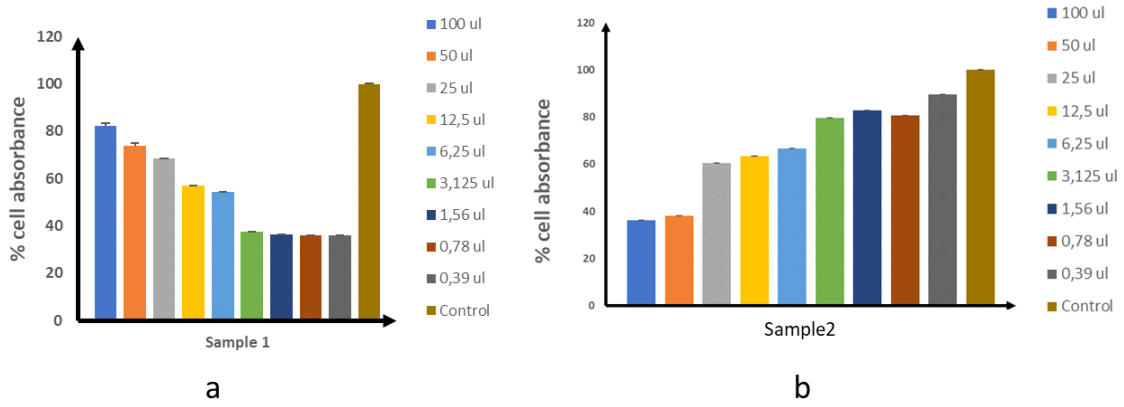

Figure 1. MTT Assay on DLD-1 cells with incubation. a) Sample 1, b) Sample 2.

3.3. Apoptosis Assay

Apoptosis was generally increased compared to the control, however, the high dose of Example 1 (200 µl) increased cell proliferation and viability, decreased late and total apoptosis and statistically decreased early apoptosis ($p < 0.003$). These are shown in the graphs in Figure 2.

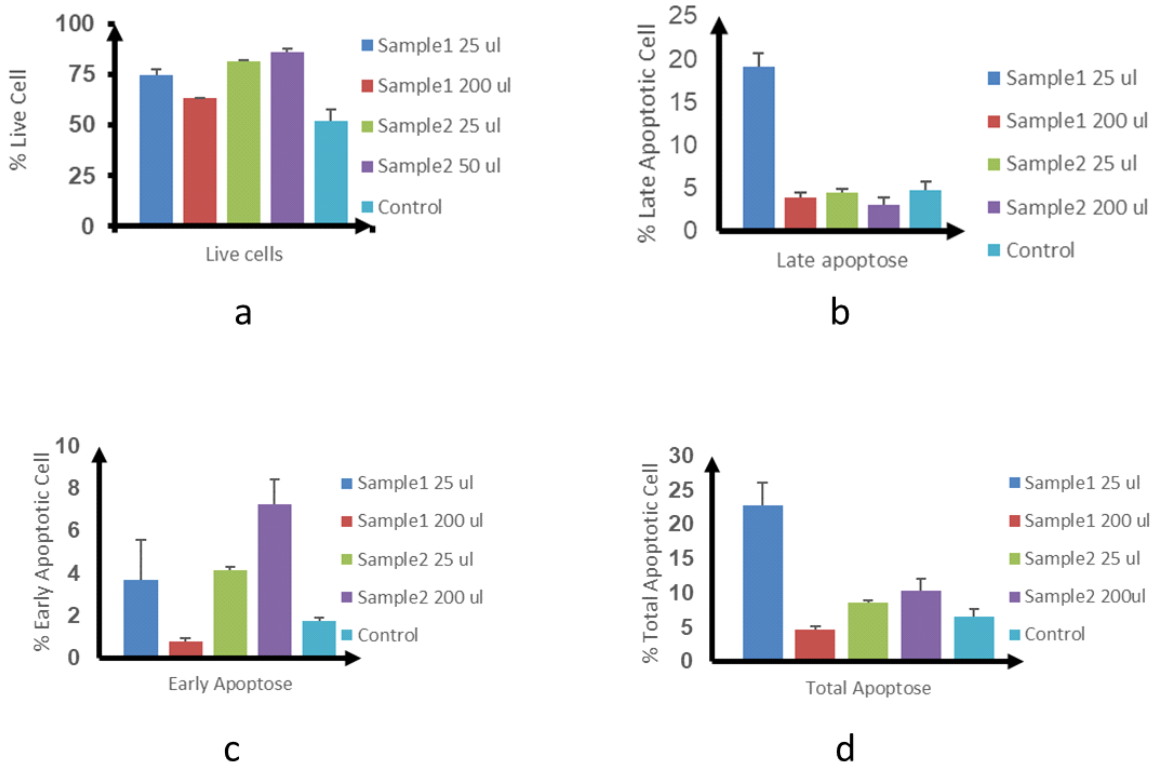

Figure 2. Demonstration of; a) % live cell profile, b) % late apoptotic cell profile, c) % early apoptotic cell profile, d) % total apoptotic cell profile.

3.4. ROS Activity Assay

As for the oxidative stress, Figure 3 shows that Sample 2 does not affect ROS activity when compared to the control, while Sample 1 has a statistically significant increase in ROS activity in cancer cells ($p_{25\mu L} < 0.002$; $p_{200\mu L} < 0.014$).

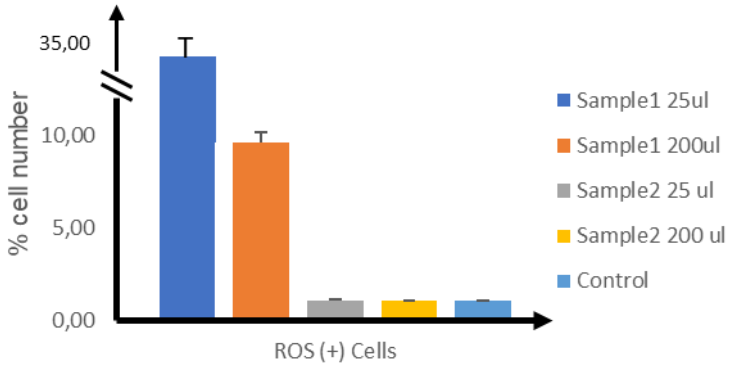

Figure 3. Demonstration of % cell number at ROS (+) cells.

3.5. Immunostaining

The immunocytochemical staining with the nuclear dye DAPI and the apoptosis indicator PI is shown in Figure 4, which shows that there were few cells stained with PI in the control group and that the cells treated with the samples and the cells treated with Cis-platin as a positive control were stained with PI like this.

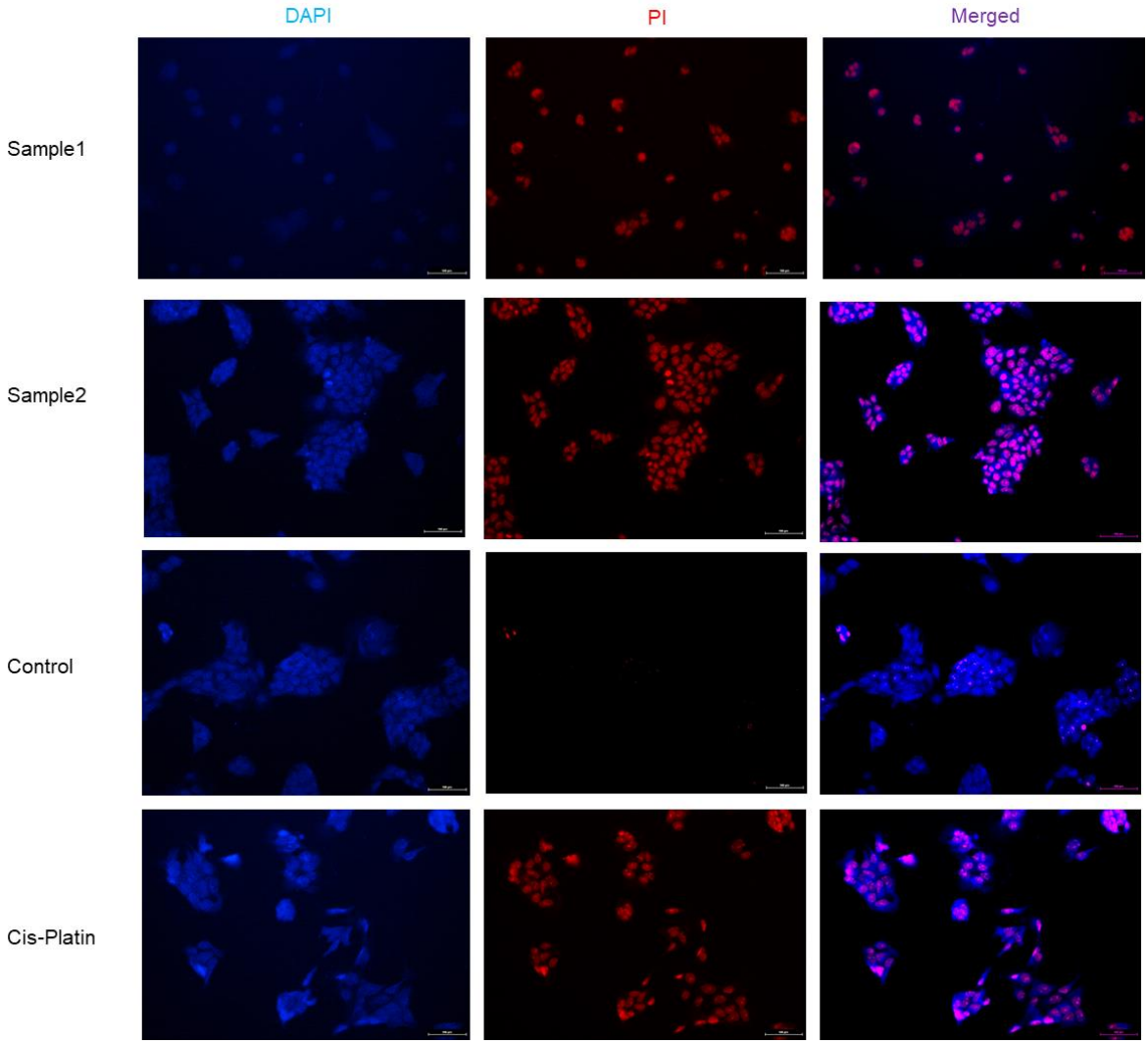

Figure 4. Experimental groups and positive and negative control groups stained with PI and DAPI (microscope magnification 10x, scale bar 100um).

3.6. GC-MS Analysis Results of Samples

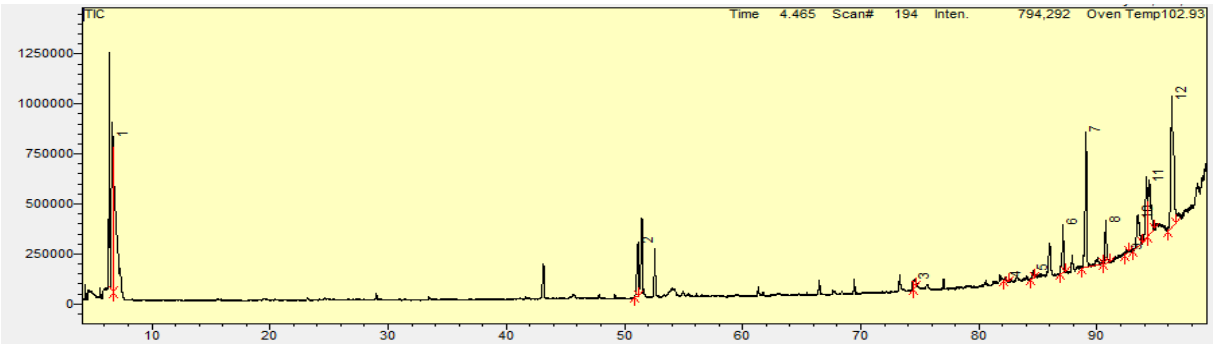

Figure 5. GC-MS spectrum of Sample 1.

The GC-MS spectrum confirmed the presence of various components with different retention times as shown in Figure 5. Large chunks of the compound are broken down into smaller compounds, which cause peaks at different m/z ratios. These mass spectra are the fingerprints of this compound that can be identified from the data library. As a result of scanning databases such as NIST11 and W9N11, the presence of 12 types of esters, alcohols, acids, ketones, and

an especially high percentage of steroidal and steroidal esters with high molecular weight were obtained and are shown in Table 1.

Table 1. GC-MS analysis results of sample 1.

No	Compound Names	% Area	Ret. Index	Molecular Weight	Molecular Formula
1	Methanesulfonamide, N-[4-(2-thienylcarbonyl) phenyl]-	6.38	2422	492.6	$C_{25}H_{20}N_2O_3S_3$
2	Benzoic acid, 2-methoxy-3-(4-methoxy-2-methyl-4-oxobutyl)-6-methyl	7.37	2076	294	$C_{16}H_{22}O_5$
3	Carbamic acid, N-[1-(3-acetylaminophenyl) ethylidenamino]-, ethyl ester	1.17	2363	263	$C_{13}H_{17}N_3O_3$
4	Cholest-5-en-3-ol (3.beta.)-, trifluoroacetate	0.66	2570	482	$C_{29}H_{45}F_3O_2$
5	Stigmastan-3-ol, 5-chloro-, acetate, (3.beta.,5.alpha.)-	0.96	2993	492	$C_{31}H_{53}ClO_2$
6	Stigmasta-5,22-dien-3-ol, acetate, (3.beta.,22Z)-	6.57	2879	454	$C_{31}H_{50}O_2$
7	Stigmast-5-en-3-ol, oleate	19.84	4469	678	$C_{47}H_{82}O_2$
8	dl-.alpha.-Tocopherol	5.80	4149	430	$C_{29}H_{50}O_2$
9	Stigmasta-5,22-diene, 3-methoxy-, (3.beta.,22E)-	0.60	2688	426	$C_{30}H_{50}O$
10	Ergost-5-en-3-ol, (3.beta.,24R)- (CAS)	7.33	2632	400	$C_{28}H_{48}O$
11	Stigmasterol	12.25	2739	412	$C_{29}H_{48}O$
12	Gamma-Sitosterol	31.07	2731	414	$C_{29}H_{50}O$

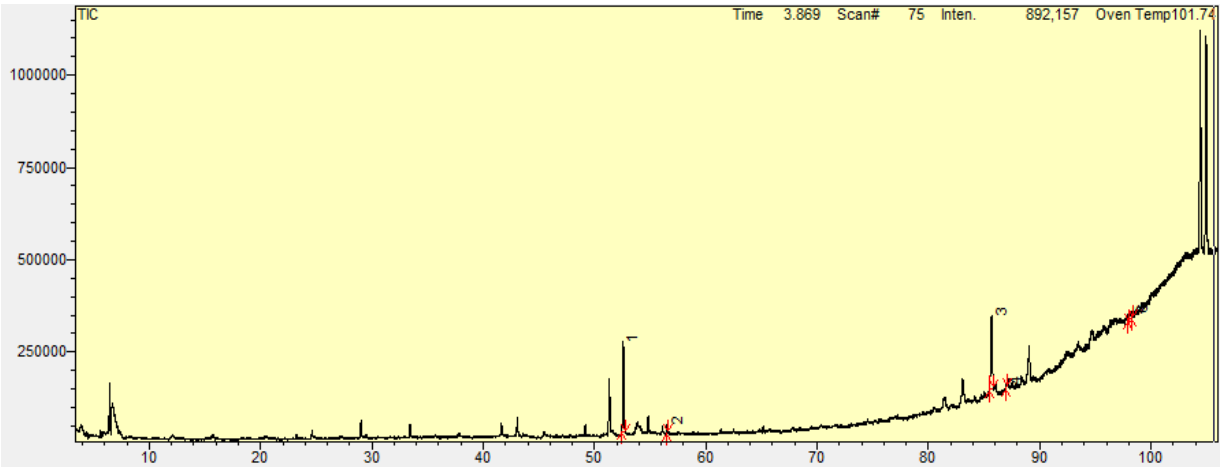

Figure 6. GC-MS spectrum of Sample 2.

The GC-MS spectrum of Sample 2 was shown in Figure 6. The presence of 6 different molecules such as amino esters, phosphonic alcohols, and naphthyls was obtained and they are shown in Table 2.

Table 2. GC-MS analysis results of Sample 2.

No	Compound Names	% Area	Molecular Weight	Molecular Formula
1	N-1-ol-8-dıphenylphosphınyl (Naphthol)	42.32	328	$C_{20}H_{23}O_2P$
2	6-Amino-5-cyano-4-isobutyl-2-phenyl-4H-pyran-3-carboxylic acid ethyl ester	2.70	326.4	$C_{19}H_{22}N_2O_3$
3	2-(1-Hydroxycyclohexylmethyl)-2'-sulphanylmethyl-1,1'-binaphthyl (Naphthyl)	49.95	412	$C_{28}H_{28}OS$
4	3-(1,2,2,6,6-Pentamethyl-1,2,3,6-tetrahydro-4-pyridinyl)-2-(2-methyl-2-aminopropyl)-1-methylindole	1.71	353	$C_{23}H_{35}N_3$
5	11-HYDROXY-3-HYDROXYMETHYLACRONINE	1.63	353	$C_{20}H_{19}NO_5$
6	1H-Pyrazole-1-acetic acid, 3-methyl-5-[[(2-oxo-2H-1-benzopyran-3-yl) carbonyl]amino]-, ethyl ester	1.69	355	$C_{18}H_{17}N_3O_5$

4. DISCUSSION and CONCLUSION

In this study, two different samples of plant origin and animal origin are discussed. The same procedures and analyses were applied to their extracts. For the determination of their biological activities, cell viability by MTT test, apoptotic cell profiles, and oxidative stress status were determined. Since the biomaterial samples used in the study contain different chemical structures, different analysis results were obtained even though the same procedures were applied to analyze the samples. Thus, the biological activity studies of the two samples were found with different contents and contrasts. In the relevant literature, the GC-MS analysis and derivatization of two samples using the method followed in this study have not been performed previously. After derivatization, red dyes were obtained with naphthol and naphthyl structures in the cochineal. The stigmasterol and sitosterol structures obtained from *Hibiscus sabdariffa* had a light color and less flavonoid content.

There have been many studies on the HPLC analysis of dried cochineal. In an article by Serrano *et al.* (2011), 7 cochineal species and 63 historical cochineal insect specimens were analyzed using the two methods. The results, mild extraction methods, and HPLC-DAD/MSn analysis produced distinctive profiles, that, in combination with a PCA reference database, provide a powerful tool for the identification of red insect dyes. New hydrophobic compounds of carminic acid, mainly used as a food dye, have been synthesized and concluded in polymeric hydrophobic matrices. Additionally, the computational investigations allowed the rationalizing of experimental data.

In a study, the degradation status in the chromatin structure was based on the results of apoptosis to explain the binding (Telford, 1992). It can be thought that substances that prevent apoptosis in cancer cells interact with the chromatin structure or DNA structure in the cell nucleus, which was observed in the immunohistochemical staining study in our specific with the growth and shape change in nuclei stained with DAPI dye. Although it is thought that the DNA in the nuclei of cancer cells incubated with Cochineal extract interacts with the extract, DNA binding and cleavage studies are required to clarify. However, substances that increase apoptosis may have therapeutic effects that support a decrease in the number of cancerous cells. It may be recommended to carry out other studies to support the use of cochineal extract as an anticancer agent. This is the first study using cochineal and *Hibiscus extracts* on DLD-1 colorectal cancer cells to our knowledge while there are many studies about Hibiscus sabdariffa extract mostly on other types of cancer (Sunkara, 2015; Adeyemi, 2014; Nguyen, 2019) as well as studies of this extract with HT-29 colon cancer cells (Czerwonka, 2017; Hsu, 2017; Kim, 2005; Li, 2013; Mata, 2016; Rouhollahi, 2015). However, there is no study of cochineal extract on DLD-1 colorectal

cancer cells. Therefore, this study fills this gap in the literature and presents a new, natural, and different source that can be used as an anticancer agent.

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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

Dilek Bahar: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing - original draft. **Nilgün Kuşçulu**: Resources, Visualization **Mehmet Çadır**: Resources, Visualization

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Research Article

Antimicrobial activities of some species in Asteraceae and Lamiaceae families from Türkiye

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Abstract: The study aimed to investigate the antimicrobial activities of the species belong to Asteraceae and Lamiaceae families collected from Çorum, Eskişehir and Kütahya provinces in Türkiye on Gram (+) and Gram (-) bacterial strains using EUCAST disc diffusion method. Ethanol (96%) and Ciprofloxacin (5mcg) were used as negative and positive controls, respectively. All plant extracts produced inhibition zones on *S. aureus* and *E. faecalis* ranged between 4.67-14.33 mm and 21.67-23.67 mm respectively. The variance in the antimicrobial activities of the plant extracts was significant between groups according to ANOVA. *L. angustifolia* samples collected from Eskişehir and Kütahya gave zone diameters close to the positive control on *S. aureus* and *E. faecalis*. It was determined that *E. coli* was the most resistant and *S. aureus* and *E. faecalis* were the most sensitive microorganisms in this study. *L. angustifolia*-E, *M. piperita* and *S. officinalis* were the species whose extracts were coming front with their high antimicrobial activities. *Pearson*'s correlation analyses displayed that the antimicrobial activity on *E. coli* was correlated positively and negatively with altitude and latitude respectively, while on *E. faecalis* was positively correlated with altitude and negatively correlated with latitude and longitude. In conclusion, the variations in the antimicrobial activities of the secondary metabolites found in the extracts of medicinal aromatic plants are important and, although the quantity, quality, and diversity of these compounds are determined according to the genotypes of plants, the environmental conditions in which the plants grow might have an impact on these differences.

1. INTRODUCTION

When we think about what life would be like without pathogenic microorganisms and the infectious diseases they cause? We would probably have a healthier and happier life than living with pathogens. Unfortunately, they exist and are part of life on earth, and we have to deal with them. No matter how much progress has been made in medicine and pharmacology in developing new drugs, especially antibiotics, antibiotic resistance remains as an important problem. In addition, it is estimated that by 2050, the number of people affected and dying from infections caused by pathogenic microorganisms that are resistant to existing antibiotics will

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exceed 10 million (Goff *et al*., 2017; Heidarian *et al*., 2022). Therefore, in order to develop alternative treatment methods to antibiotics, the use of secondary metabolites obtained from medicinal and aromatic plants has become one of the remarkable issues recently. Secondary metabolites are used not only as alternative methods in the treatment of diseases, but also in the cosmetic industry, aroma therapies, food packaging for extending shelf life, and surface disinfection products due to their antimicrobial activities.

Türkiye is one of the countries with a rich diversity in terms of medicinal aromatic plant species. Many researchers in the world and in Türkiye are conducting research on various activities of these plants such as antimicrobial, antifungal, antiviral, or antitumor. While previous studies state that the compounds that make up the chemical content of aromatic plants are controlled by their genotypes, they indicate that the environment in which they grow might also have an impact on the variations in the compounds extracted by different extraction methods from the plants growing in different environments (Burt, 2004; Saharkhiz *et al*., 2009; Andry *et al*., 2017). Increasing the number of these studies will make significant contributions to both the economy and healthy life in order to benefit from these plants more efficiently. Synthetically produced medicines also originate from the compounds found in plants in nature. Therefore, the investigation and determination of the antimicrobial activities of different medicinal aromatic plant species in this study will provide new information to the literature for more comprehensive new studies to be conducted with these plant species or other species in the future.

Some species from the Asteraceae and Lamiaceae families used in this study and previous studies:

Species belonging to the Asteraceae family: The family includes genera and species that are distributed worldwide and have economically important medicinal and aromatic properties.

Echinacea purpurea L Moench: *E. purpurea* is a herbaceous perennial with various pharmacological properties and a medicinal plant with purple flowers. It is known that the *Echinacea* genus originated in the United States and later spread to Europe (Hudson, 2012). Today, it is grown in many places in the world. There are nine different known species of *Echinacea*, and three of them (*Echinacea purpurea*, *Echinacea pallida* (Nutt.) Nutt. and *Echinacea angustifolia* DC.) are used as medicinal plants (Burlou-Nagy *et al*., 2022). It has been revealed in previous studies that the essential oils of *E. purpurea* show antimicrobial, antioxidant, anticancer and antifungal activities (Algabar *et al*., 2022).

Achillea filipendulina Lam.: *A. filipendulina* (yarrow) is an Asian species of the Asteraceae family, native to Central and Southwest Asia (Afghanistan, Iraq, Pakistan, Iran, Türkiye, Kazakhstan, and the Caucasus) (Asnaashari, 2023). *A. filipendulina*, an aromatic plant with bright yellow flowers, blooms from June to September. The extract of this plant is used to treat various diseases such as arthritis, gastrointestinal problems, blockages, and malaria. Little work has been done on the essential oils of species belonging to the *Achillea* genus (Kaur *et al*., 2017). It has been reported that the aerial parts of the *Achillea* genus and the different species it contains have properties such as antioxidant, anti-inflammatory and antimicrobial activities (Kaur *et al*., 2017).

Species belonging to the Lamiaceae *family*: Lamiaceae is one of the most widespread plant families containing a wide variety of species from an ethnomedical perspective. The Lamiaceae family contains approximately 250 genera and 6,900 to 7,200 species (Napoli *et al*., 2020).

Salvia officinalis: *Salvia* containing 900 species is the largest genus in the Lamiaceae family. *S. officinalis*, known as garden sage, is a plant native to the Mediterranean region. Garden sage is a perennial evergreen shrub with woody stems, grayish leaves, and blue to purplish flowers (Ahl *et al*., 2015). While garden sage is widely consumed as herbal tea among the people, its

essential oils are widely used in the pharmaceutical and cosmetic industries. According to previous studies, garden sage essential oils have antimicrobial activity (Abu Darwish, 2014; Sulaiman *et al*., 2023) and are used therapeutically in diseases such as antirheumatic, diuretic, expectorant, insecticide, laxative (monoterpenes), analgesic, antiarrhythmic, antiepileptic and spasmolytic (Velickovic *et al*., 2003).

Melissa officinalis L.: Lemon balm (*M. officinalis*) is a medicinal plant with high antimicrobial activity and is distributed in the Middle East, Central Asia and some parts of Europe. Previous phytochemical studies showed that this plant contains volatile compounds, triterpenoids, phenolic acids and flavonoids (Shakeri, 2016), and these metabolites have antibacterial (Hassan *et al*., 2019), antifungal (Abdel Naime *et al*., 2019; Heidarian *et al*., 2022) activities. *M. officinalis* as a medicinal plant has long been used in different ethno-medical systems in the treatment of various diseases and disorders such as headaches, irritability, colic, indigestion, heart failure, depression, and rheumatism in traditional medicine (Shakeri 2016).

Mentha piperita L.: Although peppermint (*M. piperita*) is a native species of the Mediterranean region, a medicinal aromatic plant grows in many parts of the world. Its leaves and flowers have medicinal properties. The most common compounds found in peppermint oil are menthol and menthone (Derwish *et al*., 2010). These compounds have made peppermint oil a valuable natural product preferred as a raw material in many fields such as aromatherapy and phytotherapy in the cosmetic industry. Apart from these, peppermint is a plant that is in demand as seasoning, in medicine, pharmacy, and food industry due to its smell and flavour. Additionally, according to previous studies, it has also been described that peppermint essential oil has antibacterial (Kızıl *et al*., 2010; Gruľováa 2016; Afridi *et al*., 2016) and antifungal (Kızıl *et al*., 2010; Saharkhiz *et al*., 2012) activities.

Lavandula: It is a member of the family Lamiaceae and belongs to the subfamily Nepetoideae. A number of tribes are recognized within the Nepetoideae, and Lavandula is currently treated as its own separate and isolated group, namely the *Lavanduleae* (Endl.) Boiss tribe, which contains only a single genus *Lavandula* (Lis-Balchin, 2002). The genus *Lavandula* contains approximately 39 species (Upson and Andrews, 2004). It is widespread in the Mediterranean region (Soheili and Salami, 2019), in the Canary Islands and India (Upson and Andrews, 2004), in northern, eastern and southern Africa, Bulgaria, Spain, Poland, Türkiye, France, England, Russia, Australia and the USA (Śmigielski *et al*., 2009).

Lavandula angustifolia (lavender) and *Lavandula* × *intermedia* (Emeric ex Loisel.) (lavandin): Lavender and lavandin essential oils contain various secondary metabolites such as coumarins and phenolic compounds (Panuccio *et al*., 2016). In previous studies, it has been reported that the essential oils isolated from these plants were found to have antimicrobial (Nikšić *et al*., 2017), antifungal (Slimani *et al*., 2022), antioxidant (Andrys *et al*., 2017) and herbicide (Xiaotian *et al*., 2020) activities.

The aims of this study are

i. to investigate antimicrobial activities of medicinal aromatic plants [*M. piperita* (Peppermint), *S. officinalis* (Garden sage), *M. officinalis* (Lemon balm), *A. filipendulina* (Yarrow), *E. purpurea* (Echinacea), *L. angustifolia* (lavender) and *L.* × *intermedia* (Lavandin)] on some Gram (+) [*E. faecalis* ATCC 29212, *S. aureus* ATCC 29213] and Gram (-) [*E. coli* ATCC 25922, *P. auriginosa* ATCC 27853] bacterial strains, and whether they differ in their antimicrobial activities,
ii. to find whether the antimicrobial activities of plant ethanol extracts differ according to bacterial species,
iii. to find whether plant ethanol extracts applied at three different doses (15, 20 and 25 µL) differ in their antimicrobial activities,

iv. to find whether there is a correlation between antimicrobial activities and the geological data (altitude, latitude and longitude) of the locations where the plant samples were collected, and the ethanol extracts applied at different doses.

2. MATERIALS and METHODS

1.1. Materials

In this study, the dried flowers and leaves of five different aromatic plant species [*M. piperita*, *S. officinalis*, *M. officinalis*, *A. filipendulina* and *E. purpurea*], obtained from a medicinal aromatic plant breeder (HMC Naturel) in Çorum Dodurga, and two lavandula species [*L. angustifolia* and *L.* × *intermedia*] grown by farmers, collected from Eskişehir, Çifteler, Belpınar Village, Kütahya, Merkez, Aloğlu Village, Çorum, Alaca, Gerdekkaya Village were used. Detailed information about the plants is given in Table 1. In the investigation of the antimicrobial activities of plant ethanol extracts, Gram (+) [*E. faecalis* ATCC 29212 and *S. aureus* ATCC 29213] and Gram (-) [*E. coli* ATCC 25922 and *P. auriginosa* ATCC 27853] bacterial strains were used, while ethanol (96%) and Ciprofloxacin (5mcg) antibiotic disc were used as negative and positive controls respectively.

Table 1. Detailed information about the plant material used in the ethanol extraction process (Abbreviations: A: altitude, LA: latitude, LO: longitude).

	Species Name	Local Name	Location Name	A (m)	LA	LO
1.	*Mentha piperita* L.	Tıbbi nane	Dodurga/Çorum	607	40.51	34.48
2.	*Salvia officinalis* L.	Adaçayı	Dodurga/Çorum	607	40.51	34.48
3.	*Melissa officinalis* L.	Melisa	Dodurga/Çorum	607	40.51	34.48
4.	*Achillea filipendulina* Lam.	Civanperçemi	Dodurga / Çorum	607	40.51	34.48
5.	*Echinacea purpurea* L.	Ekinezya	Dodurga / Çorum	607	40.51	34.48
6.	*Lavandula angustifolia* Mill.	Lavanta	Eskişehir, Çifteler, Belpınar Village	936	39.31	31.67
7.	*Lavandula* × *intermedia* Emeric ex Loisel.	Lavanta	Çorum, Alaca, Gerdekkaya Village	1100	40.34	35.35
8.	*Lavandula angustifolia* Mill.	Lavanta	Kütahya Merkez, Aloğlu Village	1247	39.35	29.94

1.2. Methods

1.2.1. *Extraction with Soxhlet device*

The plant materials were dried in an environment protected from sunlight, and the flower (lavandula, Echinacea, yarrow) and leaf (peppermint, garden sage, and lemon balm) parts of the plants were ground in a porcelain mortar at room temperature. The grounded plant powder was filled in a filter bag, placed in the cartridge chamber of the Soxhlet device and the extracts were obtained by using 300 mL of ethanol (96%) as the solvent and performing the extraction process for 24 hours at a temperature not exceeding the boiling point of the solvent. After the extraction finished, the extraction solution containing the extracted solutes was left to evaporate until approximately 30 mL, which was transferred to the fresh tubes (50 mL) and they were centrifuged at 2000 rpm for 20 min. The supernatant was transferred to fresh amber glass vials, evaporated until solid extracts remained, and kept at + 4°C until use. After the soluble extracts were dissolved with the same solvent (5 mL) used for extraction, the solutions were filtered through 0.22 µm pore size micro filters into capped fresh amber glass bottles and stored at +4 °C until used for antimicrobial analyses.

1.2.2. *Bacterial strains and growth media*

In the study, Gram (+) (*E. faecalis* ATCC 29,212 and *S. aureus* ATCC 29,213) and Gram (-) (*P. aeruginosa* ATCC 2753 and *E. coli* ATCC 25,922) in total four different bacterial strains were used for antimicrobial analyses. Bacterial strains were grown in different growth media

in the following order; blood agar, broth, and nutrient agar at their respective optimum growth temperatures (37°C). Luria-Bertani (LB) was used to store the bacterial strains at – 20 ºC. Bacterial suspensions were adjusted to a 0.5 McFarland standard, which is equivalent to a bacterial suspension containing 1×10^8 to 2×10^8 CFU/mL *E. coli* using sodium chloride physiological solution.

1.2.3. *Disc diffusion method*

The determination of antimicrobial activity was performed according to the EUCAST disc diffusion method (www.eucast.org, 2023). The same petri dishes were used to observe the antimicrobial activities of the plant extracts on the microorganisms and to observe the control groups. The bacterial stains were inoculated on the Müller Hinton agar medium by spreading method to determine the antimicrobial activities of the plant extracts and then, the sterile standard discs were impregnated at equal distances on the medium using a sterile forceps by pressing lightly. Application of three different doses of 15, 20, and 25 µL of plant extracts was carried out in three replicates. After the discs were placed for 15 minutes, the petri dishes were incubated in an incubator at 37 ºC for 24 hours. Commercially available standard Ciprofloxacin antibiotic disc (5mcg) was used for positive control, while ethanol (96%) was used as negative control. The evaluation was made by measuring the diameters of the inhibition zones around the discs, where the bacterial strains did not grow, in mm with a ruler. The experiments were performed in three replicates, the scores of the results were recorded for each trial, and then, the mean values of the inhibition zone diameters were used for the statistical analysis.

1.2.4. *Statistical analyses*

The mean values obtained by evaluating the antimicrobial analyses were used to determine the significance of the variance in the antimicrobial activity of the plant extracts.

One-way ANOVA: The test of Homogeneity of Variances (THV) test was applied to determine the homogeneity of sample distributions in the groups. According to the results of THV, it was determined that the sample distribution in the groups was equal, and the Sigma (*p*) value was greater than 0.05 in some groups and lower than 0.05 in some groups. Whether the variances observed in the antimicrobial activities of plant extracts on the bacterial strains were significant within and between groups was evaluated with one-way ANOVA test. Since the sample distribution was equal and homogeneous in all groups, Tukey's HSD and Sheffe *post hoc* tests (data not given) were applied along with the ANOVA.

Pearson's correlations: *Pearson*'s correlation coefficient was calculated to determine whether there was a correlation between the mean values of the antimicrobial inhibition zone diameters obtained as a result of the application of the plant extracts, ethanol (96%), and the Ciprofloxacin (5mcg) and the type of bacteria, the application of plant extracts in different volumes (15, 20, and 25 µL), and the geological data (altitude, latitude and longitude) of the locations, where the plant samples were collected. Correlation analyses were performed with IBM SPSS (ver. 22).

3. RESULTS

1.3. Antimicrobial Activities

Ethanol extracts obtained from the plants used in the analyses were applied in three replicates on four different bacterial strains in three different volumes. Ethanol was used as negative control and Ciprofloxacin antibiotic (5mcg) disc was used as positive control and standard. The mean values of the inhibition zone diameters were calculated and given in Table 2. No antimicrobial activity was observed in all negative control applications.

Table 2. The mean values of the inhibition zone diameters resulting from the antimicrobial activities of the plant extracts, the positive and negative controls.

	Species Name	*Escherichia coli* ATCC 25922			*Pseudomonas auriginosa* ATCC 27853			*Staphylococcus aureus* ATCC 29213			*Enterococcus faecalis* ATCC 29212		
		15 µL	20 µL	25 µL	15 µL	20 µL	25 µL	15 µL	20 µL	25 µL	15 µL	20 µL	25 µL
1	M*entha piperita*	0	0	2.67	2.33	7.00	8.00	12.00	14.33	14.33	6.33	9.33	11.33
2	*Salvia officinalis*	0	0	0	2.33	2.33	7.00	8.67	14.33	14.00	10.33	11.33	12.00
3	*Melissa officinalis*	0	0	0	0	2.33	7.33	6.33	9.67	11.33	7.67	5.33	7.67
4	*Achillea filipendulina*	0	0	0	0	0	4.67	9.67	6.33	11.33	0	2.67	8.00
5	*Echinacea purpurea*	0	0	0	0	0	4.67	5.67	5.67	4.67	3.00	2.33	7.67
6	*Lavandula angustifolia*-E	8.33	8.00	5.67	4.67	0	8	8.67	10.67	12	14.33	22	20.67
7	*Lavandula intermedia*- Ç	7.00	8.00	5.33	0	0	4.67	8.00	9.67	9.33	7.67	15.67	11.00
8	*Lavandula angustifolia*-K	2.33	8.00	5.67	0	0	0	7.67	10.33	11.33	9.67	20.33	18.00
N.C	Ethanol	0	0	0	0	0	0	0	0	0	0	0	0
P.C	Ciprofloxacin (5mcg)	34.00	35.00	33.67	31.67	31.33	32.00	29.00	30.00	30.00	21.67	22.00	23.67

NC: Negative control, PC: Positive control, E: Eskişehir, Çifteler, Belpınar Village, Ç: Çorum, Alaca, Gerdekkaya Village, K: Kütahya, Merkez, Aloğlu Village.

Antimicrobial activities on E. coli ATCC 25922: It was observed that the ethanol extracts obtained from *M. piperita*, *S. officinalis*, *M. officinalis*, *A. filipendulina* and *E. purpurea* plants did not have any antimicrobial activities on *E. coli* (Figure 1a). On the other hand, it was observed that the ethanol extracts of the *Lavandula* species collected from three different locations had antimicrobial activities (8.33-2.33 mm) on *E. coli*, but they showed approximately a quarter of the activity compared to the positive control (33.67-35 mm).

Antimicrobial activities on P. auriginosa ATCC 27853: When the plant ethanol extracts were applied in 15 µL and 20 µL volumes, it was observed that the *A. filipendulina*, *E. purpurea*, and *L. intermedia*-Ç samples did not show any antimicrobial activities, while the *L. angustifolia*-K did not show antimicrobial activity in all three volumes applied (Figure 1b). The inhibition zone diameters produced by *M. piperita* and *S. officinalis* in 15 µL, 20 µL and 25 µL, *M. officinalis* in 20 µL and 25 µL, *A. filipendulina* and *E. purpurea* in 25 µL, *L. angustifolia*-E in 15 µL and 25 µL ranged between 2.33-8.00 mm, 2.33-7.33 mm, 4.67 mm, and 4.67-8.00 mm respectively. It was determined that the positive control showed antimicrobial activity at approximately four times larger than the plant extracts.

Antimicrobial activities on S. aureus ATCC 29213: All the plant extracts applied in three volumes showed antimicrobial activities on *S. aureus* at rates ranging from 4.67 to 14.33 mm (Figure 1c) while inhibition zone diameters were observed in the range of 29-30 mm by the positive control. When the plant extracts were applied in 20 µL and 25 µL volumes, it was determined that *M. piperita* and *S. officinalis* showed higher activity than the other plant extracts.

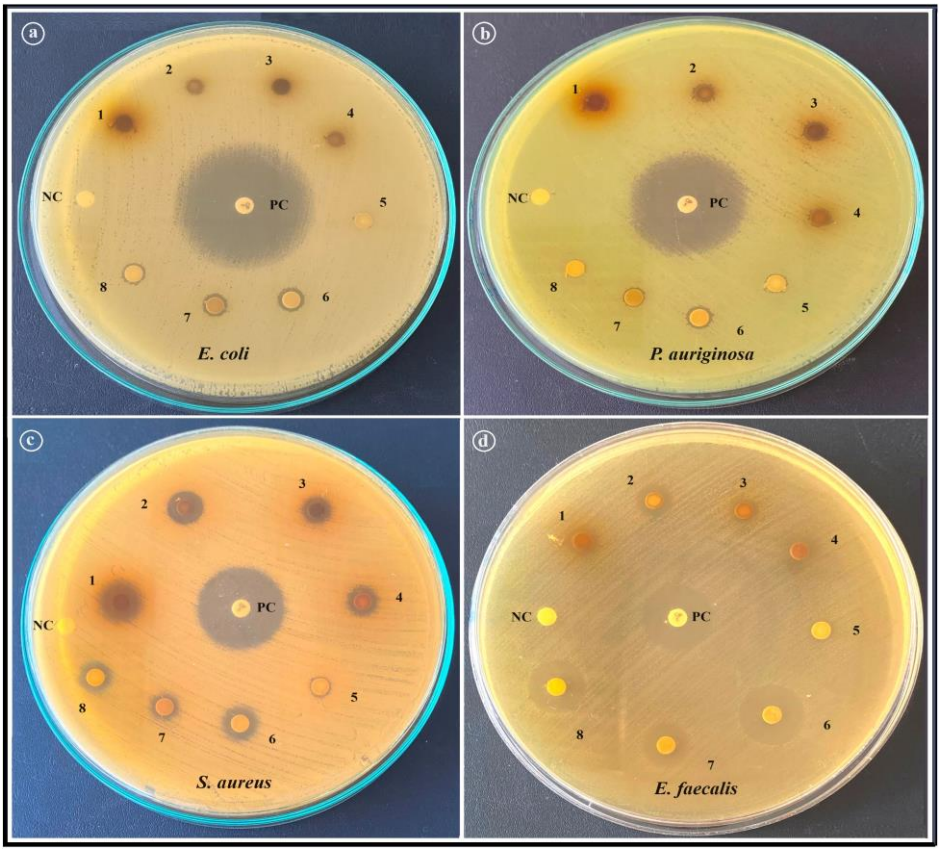

Figure 1. The inhibition zones formed by the antimicrobial activities of the aromatic plant species' extracts applied in 25 µL volumes together with positive and negative controls on the bacterial strains; a) *E. coli*, b) *P. auriginosa*, c) *S. aureus*, and d) *E. faecalis* (The numbers on the figures are representing the plant species in the same order given in Table 1, NC: Negative control, PC: Positive control)

Antimicrobial activities on E. faecalis ATCC 29212: It was determined that all plant extracts showed antimicrobial activities ranging from 2.67-20.67 mm in all three applied volumes on *E. faecalis* (Figure 1d), the positive control showed the inhibition zone diameters ranging from 21.67 to 23.67 mm. The significant point in this application is that *L. angustifolia*-E plant extract showed inhibition zone diameters ranging from 14.33 to 22 mm very close to the positive control.

1.4. Analysis of Variance According to Four Different Bacterial Groups

One-way ANOVA was applied to determine whether there was a statistically significant difference in the antimicrobial activities of the eight different plant extracts analyzed according to four different bacterial strains. According to the ANOVA results, it was revealed that the antimicrobial activities of all plant extracts and positive control showed significant differences between the groups based on *Sigma* values. ANOVA values were calculated in the range of $F = 4.52$ ($p = 0.04$ significant at the 0.05 level) to $F = 148$ ($p = 0.00$ significant at the 0.05 level) (Table 3).

Table 3. According to ANOVA, the significance of the variances of the antimicrobial activities of eight aromatic plants' ethanol extracts and positive control on the bacterial strains.

Species Name		Sum of Squares	*df*	Mean Square	*F*	Sig.
Mentha piperita	BG	256.17	3	85.39	17.36	0.00
	WG	39.36	8	4.92		
	Total	295.53	11			
Salvia officinalis	BG	314.60	3	104.87	23.22	0.00
	WG	36.14	8	4.52		
	Total	350.74	11			
Melissa officinalis	BG	145.44	3	48.48	8.68	0.01
	WG	44.67	8	5.58		
	Total	190.11	11			
Achillea filipendulina	BG	142.47	3	47.49	6.26	0.02
	WG	60.69	8	7.59		
	Total	203.16	11			
Echineceae. purpurea	BG	54.51	3	18.17	4.52	0.04
	WG	32.13	8	4.02		
	Total	86.64	11			
Lavandula angustifolia-E	BG	364.29	3	121.43	12.83	0.00
	WG	75.72	8	9.47		
	Total	440.01	11			
Lavandula intermedia-Ç	BG	159.90	3	53.30	8.19	0.01
	WG	52.04	8	6.50		
	Total	211.94	11			
Lavandula angustifolia-K	BG	414.21	3	138.07	12.81	0.00
	WG	86.22	8	10.78		
	Total	500.43	11			
Ethanol (N.C)	BG	0.00	3	0.00	-	-
	WG	0.00	8	0.00		
	Total	0.00	11			
Ciprofloxacin (5mcg)	BG	230.34	3	76.78	148.02	0.00
	WG	4.15	8	0.52		
	Total	234.49	11			

*Sigma $p < 0.05$ is significant.
Abbreviations: E: Eskişehir, Çifteler, Belpınar Village, Ç: Çorum, Alaca, Gerdekkaya Village, K: Kütahya Merkez Aloğlu Village.

Post hoc tests: When it was determined that the variance between groups was significant, Tukey's HSD and Scheffe were performed as *post hoc* tests to determine the origin of differences. According to the results of *post hoc* tests, when the groups were compared with each other, *Sigma* values showed that the antimicrobial activities of the aromatic plant extracts and positive control applied on four different bacterial strains differed between bacterial groups and this difference was significant (data not given). The least difference between bacterial groups was found after the application of *E. purpurea* extract.

1.5. *Pearson*'s Correlation

Whether there was a correlation between the antimicrobial activities of aromatic plant ethanol extracts on four bacterial strains and the geographical conditions (altitude, latitude, and longitude) of the locations, where the plant samples were collected was analyzed by calculating *Pearson's* correlation coefficients. According to the results, when the aromatic plant extracts were applied in 20 µL and 25 µL volumes, there were positive correlations between the antimicrobial activities on *E. coli* and altitude (r_P = 0.950, p = 0.000, significant at the 0.01 level and r_P = 0.896, p = 0.003, significant at the 0.05 level, respectively) (Table 4).

Table 4. *Pearson's* correlations between the antimicrobial activities of the aromatic plant extracts and the geographical conditions of the locations, where the plant samples were collected.

Bacterial strains	V		A (m)	LA	LO
Escherichia coli ATCC 25922	15 µL	r_P	0.688	-0.635	-0.301
		p	0.059	0.091	0.469
	20 µL	r_P	0.950**	-0.814*	-0.604
		p	0.000	0.014	0.113
	25 µL	r_P	0.896*	-0.790*	-0.599
		p	0.003	0.020	0.117
Pseudomonas auriginosa ATCC 27853	15 µL	r_P	-0.051	-0.401	-0.243
		p	0.904	0.325	0.561
	20 µL	r_P	-0.463	0.397	0.294
		p	0.248	0.330	0.479
	25 µL	r_P	-0.635	0.360	0.503
		p	0.091	0.381	0.204
Staphylococcus aureus ATCC 29213	15 µL	r_P	-0.131	0.058	0.070
		p	0.757	0.892	0.869
	20 µL	r_P	0.015	-0.068	-0.070
		p	0.973	0.872	0.870
	25 µL	r_P	-0.061	-0.105	-0.140
		p	0.887	0.804	0.741
Enterococcus faecalis ATCC 29212	15 µL	r_P	0.474	-0.665	-0.518
		p	0.235	0.072	0.189
	20 µL	r_P	0.824*	-0.853**	-0.690
		p	0.012	0.007	0.058
	25 µL	r_P	0.678	-0.934**	-0.826*
		p	0.065	0.001	0.011
	N		8	8	8

**Correlation is significant at the 0.01 level (2-tailed), *Correlation is significant at the 0.05 level (2-tailed).
Abbreviations: V: Volume, A: altitude, LA: latitude, LO: longitude, N: Sample size, r_P: Pearson's correlation coefficient, p: p-value.

It was determined that there were negative correlations (r_P = -0.814, p = 0.014, significant at the 0.05 level and r_P = -0.790, p = 0.020, significant at the 0.05 level, respectively) between antimicrobial activities when 20 µL and 25 µL volumes applied on *E. coli* and latitude. When the plant extracts were applied in a volume of 20 µL, the observed antimicrobial activity on *E. faecalis* was positively correlated with altitude (r_P = 0.824, p = 0.012, significant at the 0.05 level) and negatively correlated with latitude (r_P = -0.853, p = 0.007, significant at the 0.05 level). When it was applied in 25 µL volume on *E. faecalis*, there were negative correlations between antimicrobial activities and both latitude and longitude (r_P = -0.934, p = 0.001, significant at the 0.01 level and r_P = - 0.826 p = 0.011, significant at the 0.05 level, respectively).

4. DISCUSSION and CONCLUSION

The antimicrobial activities of the plant extracts obtained with ethanol on four different bacterial strains were investigated in the study. It was observed that there were variations in the antimicrobial activities of extracts from different aromatic plant species analyzed. When the mean values of inhibition zone diameters were evaluated, it was determined that plant extracts varied in terms of their antimicrobial activities on different bacterial strains. When these results were evaluated in general, it was determined that extracts of the aromatic plant species other than Lavandula species had no activity on *E. coli*, but Lavandula species showed activity, albeit lower, than the positive control. According to these results, it was found that *E. coli* was the most resistant microorganism against the antimicrobial activities of plant extracts used among the bacterial strains analyzed. Since the analyzes on *P. auriginosa* showed antimicrobial activity, albeit at low rates, depending on the application of the plant extracts in different volumes, *P. auriginosa* can be defined as the most resistant species in the second order. It was revealed that plant extracts showed intense antimicrobial activities on *S. aureus* and *E. faecalis* compared to others. When antimicrobial activities are compared, it was clearly seen that the plant extracts have higher effects on *S. aureus* than on *E. faecalis*. *A. filipendulina* and *E. purpurea* plant extracts displayed lower antimicrobial activities on the bacterial strains analyzed than the other plant species, while *Lavandula* species showed the highest activity. In addition, it was observed that the activity of *Lavandula* species on *E. faecalis* was almost the same as the positive control. These results indicate that medicinal aromatic plants potentially contain naturally occurring metabolites with high antimicrobial activity. The plant species, whose antimicrobial activities were analyzed in this study were determined to show similar activities especially on the same or different microorganisms in previous studies such as *M. piperita*, (Gruľová *et al*. 2016; Afridi *et al*. 2016), *S. officinalis* (Sulaiman *et al*. 2023), *M. officinalis* (Hassan *et al*., 2019; Heidarian *et al*., 2022), *A. filipendulina* (Kaur *et al*., 2017), *E. purpurea* (Rubinstein *et al*., 2008), *Lavandula genus* (*L. angustifolia*, and *L.* x *intermedia*) (Andrys *et al*. 2017; Nikšić *et al*. 2017).

When the contents of plant essential oils are analyzed using methods such as GC-MS, it is known that they are a mixture of many bioactive compounds. When their biological activities are investigated, it is generally unknown whether or how they interact with each other synergistically, since they are all administered together in the extract. The compositions of these essential oils may vary according to species, seasons, between populations (Vokou, 1993), and even individuals within the population (Tarayre *et al*. 1995; Gruľová *et al*. 2016).

It has also been shown in clinical studies that *M. officinalis* contains triterpenoids, phenolic acids, and flavonoids in its chemical composition and has effects on mood, cognition, and memory (Shakeri *et al*., 2016). Additionally, the compounds responsible for antibacterial and antifungal activities have been reported to be citrals (geranial and neral) and citronennal (Mimica-Dukic *et al*. 2004). Although not statistically significant, *M. officinalis* has also been reported to increase ocular pressure and inhibit thyroid hormone as side effects (Shakeri *et al*., 2016).

There are various compounds in the components of peppermint (*M. piperita*) essential oil. Gruľová *et al*. (2015) found that the components of peppermint oil were (-)-menthol (58.7-71.2%), menthone (3.5-19.6%), limonene (3.4-8.4%), menthyl acetate (1.4-17.2%) and β-caryophyllene (2.4-6.3%). It is also stated that peppermint essential oil is an effective antimicrobial and pest control agent in food plants and foodstuffs (McKay and Blumberg, 2006). In addition, it has been reported that menthol is generally responsible for the antimicrobial activity of *Mentha* x *piperita* (Iscan *et al*. 2002).

The compounds known as secondary metabolites of garden sage (*S. officinalis*) are anesthetic, antihistaminic, anti-rheumatic, diuretic, expectorant, insecticide, laxative (monoterpenes); Analgesic, antiarrhythmic, antiepileptic, spasmolytic, anthelmintic, anti-inflammatory, antitumor, hypotensive and sedative properties were reported in previous studies (Abu Darwish, 2014). Some compounds of *S. officinalis* include essential oil (alpha and beta thujone, cineole, camphor, salvia tannin, etc.), flavonoids (apigenin, luteolin, genkwanin, etc.), terpenoids (ursolic acid, picrosalvin, rosmanol, saffisinolide, etc.) and phenolic acids (rosmarinic acid, chlorogenic acid, ferulic acid, caffeic acid, etc.) have been reported to be present (Ahl *et al*., 2015).

Slimani *et al*. (2022) reported that the essential oil of *L. angustifolia* contained linalool (29.95%), linalyl acetate (18.86%), *ρ*-cymene (14.68%), and α-Campholenal (10.26%). The components of lavender essential oils are grouped as terpene hydrocarbons: monoterpenes (C10), sesquiterpenes (C15), and diterpenes (C20). Antitumor activity is associated with monoterpenes. It is stated that the complex formed by linalyl acetate, alpha-terpineol, and camphor compounds found in lavender essential oil causes inhibition of the growth of human cancer cell lines. Nikšić *et al*. (2017) also reported that lavender (*L. angustifolia*) extracts, in addition to their antibacterial and antifungal effects, have potential anti-proliferative activity against many malignant cell lines (MOLT-4, MCF-7 and H460), and the greatest effect was shown on hematological malignant MOLT-4 cells. In this study, Lavandula species displayed significant antibacterial activities that might show the high potential of the compounds harbored in the lavandula species grown by Turkish farmers. These results also indicated that the extended studies should be focused on the more detailed antimicrobial, antifungal and antitumor activities of the lavandula species grown by farmers in the globally and future in Türkiye.

According to One-way ANOVA analyses, it was observed that *E. purpurea* extract caused the lowest variance among bacterial strains. In fact, this result is consistent with the fact that the effectiveness of the antimicrobial activity of *E. purpurea* observed to be quite low compared to the other plants. It is known from previous studies that extracts obtained from medicinal aromatic plants using different solvents may contain different secondary metabolites with different concentrations. However, although the chemical compositions of plants depend mainly on the plant genotype, they may differ under the influence of environmental factors such as the plant's sun exposure time, age, seedling collection method or isolation methods of extracts (Burt, 2004; Saharkhiz *et al*., 2009; Andry *et al*., 2017). It has also been reported that chemical components vary in terms of quality and yield according to plant harvest time and different parts of the plant (Hussain *et al*., 2010).

Considering the environmental conditions in which the analyzed plants were grown, it is expected that this difference will also be reflected in their cell contents. Based on this idea, whether there was a relationship between the antimicrobial activities of the secondary metabolites stored in the plants according to the geographical conditions (altitude, latitude and longitude) where they were collected was analyzed by calculating *Pearson's* correlation coefficients. The results showed that the plant extracts showed high positive correlations with altitude in application of both volumes 20 and 25 µL, and high negative correlations with latitude. Plants have high plasticity to adapt the changes in the environmental conditions. They

can tolerate and adapt to eco-geographic changes in their environment by producing morphological changes up to a certain level, without changing their genetic structure (Nicotra *et al*., 2010). As the altitude increases, environmental conditions may become more challenging, such as the decrease in temperature and air humidity, increase in wind and water loss by transpiration. When environmental conditions are challenging, plants make some changes in their morphological structures and try to cope with these challenging conditions. One of the sources of the variances in the ethanol extracts of plants might be due to the differences in environmental conditions. Although the antimicrobial activities of these plant species were analyzed in this study, it can be thought that these metabolites also play an active role in the plant's defense mechanisms against the challenging environmental conditions. Additionally, different plant species may respond differently to altitude increases. Latitude is a factor related to light issue for plants. The plants are exposed to how much light during a daytime determines their photosynthesis rates. However, although there are not great differences in terms of latitude between the locations of the plants used in this study, it is estimated that they might cause significant changes in terms of plant cellular contents.

Extracting the secondary metabolites of medicinal aromatic plants with antimicrobial effects with various solvents such as ethanol, hexane, chloroform, and water is one of the popular topics recently. Some compounds found in these extracts are used in many areas such as cosmetics, medicine, paint, herbal teas, nutritional supplements, liquor, pesticides and fungicides, essential oil products, perfumes, flavoring liquids and cleaning products (Ahl *et al*. 2015). In order to benefit more efficiently in using the biologically active compounds found in natural plant extracts, the future studies should also focus on investigating the mechanisms of action and pharmacokinetics of these compounds. Even if the plants are not poisonous under normal conditions and are consumed naturally, the compounds they contain may have side effects. Therefore, it should not be forgotten that when using these plants in traditional treatments, the clinical effectiveness and safety of plant extracts and active compounds should be taken into account.

In conclusion;

- The different medicinal and aromatic plant species showed different antibacterial activities on the same bacterial strains.
- According to ANOVA and *post Hoc* tests, it was revealed that the variance in the antimicrobial activities of ethanol extracts of the different plant species among bacterial groups was significant.
- There were significant correlations between the conditions of the locations, where the plants were collected used in the study and the effectiveness of the antimicrobial activities of the plant extracts on *E. coli* and *E. faecalis* bacterial strains.
- Among the analyzed plant species, it was observed that the antimicrobial activities of *M. piperita, S. officinalis* and *L. angustifolia* species were significantly higher than the other species.
- The fact that *L. angustifolia*-E species gave inhibition zone diameters almost at the same rates as the positive control on *E. faecalis* bacteria showed its potential for producing natural and economical secondary metabolites with high levels of antibacterial activity that might be considered significantly for its future usage.
- As food industries tend to reduce the use of chemical preservatives in their products, essential oils of medicinal aromatic plants with potential active antimicrobial properties can be considered as a natural source to preserve or extend the shelf life of products.
- More effective secondary metabolites can be obtained from medicinal aromatic plants using different solvents and advanced extraction methods under more advanced industrial type

laboratory conditions. Since these compounds are natural, they will provide significant benefits in terms of human health and ecology.

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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.

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Both authors contributed equally to the preparation of the manuscript; Investigation, Methodology, Resources, Visualization, Software, Formal Analysis, Supervision, Validation and Writing -original draft.

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Research Article

Ultrasound and microwave extraction from *Moringa oleifera* Lam.: Characterization and antiproliferative effect

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Abstract: *Moringa oleifera* has been a focus of interest because of the different properties (anticancer, antioxidant, etc.) that have been attributed to this plant. However, the most used methodology is soxhlet, which requires long periods of reaction (18 hours), generating greater energy expenditure. Recently, green extraction technologies have been developed like ultrasound and microwaves, reducing reaction time by up to 97%. The objectives of this study were to extract and identify the polyphenolic compounds present in aqueous and hydro-alcoholic extracts from *Moringa oleifera* dried leaves using ultrasound and microwave, as well as to evaluate their in vitro cytotoxic effect using cancer and non-cancer cells. A combination of ultrasound and microwave was utilized to extract polyphenolic compounds from *Moringa* dried leaves. HPLC–MS analysis was conducted to qualitatively identify the polyphenols in the samples. The cytotoxic effect was evaluated by MTT and comet assays using non-cancer (3T3, Hek293, and Vero) and cancer (HepG2) cells lines. Results: 30 polyphenolic compounds from 9 different families were identified by HPLC. Data suggested that hydro-alcoholic extracts from *Moringa* leaves have potent cytotoxic activities in a depend-doses response. Also, compounds from aqueous extracts did not cause cell death, while polyphenol extract from hydro-alcoholic extracts decreased populations in both cancer and non-cancer cell lines measurement by MTT. HepG2 cells showed DNA damage by comet assay. The extraction using ultrasound and microwaves at 30 minutes of reaction has an antiproliferative effect through apoptosis in cancer cells, in addition ethanolic extracts have higher cytotoxicity compared to aqueous extracts.

1. INTRODUCTION

Moringa oleifera (MO) has been widely used as food or food supplement (human and animal) because of its high energy value. It is consumed as fresh, powders or aqueous extracts (Nayak *et al*., 2016) but the current worldwide boom is due to its biological effects such as antimicrobial

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activity and treatment of cardiovascular, gastrointestinal, hematological, and hepatorenal diseases (Jayawardana *et al*., 2015); urinary diseases (Nayak *et al*., 2016); activity against malaria, and even to combat the symptoms of HIV/AIDS by stimulating the immune system (Ramabulana *et al*., 2016); to treat nervous disorders (González-Trujano *et al*., 2018); hypertension, catarrh, gastric ulcers, and skin diseases (Chen *et al*., 2017), hypoglycemic (Stohs & Hartman, 2015), impotence, hemorrhoids, headaches, and gum pain (Bakre, Aderibigbe & Ademowo, 2013). In another study, it has been proven *Moringa* effects as a palliative against the pain caused by arthritis (Mahdi *et al*., 2018). The main *Moringa oleifera* phytochemicals are quercetin, alkaloids, tannins, flavonoids, and glycosides which are present in all parts of the plant. Phytochemicals generally have fewer side effects than synthetic substances (Jarriyawattanachaikul, Chaveerach & Chokesajjawatee, 2016).

It is important to note that the quantity and quality of bioactive molecules, extracted from vegetal sources depend on the optimal selection of the extraction method and solvents (Fu *et al*., 2016). There is a wide variety of extraction methods and solvents (Vongsak *et al*., 2013), from conventional techniques (soxhlet, maceration, and distillation) to the most innovative and considered green (ultrasound, microwaves, high pressure, among others) technologies (Guerrouj *et al*., 2016), which have recently increased their popularity over traditional methods because they are environmental friendly (Yang *et al*., 2011), offer savings in time and energy, applicability to smaller samples and are performed at lower temperatures, which guarantee a recovery real and effective of phytocompounds (Azmir *et al*., 2013). The most commonly used solvents are ethanol, methanol, dichloromethane, acetone, and water (Tapia-Torres *et al*., 2015). Microwave and Ultrasound Extractions have previously been done with this technology (Kayanan & Sagum, 2021) however it is the first time that its activity has been tested in both cancerous and non-cancerous cells and to know if the effect previously reported in conventional extractions is still maintained (Khor *et al*.,2018; Bhadresha *et al*., 2022).

Therefore, the objectives of this study were to identify the bioactive compounds obtained from aqueous and hydroalcoholic extracts of the MO leaves obtained using a combination of ultrasound and microwave and to evaluate their cytotoxic effect on cancer and non-cancer cells lines, besides evaluating the genotoxic behaviour of those bioactive compounds.

2. MATERIAL and METHODS

2.1. Metabolite extraction

2.1.1. *Vegetal material collection*

The vegetal material was collected from July to August 2017, from an experimental planting, at the Zaragoza Experimental Station, Zaragoza Coahuila Mexico, which belongs to the Universidad Autonoma Agraria Antonio Narro (UAAAN) and is located at -100° 55' west longitude and 28° 33' north latitude. Leaves of MO were removed from petioles and were left at room temperature for 5 days, for drying. Dried leaves were subsequently pulverized using an Oster brand industrial blender and stored under dark conditions.

2.1.2. *Extraction*

The obtained MO powder was homogenized and used to obtain five combinations of extraction variables: mass/volume ratio (g/mL) of 1:8, 1:12, and 1:16 in a mixture of EtOH / H_2O at 0, 30 and 70% at a volume of 500 mL (Table 1). Samples were sonicated at room temperature (25 ^{0}C) for 20 minutes using an ultrasound bath (Branson 5510), then subjected to a microwave treatment with a CEM Mars model 230/60, with a 5 min heating ramp until reaching 70 ° C, (power of 800 watts) that was maintained for 5 minutes. The extraction was carried out in triplicate. The obtained extracts were filtered, measuring the volume recovered, and stored at 4°C in amber glass bottles until further analysis.

Table 1. Design of extraction variables combination.

Mass/Volume ratio (g/mL)	Dissolvent EtOH-water (%)	Final ratio	Name
1:8	0/100	Mor - 1g/8 mL - 0% EtOH	Mor-8-0%
1:8	70/30	Mor- 1g/ 8mL - 70% EtOH	Mor-8-70%
1:12	30/70	Mor - 1g/12mL- 30% EtOH	Mor-12-30%
1:16	70/30	Mor - 1g/16 mL - 70% EtOH	Mor-16-70%
1:16	0/100	Mor - 1g/16 mL - 0% EtOH	Mor-16-0%

2.2. Identification of Compounds

2.2.1. *Chromatographic separation of phenolic compounds*

The separation of the polyphenolic fraction was carried out with the methodology expressed by Ascacio-Valdés *et al*., 2010. A column of Amberlite® XAD 16N resin was previously activated (10 minutes) in absolute methanol and packed as a stationary phase. Water was used as the first eluent to remove water-soluble compounds and subsequently eluted with ethanol to recover the polyphenolic fraction. Then, this fraction was placed on a glass Petri dish and dried at room temperature, without exposing it to light for 3 days, after that, the powder was collected in amber bottles and stored for later analysis.

2.2.2. *High efficiency liquid chromatography -mass spectrometry (HPLC-MS) analysis*

The compounds obtained after Amberlite XAD-16 chromatography were analyzed by HPLC (Varian Prostar), model 330 with a UV-visible diode array detector and coupled to a Varian brand mass detector, model 500-MS. A reversed-phase C18 column was used with a flow of 0.2 mL/min with a mass detection limit of 100 to 2000 m/z. For sample injection, these were prepared by weighing 10 mg of each extract and dissolving them in 1 mL of methanol. Samples were sonicated for 5 min at room temperature and finally filtered through 0.45 µm membranes.

2.3. Cytotoxicity

2.3.1. *Cell lines*

The lines used were: Hek 293 (293 [HEK-293] (ATCC® CRL-1573™)) (human cells of embryonic kidney tissue), Vero (ATCC® CCL-81™) (monkey kidney cell), NIH/3T3(ATCC® CRL-1658™) (fibroblast cell line) and Hep-G2 [HEPG2] (ATCC® HB-8065™) (transformed line, hepatocellular carcinoma). All cell lines were obtained from ATCC and were grown on Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 1% buffer HEPES, 1% non-essential amino acids, 1% penicillin/streptomycin and 1% pyruvate. The cultures with 90% confluence were treated with trypsin (5 minutes, 37°C), obtaining a cell suspension that was counted in the Neubauer chamber by the trypan blue exclusion method (Schwarzlin *et al*., 2016).

2.3.2. *Dilutions of phenolic compounds*

The tested concentrations of each phenolic compounds sample were 10, 50 and 100 µg/mL (1 mg of phenolic powder was weighed and proceeded to obtain dilutions). The phenolic powder was resuspended in 1mL of DMEM (culture medium) with 5 µL of DMSO (Houdkova et al., 2017).

2.3.3. *In vitro Cell Stimulation with Extracts and MTT Assay*

A cell density of 8.500 cells per well was plated in 96-well flat bottom plates in a volume of 100 µL medium (DMEM) per well. The cell densities were inoculated in triplicate for each cell line. Subsequently, plates were incubated for 24 h at 37°C with 5% CO_2 to allow cell adhesion.

After 24 h (incubation), the medium was replaced by 200 µL of culture medium with different concentrations of extract, then incubated for 24 h at 37°C with 5% CO_2.

After the last step, the culture medium was eliminated and 100 µL of new culture medium was added. Subsequently, 20 µL of MTT (5 mg / mL Phosphate Bufferes Saline (PBS)) were added. The plates were incubated for 4 h (37°C with 5% CO_2). Then, the supernatant was carefully removed and 100 µL of DMSO were added. The culture plates were read using a microplate reader at 570 nm (Thermo Scientific Multiskan FC). Cell viability was expressed as a percentage. The resulting values were taken as an indirect measure of viable cell mass, compared with untreated control cells. The percentage of viable cells was determined according to the following equation:

cell viability (%) = (absorbance of treated cell/absorbance of control cell) * 100.

2.3.4. *Genotoxicity*

DNA damage was determined by the comet assays (Cell Biolabs). Cells were seeded in 6-well plates (200,000 cells/well), and then incubated at 37º C and 5% CO2 for 24 h, to promote adhesion to the plate. Then, the medium was removed to place the dilutions (10, 50, and 100 µg/mL)of the extract prepared in 1 mL (final volume) of culture medium for 24 h. After that, the supernatant was removed and cells were washed twice with 1X saline solution. Subsequently, cells were removed with a scrapper, resuspended in PBS, and centrifuged, then, cells were resuspended in a final volume at a concentration of 1x10 5 cells/mL. After, cells were mixed with agarose (1:10) and 20 µL of the mixture were placed on the slide, which was incubated at 4 ºC for 15 min under dark conditions, and then submerged in the alkaline solution buffer for 1 h. Subsequently, the slides were immersed in the lysis buffer at 4 ºC for 1 h, the buffer was removed and replaced by an alkaline solution, then, the slide was left at 4ºC for 30 min. The alkaline solution was replaced twice by Tris-Borate-EDTA (TBE), and the slide was left for 5 min. Afterward, the slide was carefully transferred to the electrophoresis chamber, and covered with Tris Buffered Saline (TBS) solution, then 35 volts were applied for 15 min, at the end, the slide was kept in a horizontal position, and washed twice with water for 5 min. The slide was placed in 70% ethanol for 5 min, and green Vista Dye was added. DNA damage was assessed by fluorescence microscopy (Axioscope 5 LED, Carl Zeiss, Germany) with the specific filter for FITC. The images were analyzed with Comet Score 2.0 software to measure DNA fragmentation. Four replicates for each concentration were made.

2.4 Statistical Analysis

The means and standard errors of three replications of cell viability are shown in graphs. Differences between tested and control groups were determined by ANOVA using a statistical significance at $p < 0.05$, when it was needed, treatment mean differences were accessed using a Tukey´s multiple comparison test. Statistical analyses were performed using the SAS Software. Graph Pad Prism 6.0. was utilized to create the graphics.

3. RESULTS

3.1 Extraction and Identification of Polyphenolic Compounds

The color of the aqueous fractions varied from green to straw yellow, while the ethanolic fractions showed transparent colors with intense color saturation to transparent yellowish-green. In this study, 30 different compounds were identified with different combinations of mass/volume, ethanol relations, and a combination of ultrasound and microwaves, these compounds belong to 9 different families. Table 2 shows the distribution of the compounds from each m/v combination, as well as the retention time and compound family. It was observed that two compounds are present in all five extracts, quercetin 3-O-galactoside, and peonidin 3-O- (6 "-acetyl-galactoside), from flavanols and anthocyanidins groups, respectively.

Table 2. Polyphenolic compounds extracted from *Moringa oleifera* leaves using different combinations of mass/volume, ethanol relations and a combination of ultrasound and microwaves.

No. Compound	Family	Compound	8-0% (RT)	8-70% (RT)	12-30% (RT)	16-70% (RT)	16-0% (RT)	Reference
1	Catechins	(+)- Gallo catechin	2.26				8.89	
2		(+)- Catechin		2.22			2.49	Cuellar-Nuñez *et al*., 2018
3	Hydroxycinnamic acid	1-caffeoylquinic acid	17.37		18.87	18.67		Nouman *et al*., 2016
4		3-caffeoylquinic acid	18.07			19.22		
5		4-caffeoylquinic acid	24.90					Bing *et al*., 2015
6		Tyrosine p coumar oil	45.07					
7		Aspartate coffee oil					18.75	
8		3-p-coumaroylquinic acid					33.34	Nouman *et al*., 2016
9		Tartaricpcoumaroil acid					2.28	
10	Flavanols	Quercetin-3-O galactoside	32.93	34.88	34.58	25.48	37.32	Makita, 2017
11		Quercetin	40.77	42.27	41.99			Cuellar-Nuñez *et al*., 2018
12		Quercetin 3-O-glucoside				32.82	37.38	Nouman *et al*., 2016
13		Quercetin 3-O-acetyl-rhamnoside		37.46				
14		Quercetin 4'-O glucoside				32.82		
15		Kaempferol 3-O-galactoside-7-O- rhamnoside		20.27	23.63			
16		Kaempferol 3-O-rutinoside		29.47	25.84			Makita, 2017
17		Luteolin 7-O-rutoniside			28.57			
18		Kaempferol 3-O-rutinoside			33.50			
19	Antocianinas	Peonidin 3-O- (6 "-acetyl-galactose)	34.15	35.70	35.41	35.29	36.79	Nkechinyere Onyekwere & Felix I., 2014

20		Peonidin 3-O- (6 "-acetyl glucoside)	35.08	36.95	36.95	36.72	36.50	
21		Delphinidin 3-O galactoside				28.43		
22	Phenolic terpenes	Rosmadial					3.67	
23	Methoxy cinnamic acid dimers	5-5'-dehydro ferulic acid					29.18	
24	Metoxi flavones	Sinensetin		33.22			35.39	
25	Methoxy cinnamic acid	3-feruloylquinic acid					36.50	Nouman *et al*., 2016
26	Hydroxybenzoic acids	Gallic acid 3-O-gallate					58.66	
27	Dihydrochalcones	Phloretin			2.24			
28	Flavones	Apigenin 6-C-glucoside		33.89				Makita, 2017
29	Curcuminoids	Bisdemethoxyrcurcumin		51.56	51.46	51.35		Nurcholis *et al*. 2016
30	Tyrosols	p-HPEA-EA (possible)		20.27				

3.2. Cytotoxicity of *Moringa oleifera* Extract

To corroborate the cytotoxic effect, extracts were placed in contact with non-cancer cells (3T3, Hek293, and Vero) for 24 h and later the MTT assay was performed. The optical microscopy on 3T3 (before MTT assays) showed that control cells showed smooth and regular surfaces with normal morphology, whereas cells treated with the Mor-8-70%, Mor-12-30%, and Mor-16-70% extracts at 50 and 100 µg/mL showed changes in cell morphology due to cell death (data no shown). In Figure 1 is observed that in 50 and 100 µg/mL extracts concentration with any ethanol gradient (Mor-8-70%, Mor-12-30%, and Mor-16-70%), cell viability of three normal cells lines was affected compared to DMEM effects (assay medium with 5 microliters of DMSO) used as negative control. Extracts behaviour on Hek 293 and Vero cell lines were very similar, in this context, it is possible to observe also, that polyphenols from aqueous extracts (Mor-8-0% and Mor16-0%), which are in low or high concentrations (10-100 µg/mL) did not reduce cell populations, for the conversely, the percentage of cells viability incremented in the case of Mor-16-0%.

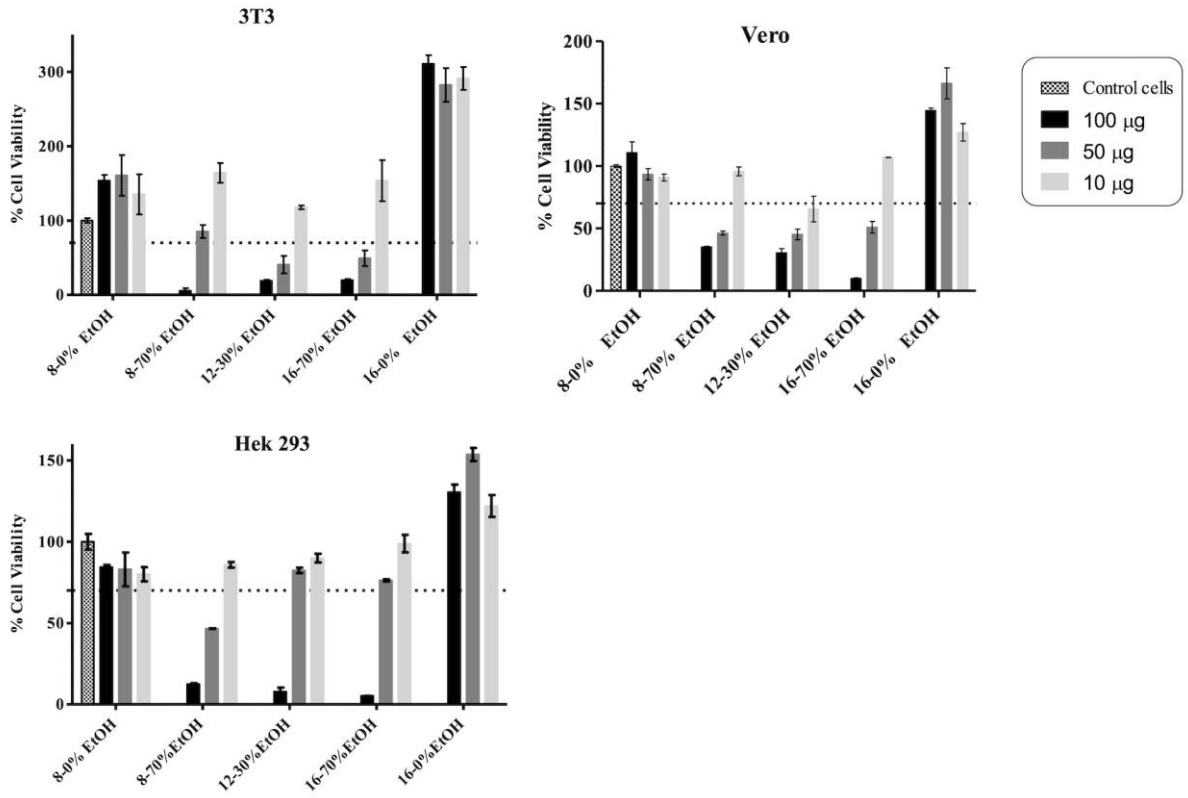

Figure 1. Cytotoxic effect of several dose (100, 50 y 10 µg) aqueous and hydroalcoholic extracts from *Moringa oleifera* on normal cells after 24 h of exposure. a) Effect on fibroblast cell (3T3), b) Effect on cells of embryonic kidney tissue (HEK-293) and c) Effect on monkey kidney cell (Vero).

Figure 2 shows the behaviour of HepG2 after 24 h of stimulation with each extract. Regarding the cells without treatment, the hydroalcoholic extracts show evident cytotoxicity at concentrations of 50 and 100 µg/mL without showing a significant difference between both doses ($p< 0.05$), while aqueous extractions do not show cytotoxicity according to the ISO standard 10993 maintaining cellular viability above 70% indicated as the permissible limit (Miller *et al*, 2017). On the contrary, the viability of the cells is increased particularly when the extraction volume is increased (16-0%).

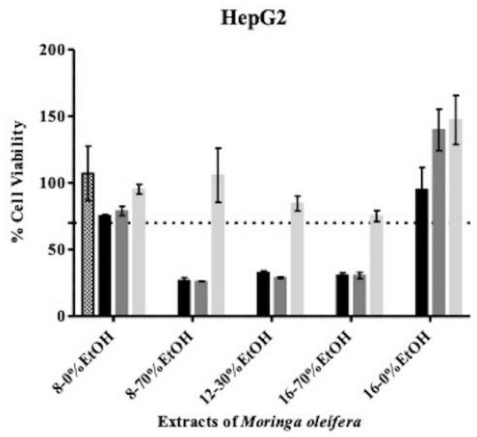

Figure 2. Effect of several doses (100, 50 and 10 µg) of aqueous and hydro-alcoholic of *Moringa oleifera* extract on Hep-G2 (transformed line, hepatocellular carcinoma) cytotoxicity after 24 h of exposure.

3.3. Hydro-Ethanolic Extracts Cause DNA Damage of HepG2 Cell Line

After learning that hydroalcoholic extracts affected cell viability, DNA damage was determined (Figure 3). Cells were exposed to 10, 50, and 100 µg/mL concentrations, with different m/v extraction ratios (8-70, 12-30, and 16-70). In all cases, greater DNA fragmentation was observed in relation to concentration, conferring a dose-response phenomenon. Regarding the % of DNA in the tail, it is observed that as the concentration increased, the percentage of DNA in the tail also increased, and this agrees with the results of the graphs of both, the length of the tail and the moment, which are parameters that are associated with the DNA fragmentation. In the 3 parameters (Tail DNA%, Length, and Moment) model, all treatments show significant differences in comparison to the control. The results obtained indicated a significant damage in the DNA of HepG2 cells after incubating with the ethanol extracts obtained from leaves.

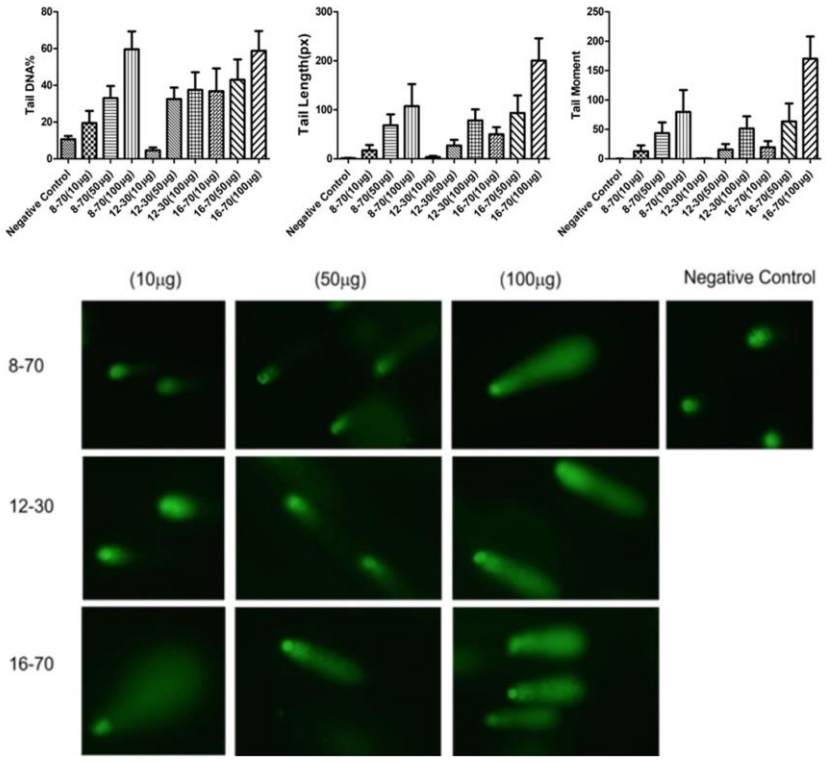

Figure 3. DNA Damage of HepG2 cell in response to the three m/v extract concentrations from *Moringa oleifera* extract.

4. DISCUSSION and CONCLUSION

The highest number and different polyphenols were extracted using a higher mass/volume ratio and low levels of ethanol (0 or 30%). The optimization of time in an extraction process is an important parameter to consider (Azmir *et al*., 2013), results showed that different polyphenols can be obtained for 30 min extraction time, compared to the literature that ranges up to 24 hours (Makkar & Becker, 1996). In this study, polyphenols were obtained under environmentally friendly extraction conditions such as water and ethanol and ultrasound and microwave technologies, contrasted with other authors who use other types of solvents and conventional technologies (Cuellar-Nuñez *et al*., 2018).

The flavonoids are the group of phytochemicals with the highest presence in the five extracts, however, they were more frequently found in the extracts with ethanol, Mor-8-70%, Mor-12-30%, and Mor-16-70% than in aqueous extracts Mor-8-0% and Mor-16-0%. In overview, the present study revealed a higher number of polyphenolic compounds in Mor-8-70% and Mor-16-0% extracts. The observed result could be due to different degrees of the polarity of the solvents used for the extraction of polyphenolic compounds and thus could contribute significantly to the cytotoxicity and pharmacology activity (Moyo*et al.*, 2012).

Quercetin 3-o-galactoside was found in all extracts. In a parallel comparison to other authors reported that this compound extracted from another plant source, and under various extraction stages, using solvents such as water, ether, and ethyl acetate provided a protective effect on PC12 cells against the cytotoxicity induced by hydrogen peroxide, and tert-butyl hydroperoxide (Liu *et al.*, 2005). Kaempferol 3-O-rutinoside was found in Mor-8-70% and Mor-12-30% extracts and these results are homologous to those reported in the literature from two *M. oleifera* species (Makita *et al.*, 2017). The extracts were obtained with ultrasound-assisted extraction (30 minutes) using 80% methanol as solvent. Several trials demonstrated the pharmacological properties of glycosides of quercetin and kaempferol, as well as the positive relationship between the consumption of vegetables with flavonoid content and a positive effect on health (Makita *et al.*, 2017). The identification of flavanols (Quercetin 3-O galactoside) in all extraction is consistent with previous reports in the literature, which is one of the most reported phytochemicals extracted from *M. oleifera* (França *et al*., 2017). Also, all hydroalcoholic or aqueous extracts contained anthocyanidins (Peonidin 3-O- (6 "-acetyl-galactoside) (Nkechinyere Onyekwere & Felix I, 2014). The compounds from the catechins group found were: gallocatechin, present in the extracts of Mor-8-0% and Mor-16-0%, and the catechin in the extracts Mor-8-70% and Mor-16-0%.

Cytotoxicity results are in concordance with the data (Mekonnen, Houghton & Timbrell, 2005), who evaluated the cytotoxicity of ethanol (80%) and aqueous extracts of *Moringa stenopetala* (Baker f.) Cufod. on HepG2 cells using the lactate dehydrogenase (LDH) test to measure cytotoxicity.

The results obtained indicated a significant decrease in HepG2 cells viability after incubating with the ethanol extracts obtained from leaves, while the aqueous extract of the leaves did not alter the levels of cellular viability, which suggests that ethanol extracts may be toxic (Mekonnen, Houghton & Timbrell, 2005). According to *in vitro* result, the aqueous extract does not cause cytotoxic damage; on the contrary, they induce cell proliferation, which may be associated with the presence of (+)-Gallocatechin, an exclusive molecule in this aqueous fraction, which has recently been reported to induce the synthesis of growth factors and collagen, promoting the regeneration of wounds (Vendidandala *et al*., 2021). These results are in agreement with literature where *Moringa* polyphenols obtained with water protect Hek293 cells against the damage generated by cadmium (Souid *et al*., 2020).

On the other hand, the presence of Bisdemethoxyrcurcumin exclusively in the ethanolic fraction suggests the participation of this molecule causing the death of cells in the 3t3 line, this agrees with the literature, since it induces apoptosis in fibroblasts (Jin *et al*., 2019), as well as Bisdemethoxyrcurcumin has been shown to induce apoptosis, and inhibit the proliferation of cancer cells (Liu *et al*., 2011; Li *et al.*, 2013).There are few reports in the literature of *in vitro* genotoxicity caused by *Moringa oleifera* extracts (Alkan *et al*., 2022), according to these results, *Moringa* extracts can cause DNA damage causing fragmentation, which is related to the mechanism of apoptosis already reported. In this study, it was found that ethanolic extracts cause DNA fragmentation, and this damage is related to the concentration of the extract.

Ultrasound and microwave technology at reaction times of 30 min demonstrated the obtaining of polyphenols from *Moringa oleifera* leaves identified by HPLC. In this study, it was observed that polyphenols obtained from aqueous extracts preserve a percentage of cellular viability higher than 70% established as the limit allowed by the Norma ISO standard 10993-5: 2009-10 test to evaluate the *in vitro* cytotoxicity of medical devices. Also, it was found that Mor-8-70%, Mor-12-30% and Mor-16-70% (hydroalcoholic extracts) had a high cytotoxic effect on all tested cell line, decreasing cell viability in a dose-dependent manner and that in HepG2 (cancer cell line) they induce apoptosis.

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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

Rosario Estrada contributes to cell experiments, **Diana Salazar** contribute extraction of polyphenols, **Aide Saenz** and **Juan Ascasio** contributed to the characterization of the compounds, **Raul Rodriguez**, **Carolina Flores** and **Cecilia Esparza** contributed to the experimental analyzes and to the writing of the article.

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Research Article

Epigenetic factors of the effect of UV-C and X-ray presowing seeds radiation exposure in *Matricaria chamomilla* L. genotypes

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Abstract: In a series of experiments using both X-ray and UV-C radiation exposure a parallel study of several pharmacological characteristics of the *Matricaria chamomilla* L. genotype group was carried out. The data concerning the changes in the productivity of pharmacological raw materials and stimulation of the synthesis of low molecular weight antioxidants as markers of secondary metabolism induction have been published earlier. In this study, the data on the relationship between the stimulation of the synthesis of secondary metabolites under different types of irradiation and the epigenetic changes in the plant organism are presented. It was shown that DNA methylation was switched to the *de novo* mode in plants of all studied genotypes of *M. chamomilla* under both types of irradiation. That indicates changes in the epigenetic program of the plant organism. Comparison of the epigenetic pattern between control and irradiated samples, based on the difference in DNA methylation patterns in terms of a statistical indicator, shows that there is no unambiguous relationship between the epigenetic changes and increasing yield of antioxidant synthesis. This is additional evidence of the diversity of metabolic rearrangements and adaptive strategies of the plant organism under radiation exposure even within one species.

1. INTRODUCTION

One of the directions of modern pharmacology is the identification of substances that are effective for medical practice and the stimulation of their formation in plant materials. The study of plant responses to stress factors has shown the possibility of stimulating the synthesis of secondary metabolic products, which include substances with antioxidant, anticancer, immunomodulatory, and anti-inflammatory effects (Dai & Mumper, 2010; Hassan *et al.*, 2017; Jan *et al.*, 2012; Kaur & Mondal, 2014; Klein *et al.*, 2018). The most effective approach is

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associated with the use of both ionizing and UV radiation with different wavelengths (Alothman *et al*., 2009; Hassan *et al*., 2017; Jan *et al*., 2012; Klein *et al*., 2018; Nocchi *et al*., 2020).

Presowing irradiation of medicinal plants has several biotechnological advantages. It is based on the systemic and long-term response of the organism to a single exposure. Presowing irradiation leads to remote effects, long-term changes in metabolism, and stimulation of the production of target substances in newly formed non-irradiated plant organs, for example, inflorescences during weeks or months after the exposure. It is crucial to utilize exposure in "low" doses that promote the production of target substances without diminishing the yield of pharmacological raw materials. Furthermore, such exposure can even stimulate it in certain species and varieties of plants (Sokolova *et al*., 2021).

A parallel study investigating various pharmacological characteristics of the *Matricaria chamomilla* L. genotype group was conducted through a series of experiments utilizing both X-ray and UV-C radiation exposure. The data concerning the changes in the productivity of pharmacological raw materials and stimulation of the synthesis of low molecular weight antioxidants as markers of secondary metabolism induction were published earlier.

Significant differences were observed in the stimulation of medicinal raw material (inflorescences) yield among different chamomile genotypes exposed to X-ray and UV-C irradiation (Sokolova *et al*., 2021). Also there was indicated a difference in the intensity of stimulation of the specific content of marker metabolites-antioxidants. This phenomenon suggests the involvement of various biophysical and biochemical mechanisms that influence sensitivity to specific types of radiation, along with potential variations in the development of protective reactions (Zhuk *et al*., 2021).

Recent-year research has significantly changed our understanding of the formation of the metabolic organism response to radiation. Switching of the activity of dozens and even hundreds of genes in the first minutes after the radiation exposure was established (Coleman *et al*., 2005). Significant rearrangements of the methylome under different modes of irradiation (Sokolova *et al*., 2013; Kravets *et al*., 2013, Kravets & Sokolova, 2020), large-scale changes in the proteome, stimulation of not only reparative and protective mechanisms, but also restructuring of the entire metabolon including various blocks of both primary and secondary metabolism (Danchenko *et al*., 2009; Klubicova *et al*., 2013) became known with the use of "omic" technologies in a radiobiological experiment. These facts not only have made a significant contribution to the transformation of the radiobiological paradigm, but are also important from a practical point of view.

The question arises as to the extent to which global metabolic changes in response to stress are linked to the release of practically valuable substances. Hence, one of the objectives of the research was to evaluate the correlation between the extent of generalized metabolic switching during pre-sowing irradiation of medicinal plant seeds and the yield of target metabolites. One possible approach to addressing this issue was to assess the overall epigenetic differences between control and irradiated samples, followed by comparison with antioxidant yield.

Currently, research on epigenetic mechanisms, such as gene expression mechanisms, is being conducted in various directions: from studying changes in chromatin organization to exploring mRNA translation and interference mechanisms under the influence of climatic factors and various stresses (Flores *et al*., 2013; Ng & Bird, 1999; Teif, 2015). DNA methylation stands out as the most studied chromatin modification, serving as a key factor in gene expression control and the primary mechanism of transgenerational "epigenetic memory" (Hauser *et al*., 2011). This process constitutes a crucial, inseparable component of the multilevel epigenetic regulation system (Flores *et al*., 2013; Xu *et al*., 2019). Consequently, in addressing several practical issues, the "distance" between methylation patterns is utilized as a measure of the impact on epigenomes by environmental or stress factors (Flores *et al*., 2013).

The aim of this research was to investigate changes in the methylome under radiation exposure of different physical natures and to evaluate the relationship between these changes and the yield of secondary metabolites. In addition to a visual analysis of electrophoregrams, valuable information was derived from the assessment of their quantitative characteristics, illustrating differences in the distribution of ITS-ISSR-PCR amplicons as restriction products in control and exposed variants.

2. MATERIAL and METHODS

Research was carried out on 8 genotypes of chamomile: 1 - generative breed of the mutant Perlyna Lisostepu (Ukraine); 2 - Quedlinburg variety (Germany); 3 - Goral variety (Slovenia); 4 - variety Azulena (Russia); 5 - Zlaty Lan variety (Poland); 6 - Perlyna Lisostepu variety (Ukraine). Additionally, unsorted material, specifically the edaphic ecotypes, were included in the study: 7- from the Golden Garden supplier (Ukraine), hereinafter referred to as the Golden Garden ecotype; 8 – from the supplier Seed Era (Ukraine), hereinafter referred to as the Seed Era ecotype. The varietal material was obtained from the Central Research Station of Medicinal Plants of the Institute of Agroecology and Nature Management of the National Academy of Sciences of Ukraine in Lubny. The experiment was repeated three times. Dry seeds were exposed using the RUM-17 X-ray installation (Russia) at a dose of 10 Gy, with a dose rate of 1.42 cGy/s. The choice of the X-ray irradiation dose was based on the authors' results, confirmed by a patent (Shilina *et al*., 2018). UV-C exposure was conducted at a dose of 10 kJ/m² using an OBM-150 M installation (Ukraine) with two Philips Special TUV 30 W lamps (Netherlands). The choice of the UV-C irradiation dose was based on preliminary studies, also confirmed by a patent (Kravets *et al*., 2021). DNA methylation research was carried out through restriction analysis followed by ISSR-ITS-PCR (Ausubel, 2004; Hernández *et al*., 2013).

DNA was isolated from the plant vegetative mass during the flowering phase using a set of reagents ZymoResearch (Quick-DNA Plant/Seed Miniprep Kit) according to the manufacturer's protocol. The nativeness of the isolated DNA was checked in a 1.7% agarose gel with TBE buffer in the presence of ethidium bromide and visualized on a UV transilluminator (Figure 1) When setting up electrophoresis 5 µL of DNA solution was put into the "pocket" of the gel. GeneRuler 50 bp was used as a molecular weight marker. Three types of markers were used for PCR: ISSR-5 (CAC-ACA-CAC-ACA-CAC-AAC), ITS (ITS1 (TCC-GTA-GGT-GAA-CCT-GCG-G) and ITS4 (TCC-TCC-GCT-TAT-TGA-TAT-GC) and ready-to-use PCR MIX 2x-R («Neogene», Ukraine).

ISSR–PCR reaction mix 25 µL volume included: 12.5 µL PCR MIX 2x-R), 1.75 µL ISSR/OPA09 markers, 5.75 µL deionised water and 5 µL genome DNA. ITS-PCR reaction mix 25 µL volume included: 12.5 µL PCR MIX 2x-R), 0.6 µL ITS1 marker, 0.6 µL ITS4 marker, 5.75 µL deionised water and 5 µL genome DNA. Amplification with ISSR-primers included stages: initial denaturation 4 min under 94 ºC, 40 cycles; denaturation under 94 ºC – 45 sec., annealing under 52 ºC – 45 sec., elongation under 72 ºC – 45 sec.; final elongation 7 min under 72 ºC. Amplification with OPA09-primers included stages: initial denaturation 5 min under 94 ºC, 40 cycles; denaturation under 94 ºC – 40 sec., annealing under 36 ºC – 40 sec., elongation under 72 ºC – 2 min.; final elongation 10 min under 72 ºC. Amplification with ITS-primers included stages: final denaturation 5 min under 94 ºC, 40 cycles; denaturation under 94 ºC – 40 sec., annealing under 52 ºC – 40 sec., elongation under 72 ºC – 2 min; final elongation 10 min under 72 ºC.

For restriction analysis two types of restrictase-isoschizomers were used: MspI (C…C*GG and C…CGG) and HpaII (C…CGG), (Fermentas, Litva). Enzymes HpaII and MspI cleave the CCGG sequence, but the action of HpaII is directed only at unmethylated cytosine. MspI, as an isoschizomer of HpaII, cleaves both methylated and unmethylated sites. Restriction reaction with endonuclease MspI was in volume 20 µL, included 0.6 u of enzyme (0.6 µL), 6 µL

10xBuffer Tango, 500 ng genome DNA (5 µL), and 5.75 µL deionised water. Reaction mix for the restriction with HpaII in volume 20 µL included 0.2 u of enzyme (0.2 µL), 6 µL 10xBuffer Tango, 500 ng genome DNA (5 µL), 8.8 µL of deionised water. The reaction was done 3 h under 37 ºC, termination of the reaction – 20 min under 65 ºC (for HpaII) and 20 min under 80 ºC (for MspI).

The products of PCR and restriction were separated in 1.7% agarose gel with TBE buffer in the presence of ethidium bromide and visualized on UV transilluminator. When setting up electrophoresis the same volume of PCR and restriction products (5 µL) was add into the gel "pocket". GeneRuler 50 bp was used as a molecular weight marker.

As an indicator of the difference between the set of electrophoregrams of control and exposed samples the indicator D = epigenetic distance (hereinafter - ED) was used. It was calculated similarly to the estimation of the genetic distance according to Nei (Nei, 1974).

At the same time D = 0 with absolute coincidence between the set of bands in the electrophoretic spectra that are compared; D = 1 under conditions of complete difference between the set of amplicons in the electrophoretic spectra.

3. FINDINGS

Assessment of the isolated DNA's quality confirms its high purity and integrity (Figure 1) in both control and exposed experimental variants, enabling further restriction analysis. Analysis of the data obtained revealed that both ionizing and UV-C pre-sowing irradiation of seeds led to a transition in the DNA methylation process from maintenance to de novo mode across all genotypes during plant formation (Figure 2 and 3).

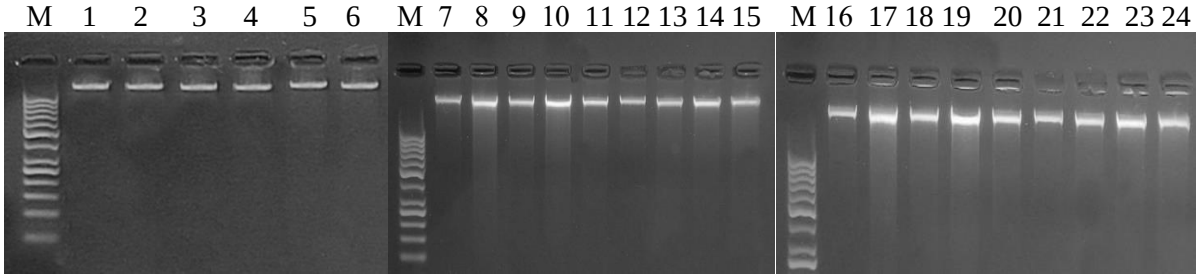

Figure 1. Electrophoregram of the isolated DNA nativity.
M – molecular weight marker GeneRuler 50 bp, 1 – genotype 1, control; 2 – genotype 1 + UV-C; 3 – genotype 1+ x-ray; 4 – genotype 2, control; 5 – genotype 2+ UV-C; 6 – genotype 2+ x-ray; 7 – genotype 3, control; 8 – genotype 3+ UV-C; 9 – genotype 3+ x-ray; 10 – genotype 4+ control; 11 – genotype 4+ UV-C; 12 – genotype 4+ x-ray; 13 – genotype 5, control; 14 – genotype 5+ UV-C; 15 – genotype 5+ x-ray; 16 – genotype 6, control; 17 – genotype 6+ UV-C; 18 – genotype 6+ x-ray; 19 – genotype 7, control, 20 – genotype 7+ UV-C, 21 – genotype 7+ X-ray; 22 – genotype 8, control; 23 – genotype 8, UV-C; 24 – genotype 8 + x-ray

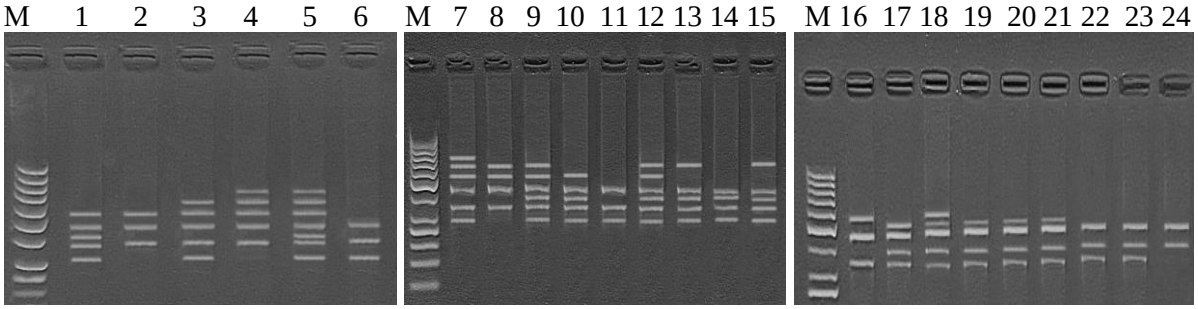

Figure 2. Amplification of HpaII restricts with ISSR markers (Designation as in Figure 1).

Significant changes in methylation of HpaII restriction sites were observed for minisatellite sequences (Figure 2) across all genotypes. In the mutant Perlyna Lisostepu variety, methylation patterns (Figure 2, positions 1-3) were altered relative to the control variant under both UV-C and X-ray exposure. Notably, relatively large amplicons of 600, 500, and 400 bp were maintained for all variants. However, amplicons of 450 and 300 bp disappeared under UV-C exposure, indicating the removal of methylation in specific areas of the minisatellite sequence. Upon exposure to X-rays, a "short" amplicon of 300 bp was retained, while a relatively "long" one of 700 bp appeared, also suggesting the removal of methylation in certain areas of the minisatellite sequence, given that HpaII restrictase cleaves sites with unmethylated cytosine (C…CGG).

Similarly, changes in methylation of HpaII restriction sites were observed for minisatellite sequences in the Quedlinburg variety (Figure 1, positions 4-6). Amplicons of 500 and 400 bp were retained for both control and exposed variants. Under UV-C exposure, "long" amplicons of 600-800 bp were maintained, and "short" ones of 300 and 450 bp appeared. Additionally, amplicon 300 bp appeared under X-ray exposure, although with this type of irradiation, all "long" amplicons disappeared.

For the Goral variety (Figure 2, positions 7-9), changes in HpaII restriction site methylation within minisatellite sequences under UV-C and X-ray irradiation were also noted. Across all variants, amplicons of 700, 600, 500, and 350 bp were maintained.

The most significant difference was observed for the Azulena variety under UV-C exposed variants (Figure 2, positions 10-12). Amplicons of 500, 350, and 300 bp were present for both control and exposed variants. Under UV-C exposure, the longest band of 600 bp disappeared, reflecting an increase in methylation yield in the satellite DNA sequence. Conversely, under X-ray exposure, a “long” amplicon of 700 bp appeared, indicating the removal of methylation in certain areas of the minisatellite sequences.

Similarly, Zlaty Lan variety plants reacted to irradiation, with the largest change in methylome observed under UV-C exposure. Across all variants, only amplicons of 500, 400, 350, and 300 bp were retained (Figure 2, positions 13-15). Perlyna Lisostepu variety was characterized by the appearance of additional amplicons of 550 and 400 bp under both types of exposure (Figure 2, positions 16-18) (Figure 2, positions 16-18).

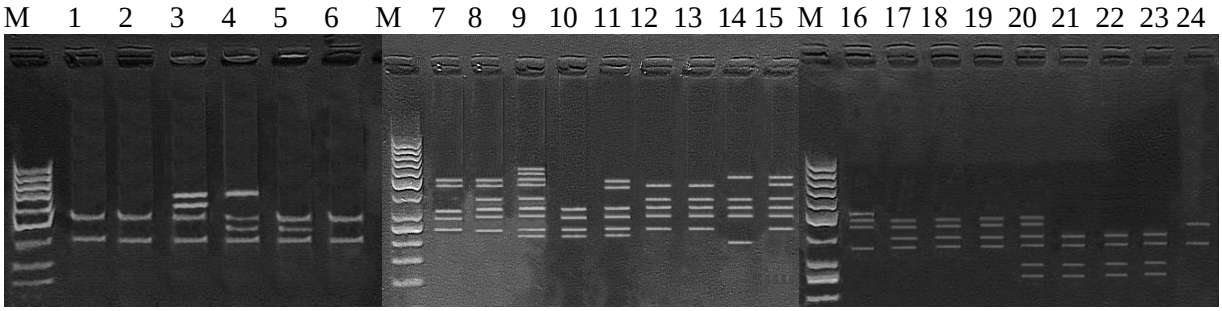

Figure 3. Amplification of MspI restricts with ISSR markers (Designation as in Figure 1).

There were no changes observed in the methylome of the Golden Garden variety under both types of radiation exposure (Figure 2, positions 19-21). The Seeds Era variety demonstrated the same set of amplicons under UV-C exposure as the control, but under X-ray exposure, the 350 bp amplicon disappeared (Figure 2, positions 22-24). Amplification of HpaII restricts with ITS markers did not indicate any difference between the amplicon sets in both control and exposed variants.

Significant changes in methylation of MspI restriction sites were also observed within minisatellite sequences (Figure 3). For example, in the mutant Perlyna Lisostepu variety, amplicons of 550 and 400 bp were maintained for all variants, and two "long" amplicons of 800 and 700 bp appeared additionally after X-ray exposure (Figure 3, positions 1-3). Similarly, for the Quedlinburg variety, amplicons of 550 and 400 bp were maintained for all variants. However, the "long" amplicon of 800 bp disappeared under both types of exposure, and the 450 bp amplicon disappeared only under X-ray irradiation (Figure 3, positions 4-6).

Significant rearrangements of the methylome were observed for the Goral variety, where amplicons of 550, 500, 350, 300, and 250 bp were detected for all variants (Figure 3, positions 7-9).

For the Azulena variety, the most significant changes (Figure 2, positions 10-12) relative to the control were observed under UV-C irradiation. Amplicons of 500, 350, and 300 bp were presented in both control and irradiated variants. Under UV-C exposure, the longest amplicon of 600 bp disappeared, indicating an increasing methylation level for this satellite sequence. Conversely, under X-ray exposure, a long amplicon of 700 bp appeared, indicating the removal of methylation in certain areas of the minisatellite sequences.

For the Zlaty Lan variety, general amplicons of 400, 350, and 300 bp were observed for both control and exposed variants. Under UV-C exposure, a new band of 550 bp appeared (Figure 3, positions 13-15). The 600 bp amplicon appeared only in the control variant of the Perlyna Lisostepu variety and disappeared under both types of irradiation. Moreover, bands of 550, 500, and 350 bp were common for all variety variants (Figure 3, positions 16-18).

For the Golden Garden ecotype, amplicons of 400 and 350 bp were common for all variants of the experiment. Under UV-C irradiation, amplicons of 550, 500, 400, and 350 bp were maintained, and amplicons of 300 and 250 bp appeared in the control variant. The same amplicons also appeared under X-ray irradiation. Longer amplicons presented in control and UV-C exposed variants disappeared (Figure 3, positions 19-21).

The 350 bp amplicon was common for all variants of the Seeds Era variety. Under UV-C exposure, there were no changes within the set of 4 amplicons relative to the control variant, but under X-ray exposure, the additional 500 bp amplicon appeared (Figure 3, positions 21-24).

Thus, no changes in the methylation pattern of transcribed DNA sequences were detected for most Perlyna Lisostepu genotypes. The appearance of a new 600 bp amplicon during amplification of MspI restricts with ITS markers was detected only under UV-C exposure of the Perlyna Lisostepu variety.

At the same time, changes in DNA methylation patterns, mostly through satellite DNA sequences, have been significant for gene expression regulation. Depending on current data, satellite DNA is considered to play an important role in chromatin remodeling, altering its effect on denaturation, and increasing the availability of transcription and repair factors. Thus, it could immediately affect DNA sensitivity to radiation damage and determine epigenetic control of metabolic processes (Flores *et al*., 2013; Kravets & Sokolova, 2020; Ng & Bird, 1999; Teif, 2015; Xu *et al*., 2019).

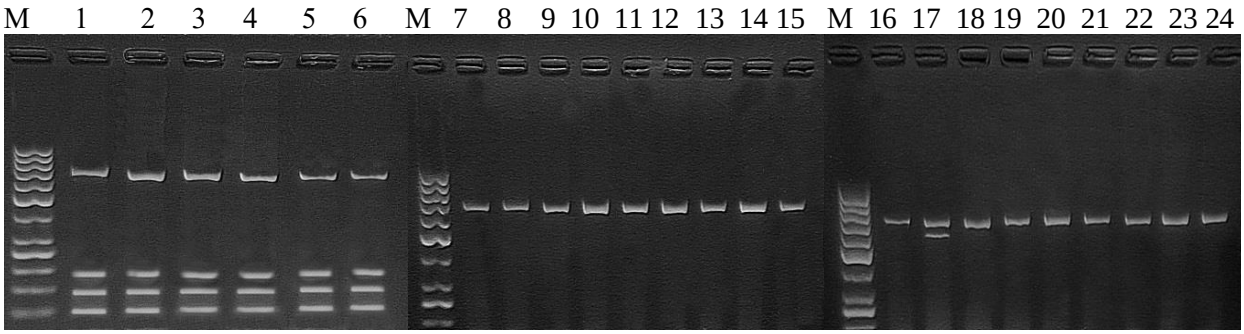

Figure 4. Amplification of MspI restricts with ITS markers (Designation as in Figure 1).

The restriction analysis with restrictases - isoschizomers did not indicate any general trend of changes in the overall DNA methylation yield under UV-C or X-ray exposure across the studied genotypes, which could have been reflected in a common increase or decrease in the yield of "long" amplicons. However, significant changes in the DNA methylation pattern were observed. These results confirm our previous findings and those of other studies, indicating that phenotypic variation is not attributed to the overall DNA methylation yield but rather to its pattern (Xu *et al*., 2019; Kravets & Sokolova, 2019; Kravets & Sokolova, 2020).

4. DISCUSSION and CONCLUSION

The comparison of the epigenetic distance between control and exposed variants with the experimental results of pharmaceutical raw material yield and antioxidant content is presented in Table 1 (Sokolova *et al*., 2021; Zhuk *et al*., 2021). The highest deviation rates from the control methylation pattern under both types of exposure were observed for the mutant of the Perlyna Lisostepu variety. Additionally, this genotype exhibited greater responsiveness to UV-C irradiation in terms of pharmaceutical productivity.

Table 1. Epigenetic distance of exposed variants relative to the control.

Variety	D-epigenetic distance of exposed variants relative to the control		Variety	D- epigenetic distance of exposed variants relative to the control	
	10 Gy	10 kJ/m^2		10 Gy	10 kJ/m^2
Mutant of Perlyna Lisostepu variety	0.083	0.04	Zlaty Lan	0.01	0.01
Quedlinburg	0.01	0.04	Perlyna Lisostepu	0.04	0.04
Goral	0.02	0.03	Golden Garden	0.02	0.01
Azulena	0.04	0.01	Seed Era	0	0.03

For the Quedlinburg variety, a higher distance between the methylation patterns of the control and exposed variants was observed under UV-C exposure. The yield of inflorescences was higher under X-ray exposure, and an increase in the specific yield of flavonoids was observed under both types of irradiation.

In the case of the Azulena variety, there is a correlation between a high rate of DNA methylation pattern deviation under X-ray exposure relative to the control (0.04) and an increase in the yield of both inflorescences and flavonoids. The Perlyna Lisostepu variety exhibited the highest specific content of flavonoids in the control variant. A significant increase in the indicator and the yield of inflorescences was observed under both types of exposure, accompanied by similar deviations in the DNA methylation pattern between exposed variants and the control.

The comparison of the degree of deviation in the methylation pattern with indicators of pharmaceutical productivity indicates the absence of a straightforward relationship between

these quantitative characteristics. This result suggests a diversity even within one species. In the context of biotechnological implementation of various types of irradiation, this diversity should be considered as a basis for exploring a wider range of useful substances that could be produced by plants under stress exposure.

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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

Daryna Sokolova: Investigation, Methodology, Visualization, Software, Writing original draft. **Vladyslav Zhuk**: Investigation, Methodology, Visualization, Software. **Ludmila Hlushchenko**: Resources. **Alexandra Kravets**: Investigation, Methodology, Formal Analysis, Validation and Writing original draft, supervisor.

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Research Article

Chemical composition of the essential oils isolated from *Phlomis olivieri* Benth (Lamiaceae) in four western provinces in Iran

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Abstract: *Phlomis olivieri* Benth is a valuable medicinal plant in the flora of Iran and can be collected in different parts of the country. To date, no comprehensive phytochemical research has been done on it in different parts of Iran. In this research, the essential oils of this medicinal plant were investigated in eight locations of western provinces of Iran. For this, aerial parts of the plant were collected in its natural habitats, dried under the shade condition (approximately 25°C), and then powdered. The essential oil was isolated by Clevenger apparatus and chemically analyzed by a Gas Chromatography (6890N)-Mass Spectroscopy (5973N) device in Payame Noor University (PNU), Hamedan, Iran. Except for some cases (EC, TNV, and K) there were no significant differences in the characteristics of the soil of the investigated areas. In the chemical structure of this plant, 17 and 11 constituents were identified in A1 and A2, 17 and 18 in B1 and B2, 17 and 15 in C1 and C2, and 21 and 15 in D1 and D2 locations of four western provinces in Iran. The results showed that caryophyllene (A1, A2, B1 and B2), 1H-cyclopenta [1, 3] cyclopropa [1, 2] benzene (C1), naphthalene, decahydro-4a–methyl (C2), estra-1, 3, 5(10)-trian-17a-ol (D1), and n-hexadecanoic acid (D2) were dominant constituents. Therefore, this valuable medicinal plant has diverse chemical constituents in the studied locations in Iran which should be considered from different aspects.

1. INTRODUCTION

Various medicinal plants are used by people in developed and developing countries all over the world. For this reason, they have great therapeutic value. People around the world use 50,000 to 80,000 flowering plants for medicinal purposes. In other words, medicinal and aromatic plants are consumed by 70%-80% of the world's population (Uritu *et al.*, 2018). *Phlomis* L. is a large genus in the Lamiaceae, with over 100 species distributed throughout Euro- Asia and North Africa continents. This genus represented by 17 species, witch 10 species are endemic in Iran (including *P. olivieri*). *P. olivieri* grows wildly in north, west and center of Iran . In differend countries, they have various uses for human health. This plant is generally used as an herbal tea to treat gastrointestinal troubles and promote good health by protecting the liver, kidney, bone and cardiovascular systems (Amor *et al.*, 2009). Many of them are fragrant in all

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parts and contain widely used medicinal herbs including basil, mint, rosemary, sage, savory, marjoram, oregano, hyssop, thyme, lavender and perilla. Some species of this family are shrubs and trees and rare of them are vines (Raja, 2012).

Phlomis olivieri (Figure 1) is an herbaceous and perennial plant with height between 25-65 cm. Its stem is multiple and branched with a white or yellow coating. The leaves are simple, opposite, and their base has a more or less broad petiole up to 10 cm long on a shorter stem. The basal and middle leaves are ovate or oblong with a cordate base or a cordate cut with a flat tip. The density of hair is higher on the lower surface of the leaves, the color of which is variable, and the upper leaves are pointed and slightly curved at the edge. The terminal leaves are small, short or wedge-shaped at the base and have reticulated veins. Its inflorescence is a spike and includes flower cycles with multiples of 2 to 10. The lower leaves of the plant are longer than the flowers. The length of the leaves is 5 to 9 mm. The calyx is tubular, swollen with prominent veins with 14-20 mm long. In calyx the length of the teeth is 4-6 mm. The calyx has two yellow edges and 25-35 mm long. The upper edge of the flower cup has two lobes and the lower one has three. The outer surface of the flower cup is covered with star-shaped felt hairs on the upper lip, and inside of them covered with a ring of hairs. The fruit of this medicinal plant has four seeds with a three-sided and round tip (Ghassemi *et al.*, 2001; Mohammadifar *et al.*, 2015).

Figure 1. General appearance of *P. olivieri*.

Essential oils are concentrated hydrophobic liquid contains volatile plant chemical compounds that evaporate easily at normal temperatures. The terpene derivatives present in essential oils are essentially hemiterpenes, monoterpenes, and sesquiterpenes which can be little or very volatile and thermolabile and may be easily oxidized or hydrolyzed depending on their respective structure (Turek & Stintzing, 2013). An essential oil contains aroma of plants whose properties are derived. Essential oils are generally isolated by hydro-distillation method often by using steam. Other processes include expression, solvent extraction, sumatra, absolute oil extraction, resin tapping, wax embedding, and cold pressing (Hanif *et al.*, 2019). Essential oils have been produced in plants for many years and are also called as plant secondary metabolites. These compounds have contact, fumigant, repellent, and anti-nutritional effects (Asadi *et al.*, 2019). For years, plants have used these compounds during co-evolution to repel invading insects. Plant essential oils can have changes in different conditions. One of the factors that can be very involved in this field is the type of weather and region (Figueiredo *et al.*, 2008; Mehalaine *et al.*, 2021). Soil characteristics are influential in this field, and in rich soils, the number and variety of secondary compounds is greater (Khalid & Ahmed, 2021). These factors have significant effects on the quality and quantity of plant essential oils. For this purpose, this

study was conducted in different western provinces of Iran in order to investigate the changes of secondary compounds in this important medicinal plant.

2. MATERIAL and METHODS

2.1. Identification of Species

Identification of the species for the medicinal plant *P. olivieri* was done by sending its mature sample to one of the botany specialists in the Herbarium of Payame Noor University (PNU), Hamedan, Iran.

2.2. Soil Sampling

The soil of different localities from natural habitats of *P. olivieri* was removed from a depth of 30 cm. After mixing the samples with each other and separating the pebbles, finally 2 kg sample was separated from each area and sent to the soil science laboratory of research center and natural resources of Hamadan province (Carter & Gregorich, 2007). In this way, the physical and chemical characteristics of the soil from each locality were determined.

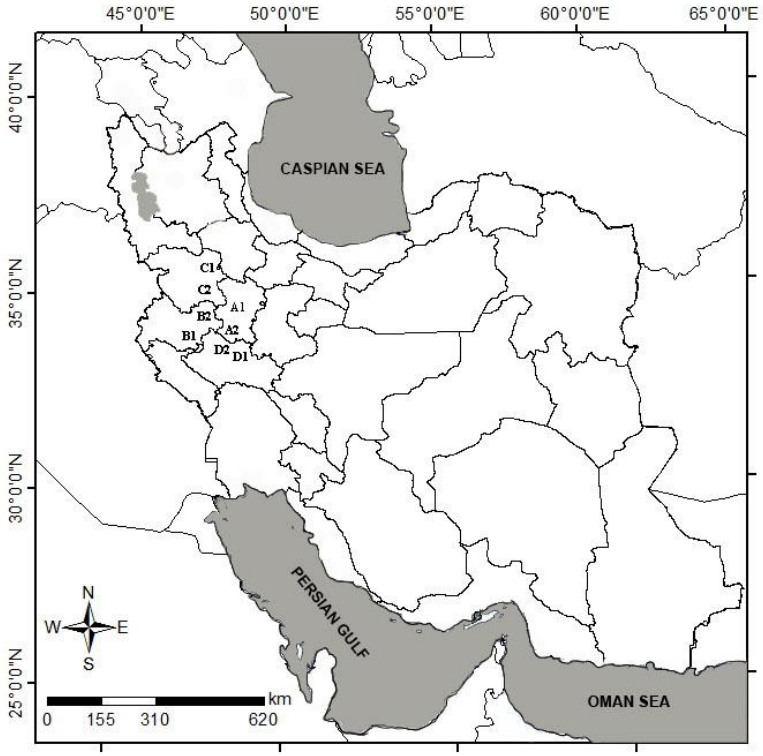

Figure 2. Different localities of studied regions in western of Iran.

2.3. Isolation of Essential Oil

In this research, the aerial parts of *P. olivieri* were collected from its natural habitats in eight localities of four western provinces in Iran, during 2022 (Figure 2) (see Table 1). After drying the collected plants at a temperature of 25°C, they were transferred to the laboratory and their essential oils were isolated. For this purpose, the collected samples were powdered. Then 50 g of their powder was added along with 500 ml of the deionized water inside balloon of the Clevenger apparatus (1 liter) (Figure 3) (Babaee Ghaghelestany *et al.*, 2020). About four hours after starting the heating, the essential oils formed as a light green layer on top of water. Na_2So_4 (sodium sulfate compound) was used to remove water and purify the essential oils (Asadi, 2022). Finally, the purified essential oil was stored in special 5 ml microtubes covered with aluminum foil inside a refrigerator (about 4 °C) until GC-MS analysis (Asadi *et al.*, 2018; Negahban *et al.*, 2007; Parsia Aref & Valizadegan, 2015; Samsam Shariat 2007).

Table 1. List of localities which *P. olivieri* collected of them.

Country	Locality	Latitude	longitude	Altitude (m)	Voucher
Iran	A1	34° 45' 46" N	48° 26' 22" E	2500	Asgari (HPNU) 35140
	A2	34° 11' 18" N	48° 22' 37" E	2200	Asgari (HPNU) 35141
	B1	34° 37' 43" N	47° 32' 28" E	1800	Asgari (HPNU) 35142
	B2	34° 28' 52" N	47° 41' 36" E	1380	Asgari (HPNU) 35143
	C1	35° 17' 47" N	46° 57' 18" E	2300	Asgari (HPNU) 35144
	C2	34° 58' 42" N	47° 54' 53" E	2450	Asgari (HPNU) 35145
	D1	33° 26' 58" N	49° 12' 50" E	1540	Asgari (HPNU) 35147
	D2	33° 51' 51" N	48° 15' 45" E	1750	Asgari (HPNU) 35146

Figure 3. Essential oil isolation by Clevenger apparatus.

2.4. Chemical analysis

Quantitative and qualitative components of the essential oils were detected by Agilent technology gas chromatography (6890N)-Mass Spectroscopy (5973N) (made in the USA) with the following specifications (Figure 4):

Program:
- Mode: Splitless
- Gas: Helium
- Heater: 220
- Split ratio: 0

Column:
- Mode: Const Flow
- Detector: MSD
- Flow:1ml/min

Oven:
- C/min (--), Next C (60), and holdmin (3)
- C/min (20), Next C (220), and holdmin (3)

Aux:
- Setpoint: 280

MS:
- Mode: Scan 2-800

Identification of the components was based on comparison of their mass spectra with those of internal Wiley GC-MS spectral library, or with published mass spectra (Adams *et al.*, 2001).

Figure 4. GC-MS analyzer device in Razi University of Kermanshah, Iran.

3. RESULTS

3.1. Soil features

In examining the soil of different regions, the differences were observed which are given in Table 2. In the case of EC, the difference between A2 location and the rest was evident, while there was no significant difference about this parameter in the other seven regions. In terms of pH, there was no clear difference among eight studied locations. About TNV, the highest and lowest percentages were determined in D2 and B1 locations with 47% and C2 (2%) respectively. In organic compound (OC), the highest and lowest values were observed in C1 and A2. The highest soil phosphorus (P) was determined in C2 with 51 ppm and D2 with 9 ppm. In the case of potassium (K), the highest and lowest values were determined as 512 ppm and 222 ppm in C2 and D2. About the total nitrogen (N), there was no difference among the locations studied. On the soil texture, except for two localities D1 and D2, the rest had clay-loam texture.

Table 2. Soil characteristics of the investigated localities.

Locality	EC (ds/m)	pH	%TNV	%OC	P (ppm)	K (ppm)	Total N (%)	Texture
A1	2.70	8.02	5	0.31	11	246	3	Clay loam
A2	22.2	7.98	7	0.20	16	282	2	Clay loam
B1	1.93	8.16	47	0.62	10	246	6	Clay loam
B2	2.44	8.07	39	0.23	18	307	2	Clay loam
C1	2.40	8.00	14	0.7	27	331	7	Clay loam
C2	2.64	7.95	2	0.51	51	512	5	Clay loam
D1	0.80	8.05	24	0.31	18	464	3	Clay
D2	2.63	7.94	47	0.35	9	222	3	Clay

3.2. Localities A1 and A2

In locality of A1, 17 constituents were identified (Table 3) while caryophyllene was dominant (peak 4, retention time of 9.585 min, 24.093% of total). Also, 12-methyl-E, E-2, 13-octadecadien-1-ol (peak 16, retention time of 11.447 min, 0.290% of total) was minimum constituent. Furthermore, 11 constituents were detected in locality of A2 (Table 4). Accordingly, caryophyllene was determined as main constituents (peak 2, retention time of 9.585 min, 30.371% of total). In opposite, Azulene, 1, 2, 3, 3a, 4, 5, 6, 7-octahydro in peak 6

with retention time of 10.047 min and 2.327% on total was detected as minimum of them. According to this, caryophyllene was a dominant constituent in two localities of A1 and A2. Their chromatogram is shown in Figure 5.

Table 3. Chemical compounds in essential oil of *P. olivieri* collected from locality A1.

Peak	Compound	Retention time (min)	Peak height	Percentage of total
1	Decane, 2, 4, 6-trimethyl-	5.810	189111	1.223
2	Heptadecane, 2, 6, 10, 14-tetramethyl-	6.861	121985	0.836
3	1H-Indene, 1-ethyloctahydro-7a-methyl	8.105	110468	0.514
4	Caryophyllene	9.585	3648417	24.093
5	(E)-â-Famesene	9.689	3648417	18.445
6	Humulene	9.823	432464	3.551
7	Germacrene D	9.997	2295870	15.489
8	1S,2E,6E,10R)-3, 7, 11, 11-Tetramethylbicyclo	10.091	951466	8.382
9	1-Isopropyl-4, 7-dimethyl-	10.223	201134	3.057
10	Formic acid, 3, 7, 11-trimethyl-1	10.456	104357	1.079
11	Dodecanoic acid	10.531	557774	6.195
12	n-Hexadecanoic acid	10.575	323764	2.989
13	Caryophyllene oxide	10.769	599228	6.370
14	1-Heptatriacotanol	11.212	100059	1.541
15	Cholestan-3-ol, 2-methylene	11.333	99966	2.099
16	12-Methyl-E,E-2,13-octadecadien-1-ol	11.447	29663	0.290
17	Tetradecanoic acid	12.191	260947	3.848

Table 4. Chemical compounds in essential oil of *P. olivieri* collected from locality A2.

Peak	Compound	Retention time (min)	Peak height	Percentage of total
1	â-Ylangene	9.322	181133	2.921
2	Caryophyllene	9.585	2762424	30.371
3	cis-â-Farnesene	9.687	172618	3.030
4	Humulene	9.822	506214	5.282
5	Germacrene D	9.997	2086704	21.329
6	Azulene, 1, 2, 3, 3a, 4, 5, 6, 7-octahydro	10.047	231596	2.327
7	(1S, 2E, 6E, 10R)-3, 7, 11, 11-Tetramethylbicyclo	10.091	238751	2.381
8	1S, 2S, 5R-1, 4, 4-Trimethyltricyclo	10.537	342454	6.130
9	n-Hexadecanoic acid	10.651	412835	14.526
10	Caryophyllene oxide	10.768	460229	8.787
11	9, 12-Octadecadienoic acid	12.461	33581	2.915

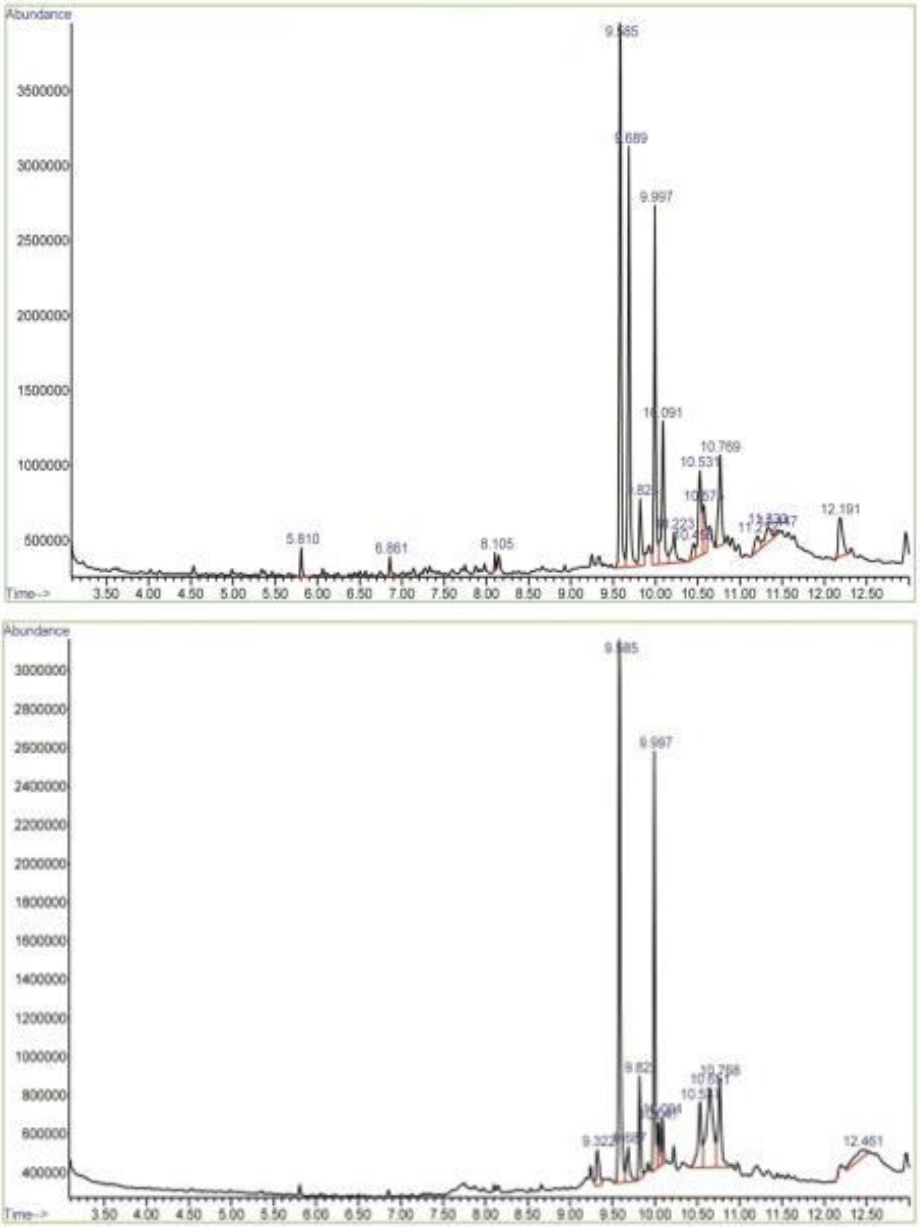

Figure 5. Chromatogram of chemical compounds from *P. olivieri* in localities of A1 and A2.

3.3. Localities B1 and B2

Totally, 17 constituents were identified in locality of B1 (Table 5), among which caryophyllene was dominant (peak 6, retention time of 9.583 min, 18.550% of total). Also, Heptadecane, 2, 6-dimethyl in peak 2 with retention time of 6.860 min and 1.244% on total being minimum constituent. Futhermore, 18 constituents were detected in locality of B2 (Table 6), among which caryophyllene being a dominant constituent (peak 7, retention time of 9.582 min, 25.905% of total) while tetradecane, 2, 6,10-trimethyl detected in peak 10 with retention time of 9.891 min and 1.720% on total was minimum of them. Accordingly, caryophyllene was dominant compound in two locations of B1 and B2. The chromatogram of these two localities is also shown in Figure 6.

Table 5. Chemical compounds in essential oil of *P. olivieri* collected from B1.

Peak	Compound	Retention time (min)	Peak height	Percentage of total
1	Decane, 2, 5, 6-trimethyl	5.809	137139	1.685
2	Heptadecane, 2, 6-dimethyl	6.860	95155	1.244
3	1H-Indene, 1-ethyloctahydro-7a-methyl	8.105	112105	1.437
4	2(1H)-Naphthalenone, octahydro-8a-methyl	8.149	102825	1.730
5	Tetradecane, 2, 6, 10-trimethyl	9.252	115980	1.354
6	Caryophyllene	9.583	1482750	18.550
7	cis-â-Farnesene	9.688	1160083	14.703
8	Humulene	9.823	188731	3.070
9	(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl	9.995	1000903	12.902
10	(1S,2E,6E,10R)-3, 7, 11, 11-Tetramethylbicyclo[	10.090	428373	7.287
11	Phenol, 3,5-bis(1,1-dimethylethyl)-	10.199	142961	4.456
12	Estra-1, 3, 5(10)-trien-17â-ol	10.578	217113	3.545
13	n-Hexadecanoic acid	10.646	157284	5.633
14	Caryophyllene oxide	10.767	378527	9.048
15	Olean-12-ene-3,28-diol, (3â)-	11.180	90074	3.179
16	7-Hexadecenal, (Z)-	11.352	111279	4.764
17	Octadecane, 3-ethyl-5-(2-ethylbutyl)	11.447	136044	3.137

Table 6. Chemical compounds in essential oil of *P. olivieri* collected from B2.

Peak	Compound	Retention time (min)	Peak height	Percentage of total
1	Decane, 2, 5, 6-trimethyl	5.811	164674	3.301
2	Decane, 2, 4, 6-trimethyl	6.861	103008	2.010
3	1H-Indene, 1-ethyloctahydro-7a-methyl- (1á,3aâ,7aá)	8.105	113156	2.253
4	1,7-Dodecadiene	8.150	101600	2.712
5	Tetradecane, 2,6,10-trimethyl	9.252	133034	3.348
6	(2E,4S,7E)-4-Isopropyl-1, 7-dimethylcyclodeca-2,7-dienol	9.322	73671	2.740
7	Caryophyllene	9.582	1172705	25.905
8	Formic acid, 3, 7, 11-trimethyl-1,6,10-dodecatrien-3-yl ester	9.687	90132	4.386
9	Humulene	9.822	202530	4.676
10	Tetradecane, 2, 6, 10-trimethyl	9.891	56082	1.720
11	Germacrene D	9.994	913151	18.429
12	á-aAcorenol	10.046	91238	1.851
13	(1S,2E,6E,10R)-3,7,11,11-Tetramethylbicyclo	10.090	96865	2.515
14	Ledol	10.537	146094	4.342
15	Caryophyllene oxide	10.768	154314	4.643
16	Olean-12-ene-3, 28-diol	11.178	55743	2.034
17	Isopropyl linoleate	11.368	67368	5.312
18	Tetradecane, 2, 6, 10-trimethyl	11.447	90529	3.937

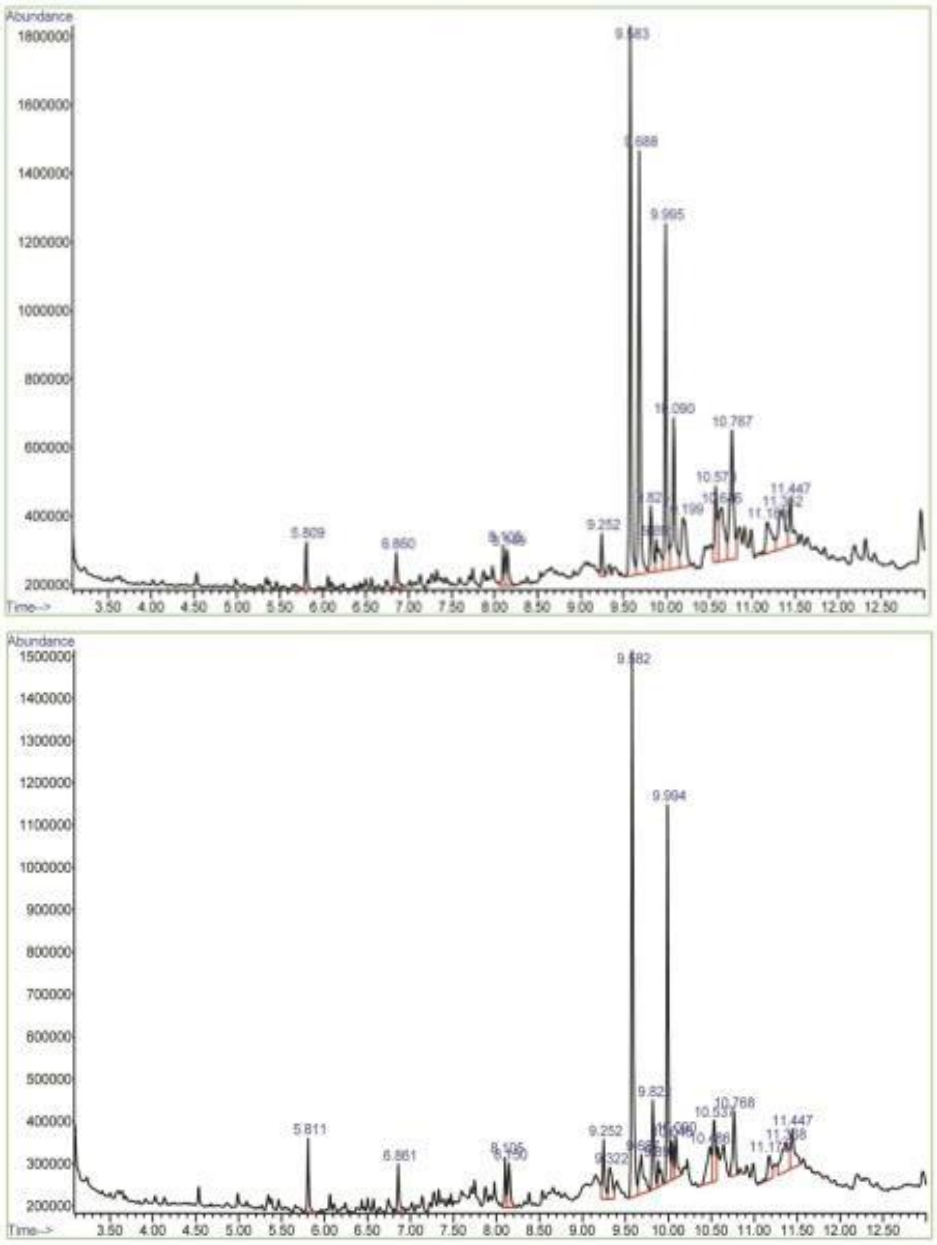

Figure 6. Chromatogram of chemical compounds from *P. olivieri* in localities of B1 and B2.

3.4. Localities C1 and C2

In total, 17 constituents were identified in locality of C1 (Table 7), among them 1H-cyclopenta [1, 3] cyclopropa [1, 2] benzene, octahydro-7-methyl was determined as dominant constituent (peak 12, retention time of 9.994 min, 16.137% from total). Also, 2(1H)-naphthalenone, octahydro-8a-methyl-trans in peak 3 with retention time of 8.106 min and 1.044% of total being a minimum compound. Futhermore, 15 compounds were detected in locality of C2 (Table 8), among which naphthalene, decahydro-4a-methyl-1-methylene-7 being a dominant (peak 8, retention time of 10.000 min, and 19.990% on total) when tetradecane, 2, 6, 10-trimethyl in peak 2 with retention time of 9.253 min and 1.200% on total was determined as minimum constituent. Their chromatogram is shown in Figure 7.

Table 7. Chemical compounds in essential oil of *P. olivieri* collected from C1.

Peak	Compound	Retention time (min)	Peak height	Percentage of total
1	Hydroxylamine, O-decyl	5.811	101504	1.602
2	Z-2-Dodecenol	7.752	92234	2.684
3	2(1H)-Naphthalenone, octahydro-8a-methyl	8.106	60509	1.044
4	Tetradecane, 2, 6, 10-trimethyl	9.252	89011	2.318
5	Isocaryophillene	9.488	526353	11.223
6	Caryophyllene	9.583	580657	12.726
7	cis-â-Farnesene	9.634	176649	4.538
8	(E)-â-Famesene	9.688	171517	4.413
9	Humulene	9.749	87983	1.956
10	Nerolidyl acetate	9.822	118973	2.775
11	(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl	9.938	659231	15.084
12	1H-Cyclopenta [1, 3] cyclopropa[1,2]benzene	9.994	734154	16.137
13	(1S,2E,6E,10R)-3, 7, 11, 11-Tetramethylbicyclo	10.042	191288	4.641
14	Geranyl isovalerate	10.566	97546	7.082
15	Estra-1, 3, 5(10)-trien-17â-ol	10.641	65326	2.857
16	Caryophyllene oxide	10.739	128211	5.353
17	Heptadecane, 9-octyl	11.442	110011	3.566

Table 8. Chemical compounds in essential oil of *P. olivieri* collected from C2.

Peak	Compound	Retention time (min)	Peak height	Percentage of total
1	2-Pentadecanone, 6, 10, 14-trimethyl-	9.003	157379	1.919
2	Tetradecane, 2, 6, 10-trimethyl-	9.253	212731	1.200
3	6-epi-shyobunol	9.328	213388	1.502
4	Caryophyllene	9.586	3685264	16.993
5	cis-â-Farnesene	9.689	1119730	9.005
6	Humulene	9.824	722638	4.715
7	Tetradecane, 2, 6, 10-trimethyl-	9.894	305912	3.340
8	Naphthalene, decahydro-4a-methyl-1-methylene	10.000	4313200	19.990
9	(1S, 2E, 6E,10R)-3, 7, 11, 11-Tetramethylbicyclo [8.1.0]	10.050	596229	2.749
10	(3R, 3aR, 3bR, 4S, 7R, 7aR)-4-Isopropyl	10.093	838145	5.417
11	Dodecanoic acid	10.225	324779	4.647
12	Caryophyllene oxide	10.534	576183	6.817
13	Germacrene D	10.769	644367	5.724
14	n-Hexadecanoic acid	11.663	501210	13.678
15	Tetradecanoic acid	12.194	213502	2.304

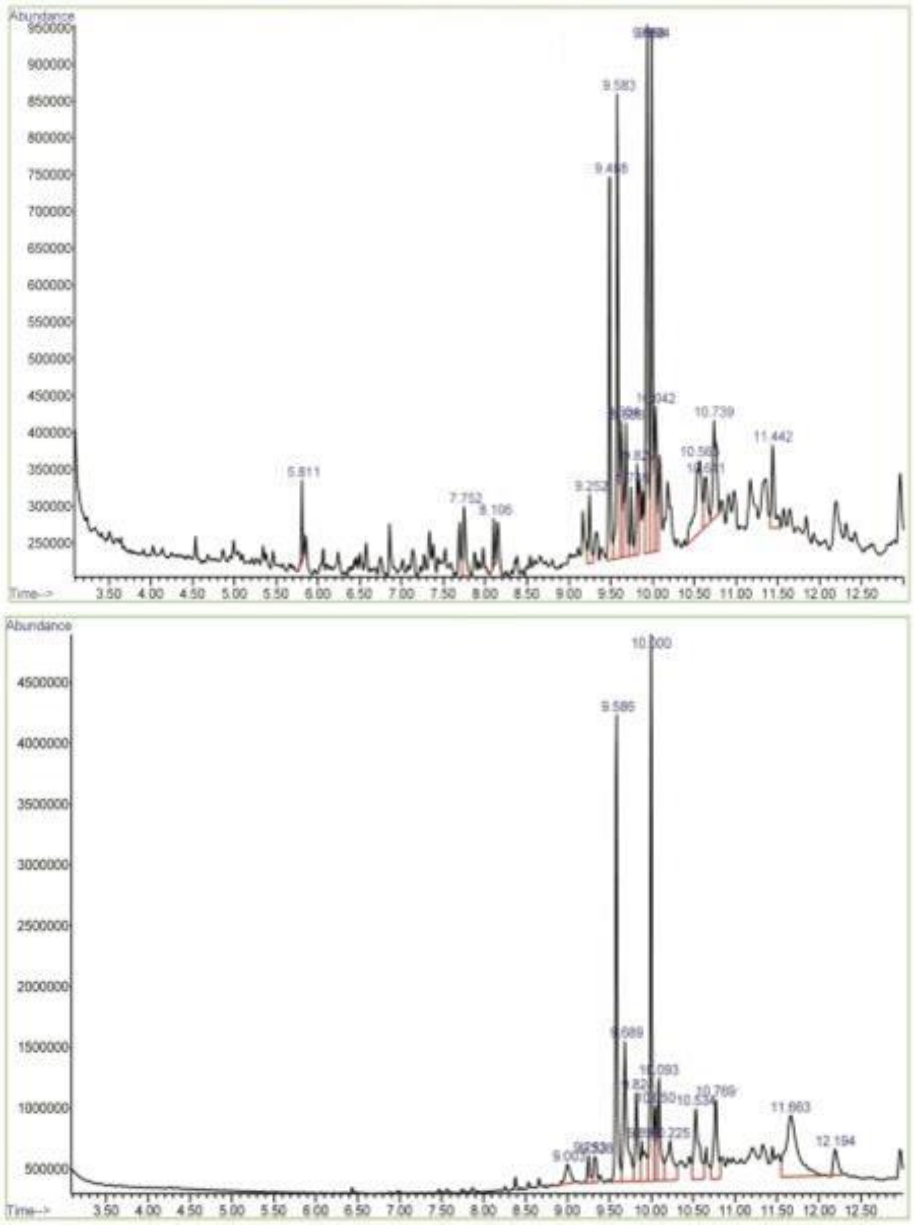

Figure 7. Chromatogram of chemical compounds from *P. olivieri* in localities of C1 and C2.

3.5. Localities D1 and D2

In locality of D1, 21 compounds were detected (Table 9), among which estra-1, 3, 5 (10)-trien-17â-ol being dominant compound (peak 17, retention time of 10.639 min, 18.320% of total) when chloromethanesulfonyl-dichloromethanesulfonyl chloride detected in peak 1 with retention time of 4.538 min and 1.075% on total being minimum of them. In locality of D2, 15 constituents were identified (Table 10), among which n-hexadecanoic acid being dominant compound (peak 12, retention time of 10.646 min, and 29.363% of total) when ã-elemene (peak 6, retention time of 9.615 min, and 1.668% of total) was minimum of them. The chromatogram of these two localities is also shown in Figure 8.

Table 9. Chemical compounds in essential oil of *P. olivieri* collected from D1.

Peak	Compound	Retention time (min)	Peak height	Percentage of total
1	Chloromethanesulfonyl-dichloromethanesulfonyl chloride	4.538	35939	1.075
2	Decane, 2, 4, 6-trimethyl	5.811	129700	3.727
3	Hydroxylamine, O-decyl	6.861	80224	2.468
4	2(1H)-Naphthalenone, octahydro-8a-methyl	7.878	45319	2.014
5	1H-Indene, 1-ethyloctahydro-7a-methyl	7.979	50343	2.331
6	Hexen-1-ylcyclohexane	8.106	89321	2.471
7	2(1H)-Naphthalenone, octahydro	8.151	79469	2.699
8	9,9-Dimethoxybicyclo [3.3.1] nona-2,4-dione	8.602	47227	4.852
9	Octadecane, 6-methyl	9.164	42513	4.394
10	Tetradecane, 2, 6, 10-trimethyl	9.252	75446	2.547
11	Hexadecanoic acid, methyl ester	9.418	38221	2.806
12	7-epi-cis-sesquisabinene hydrate	9.582	75308	1.923
13	Formic acid, 3, 7, 11-trimethyl-1,6,10-dodecatrien-3-yl ester	9.615	72468	2.526
14	Heptadecane, 2, 6, 10, 15-tetramethyl	9.668	57578	3.123
15	â-Copaene	9.993	432025	11.297
16	á-Acorenol	10.089	83730	2.993
17	Estra-1, 3, 5(10)-trien-17â-ol	10.639	154200	18.320
18	Methyl 16-hydroxy-hexadecanoate	10.813	129032	8.483
19	1-Ethyl-3-propyl-5-(propene-1-yl)adamantine	11.174	129032	1.956
20	Tetradecane, 2, 6, 10-trimethyl	11.447	35115	1.051
21	Tetradecanoic acid	12.204	45712	2.894

Table 10. Chemical compounds in essential oil of *P. olivieri* collected from D2.

Peak	Compound	Retention time (min)	Peak height	Percentage of total
1	Decane, 2 ,6, 7-trimethyl	5.811	154663	2.668
2	Decane, 2, 4, 6-trimethyl	6.862	92784	1.723
3	2(1H)-Naphthalenone, octahydro-8a-methyl-, trans	8.106	108447	1.855
4	1H-Indene, 1-ethyloctahydro-7a-methyl	8.150	101381	2.309
5	Tetradecane, 2, 6, 10-trimethyl	9.252	114629	2.033
6	ã-Elemene	9.615	94262	1.668
7	Tetradecane, 2, 6, 10-trimethyl	9.667	179329	3.036
8	Germacrene D	9.995	1493761	26.558
9	7-epi-cis-sesquisabinene hydrate	10.050	123410	1.956
10	(1S,2E,6E,10R)-3, 7, 11, 11-Tetramethylbicyclo	10.090	285307	5.291
11	á-Acorenol	10.538	245616	9.656
12	n-Hexadecanoic acid	10.646	434435	29.363
13	Estra-1,3,5(10)-trien-17â-ol	10.744	434435	4.884
14	9,12-Octadecadienoic acid	11.367	64427	4.153
15	7-Hexadecenal	11.444	80542	2.846

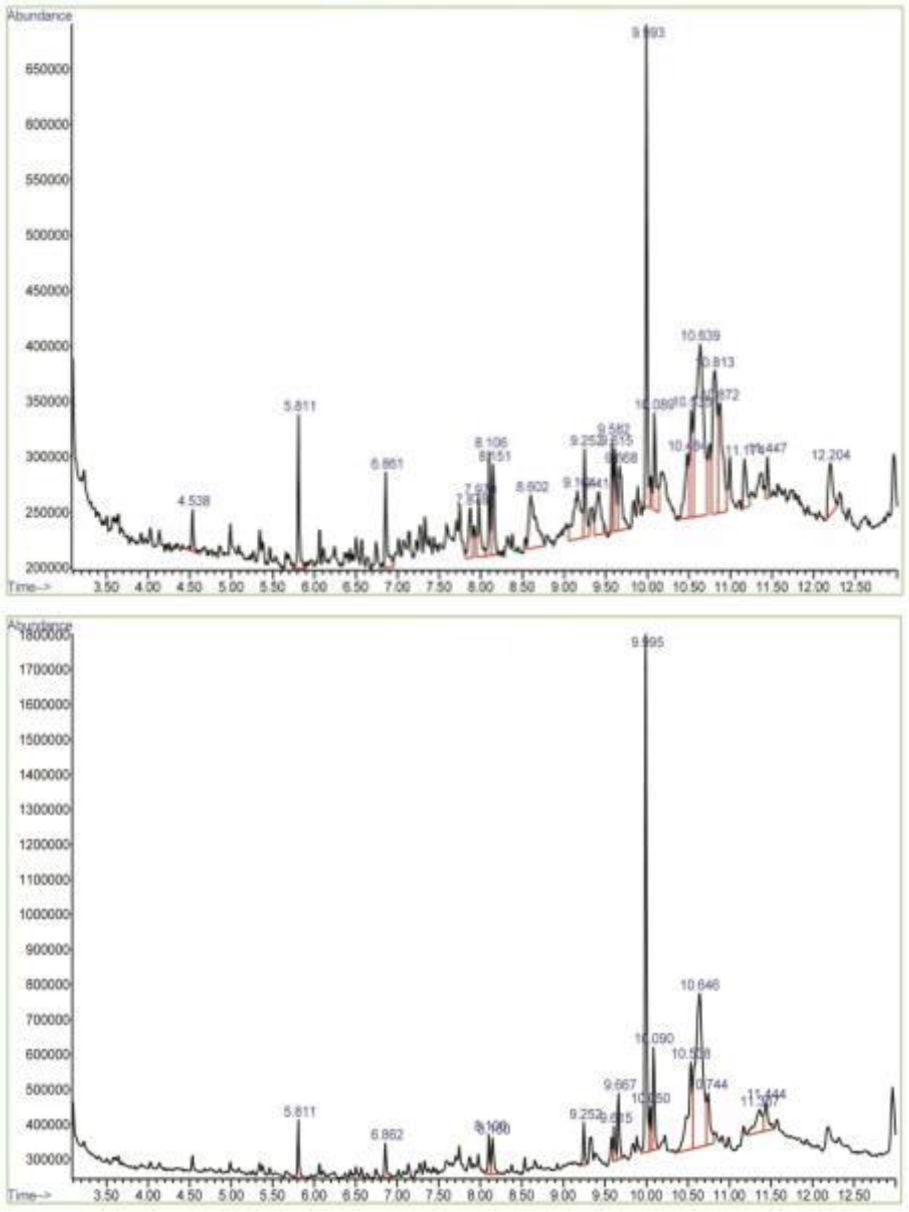

Figure 8. Chromatogram of chemical compounds from *P. olivieri* in localities of D1 and D2.

4. DISCUSSION and CONCLUSION

Regarding the investigation on this valuable medicinal plant, various studies have been conducted in Iran. We indicate some of them in the following and explain their results in comparison with ours. Mirza and Baher Nik (2003) studied the essential oil of *P. olivieri* in Iran and among 15 identified constituents, germacrine D (28.1%), β-caryophyllene (16.1%), α-pinene (11.7%), and β-seleline (10.2%) were dominant constituents. Similarities among the dominant compounds can be confirmed in two studies, which indicate similar conditions in production of secondary compounds. Sarkhail *et al.*, (2006) found that 22 constituents from this plant essential oil made up 93.6% of total volume when main constituents were germacrene D (66.1%), β-seline 5.1%, β-caryophyllene (4.2%), and α-pinene (4.2%). The results of previous studies in comparing the constituents of essential oil of *P. olivieri* in different regions showed that germacrene D and β-caryophyllene were main compounds, that our studies confirm it. In our investigation, caryophyllene had a significant percentage in majority of essential oils isolated from different localities shows that the essential oils of this plant in the northern regions of Iran have similarities with western regions. Although, quality of collected medicinal plant,

method of isolation, and analyzer device of essential oil are also effective agents in this direction. β-caryophyllene (BCP) is a natural bicyclic sesquiterpene and a common constituent of the essential oils of numerous spice and food plants. Anti-inflammatory agents that are non-steroidal in nature. In addition to anti-inflammatory actions, they have analgesic, antipyretic, and platelet-inhibitory actions (Amor *et al.*, 2009).

The reaction mechanism of all sesquiterpene synthases starts with the ionization of farnesyl diphosphate. The resulting carbocation can undergo a range of cyclizations. The 11,1-closure yields the 11-membered ring humulyl cation. Then, deprotonation followed by cyclobutane ring formation leads to b-caryophyllene production. Otherwise, the 10,1-closure originates the germacrenyl cation, which can be converted to germacrene D. Thus, the precursors flow toward one of these constituents can reduce the amounts of the other (Degenhardt *et al.*, 2009).

Tajbakhsh *et al.*, (2007) investigated the essential oils isolated from leave, flower, stem, and root of *P. olivieri*. They identified thirteen components in the oil of leaf that germacrene D (58%), α-pinene (10.4%), and bicycle germacrene (4.4%) were major components of them. In flowers, 10 compounds were identified and germacrene D (48%), α-pinene (19.7%), and β-selinene (10.1%) made up its majority. Moreover, 10 compounds characterized stem essential oil and germacrene D (57%), α-pinene (8.6%), and 2-pentadecanone (6.5%) were dominant constituents. In the essential oil of root, germacrene D (35%), α-pinene (12.6%), and spathulenol (6.6%) were major. Different parts of the plants can have differences in terms of chemical compounds. In our study, we examined the aerial parts including leaves and stem and flowers in a mixed form , which have similarities with this study; but, there are obvious differences in other parts, and it is not possible to make a direct examination. Javidnia *et al.*, (2010) studied essential oil composition of two species from the genus *Phlomis* including *P. aucheri* Boiss. and *P. elliptica* Benth. in Iran. They found 39 compounds of *P. aucheri* and 58 from *P. elliptica* containing 91.2% and 90.1% of total. Moreover, caryophyllene-type compounds contained 63.8% of *P. aucheri* and the main of them were caryophyllene oxide (33.5%), β-caryophyllene (27.0%) and β-selinene (10.2%). In apposite for *P. elliptica*, essential oil structure included aliphatic hydrocarbons with hexadecanoic acid (19.1%), linoleic acid (10.2%) and β-selinene (9.9%) as main constituents. One of the important factors in modifying the composition of plant essential oils is the studied species. However, in the species within the same genus, the differences in secondary metabolites are insignificant. In this study, minor changes were determined compared to the species under our study, the differences were not overall. In different genus, variation among the compounds will definitely be a lot of.

Jamzad *et al.*, (2013) studied the essential oil from leaves and flowers of *P. persica* Boiss. and *P. olivieri* from Iran and concluded that in *P. persica*, 98.5 % of essential oil obtained from the leaves and 89.8 % from flowers. Also, in *P. olivieri*, 98.9 % of the essential oil isolated from leaves and 99.2 % from flowers. The essential oils were rich in sesquiterpenes and germacrene D being major constituent in both (26.5 %, 19.5 %) and (37.6 %, 19.5 %), in the leaves and flowers of *P. persica* and *P. olivieri*. Bicyclo germacrene was abundant in leaves and flowers of two species (18.7%, 20.4%) and (8.0%, 5.7%). In another study, Mohammadifar *et al.*, (2015) studied chemical analysis of *P. olivieri* and *P. persica* essential oils and found that 46 compounds were available in both essential oils. Among them, β-caryophyllene (25.7%) and germacrene D (19.5) in *P. olivieri* and germacrene D (17.2%) and γ-elemene (15.4%) in *P. persica* were dominant. Essential oils of *P. olivieri* and *P. persica* being rich of sesquiterpens. The chemical constituents of the analyzed essential oils were also found to be different from those reported in other regions of Iran, which may be due to the existence of possible different chemotypes between populations of these two species.

Mohammadifar *et al.*, (2015) studied constituents of *P. olivieri* essential oil and found that one caffeoylquinic acid derivative, chlorogenic acid (1), one iridoid glycoside, ipolamiide (2),

two phenylethanoid glycosides, phlinoside C (3), and verbascoside (5), along with two flavonoids, isoquercetin (4), and naringenin (6) were identified from this medicinal plant. Their results study indicated that *P. olivieri* is a medicinal plant with suitable biological and pharmacological properties. In this study, type of analyzer device was different from ours, and for this reason, some differences were observed in the results. Therefore, the method of analysis is one of the influential factors in secondary metabolites and plant volatiles, which should be carefully considered. Bajalan *et al.,* (2017) studied the essential oil of wild populations from *P. olivieri* and concluded that 27 compounds containing 90.52- 98.51% of total. Results indicated that major constituents were germacrene D (26.54-56.41%), bicycle germacrene (6.38-30.55%), β-caryophyllene (5.32-24.52%), and α-pinene (1.29-15.53%). The results of their study showed new insights for cultivation and industrial uses of this medicinal plant in Iran; because, this valuable medicinal plant contains numerous compounds with abundant medicinal properties that have received little attention.

Khalilzadeh *et al.,* (2005) investigated essential oils of *P. persica* and *P. olivieri* from Iran when found that *P. persica* contained germacrene D (38.2%), bicycle germacrene (16.3%), and α-pinene (13.3%) as major of them. The oil of *P. oliveri* was also characterized also by higher amount of germacrene D (26.4%) and bicycle germacrene (12.7%). Both oils consisted mainly of sesquiterpene hydrocarbons. There are no obvious differences in secondary compounds in closely related species, and the results of this study confirm this subject.

Fattahi and Najjari (2022) studied the essential oil composition from three species of *Phlomis olivieri*, *P. persica*, and *P. herba-venti* collected from northwest of Iran. They found that 38 compounds were available in each essential oil when the main compounds of *P. olivieri* included as dioctyl phthalate (69.71%), gamma-almen (7.93%), and germachron-di (7.44%). Also, napthalactone (45.24%), bis(2-ethylhexyl) phthalate (32.72%), and Germacron-D (5.59%) was major in *P. herba-venti*, Moreover, in essential oil of *P. persica*, dioctyl phthalate (49.74%), alpha-murolene (16.18%), and hexa hydrofarnesyl acetone (7.82%) being dominant. Comparing the constituents identified in the essential oil of three species showed a higher percentage of dioctyl phthalate in *P. olivieri* (69.71%) compared to the other species. The highest amount of total phenol (136.34 mg of gallic acid per gram of dry weight), total flavonoid (49.29 mg of quercetin per dry weight), and antioxidant activity (48.68%) related to *P. persica*. Different species within the same genus have differences in the essential oil structure, which should be taken into account in different studies. However, due to the differences in these compounds, occurrence of biological differences is a natural subject. Regarding the species in two studies, *P. olivieri*, a certain difference in the dominant compounds and total number of them observed when weather can be considered an effective factor for the differences.

Salehi and Kalvandi (2022) investigated essential oil of different *P. olivieri* populations in Hamedan province and found that 31 compounds were identified when caryophyllene, germacrene D, and (E)-b-farnesene had the highest percentage compared to the other essential oil constituents. Also, the highest essential volume of essential oil was obtained in the Koohani population which had the lowest altitude among other populations. In our study, caryophyllene in the essential oil of both regions had the highest percentage in essential oil, which confirms each other. Although, we have investigated A1 and A2 regions in this province. Despite the presence of these regions within the same province and slight differences in climate, no drastic changes were observed in the secondary compounds. Salehi and Kalvandi (2023) investigated chemical structure of essential oils from *P. olivieri* when found that variation was estimated in samples collected from 11 different regions of Hamedan province. The major constituents contained sesquiterpenes including germacrene D, (E)-β-caryophyllene, and (E)-β-farnesene. Germacrene D differed from 5.3% to 36.9%, (E)-β-caryophyllene from 6.3% to 61.9%, and (E)-β-farnesene from 5.1% to 18.4%, suggesting occurrence of three chemotypes in this province. Ghavam (2023) studied essential oil analysis from of *P. olivieri* in Iran when the main

compounds included sesquiterpenes such as germacrene D (26.43%), β-caryophyllene (20.72%), elixene (6.58%), β-trans-farnesene (6.17%), β-cyclo germacrane (5.04%), germacrene B (4.73%), α-humulene (4.22%), and monoterpene α-pinene (3.22%).

There are very limited studies about the effect of ecological factors on the changes of plant essential oils. In this direction, Mahdavi *et al.*, (2014) investigated these factors on the essential oil of *Phlomis cancellata* Bunge in Mazandaran province (Iran). They found that the efficiency was high in 2400 m when percentage of main component was higher at altitude of 2000 m. Due to the high efficiency, large number of components, and valuable amount of main constituents, the essential oil isolated from height of 2000 m has the most favorable quality for use in various industries especially pharmaceutical industries. The results of their study are consistent with ours; although the examined species was different. In the area where this study was conducted, since the ecological conditions were better in terms of altitude, the variety and number of secondary compounds were identified and the difference between dominant constituent with the others was obvious. Unfortunately, there is no direct study on other parameters including soil characteristics of each region on the changes of these compounds. The current research is considered as new step in this field and must be considered.

In general, it can be said that this plant has valuable medicinal effects with good diversity and proper distribution in different parts of Iran; but, it has received less attention. With supplementary and comprehensive studies, we can obtain localities that that this medicinal plant has more compounds with better quality. There are many studies about this valuable medicinal plant; but, the effects of its various medicinal compounds have not been comprehensively investigated. The authors of this article encourage other researchers to conduct more studies on this valuable plant and to examine the effects of the various factors on it. Later, if the compounds of this medicinal plant are suitable, it is possible to isolate and commercialize the compounds contained in it for medicinal usages.

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

Mahtab Asgari Nematian: Investigation, Resources, Visualization, and Software. **Behjat Bahramynia**: Formal Analysis and Writing original draft. **Zahra Baghaeifar**: Methodology, Supervision, and Validation.

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Research Article

Changes in antioxidant properties of pepper leaves (*Capsicum annuum* L.) upon UV radiation

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Ultraviolet radiation,
Bell pepper.

Abstract: Bell pepper (*Capsicum annuum*) is one of the most popular vegetables consumed worldwide. The leaves of pepper are rich in phenolics, including phenolic acids and flavonoids. These compounds are well known for their ultraviolet (UV) absorbing and antioxidant properties. While the change of the phenolic pattern is an intensive research subject, it is not yet well-known in pepper leaves, particularly in outdoor conditions. In this experiment, we examined the effect of UV radiation on the leaves of outdoor grown peppers, focusing on the UV-absorbing properties and antioxidant capacities. Three different total antioxidant capacity (TAC) measurements have been compared: (I) Folin-Ciocalteu Reactivity (FC), (II) Ferric Reducing Antioxidant Power (FRAP) and (III) Trolox Equivalent Antioxidant Capacity (TEAC). Moreover, non-enzymatic hydrogen peroxide scavenging antioxidant capacity was measured. Significant increase was detected only in FRAP, suggesting an elevation exclusively in the level of phenolic acids in case of UV exposed outdoor grown pepper leaves.

1. INTRODUCTION

Phenolic compounds like phenolic acids and flavonoids are synthetized via the shikimic acid pathway (Casañal *et al*., 2013), and are also referred to as phenylpropanoids (Deng & Lu, 2017). Phenolics usually occur mainly as flavonoid glycosides in plant tissues (Yang *et al.,* 2018). These metabolites protect the plants against ultraviolet (UV) radiation and act as UV-screeners because of their partly different UV absorption spectra (Csepregi & Hideg, 2018). UV radiation, especially UV-B radiation is a potential stressor (Ballaré, 2003). Depending on irradiance and dose, UV-B can induce reactive oxygen species (ROS) and the cellular hydrogen peroxide level (Czégény *et al*., 2014). ROS compounds can cause cell damage through the oxidation of DNA, lipids, and proteins (Hernández *et al*., 2009). However, UV-B can also elicit increase the production of phenolic compounds an effect which has been demonstrated across many species including, *Arabidopsis thaliana* (Hectors *et al*., 2014), *Betula pendula* (Morales *et al*., 2010), *Hordeum* spp. (Klem *et al*., 2015) and *Capsicum* spp. (Rodríguez-Calzada *et al*., 2019). The induction of phenolic compound biosynthesis is correlated with the stimulation of the UVR8 photoreceptor by UV-B (Jenkins, 2014; Morales *et al*., 2013; Rizzini *et al*., 2011) and the cryptochrome photoreceptors by UV-A (Siipola *et al*., 2015). Typically, UV enhances the total

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concentration of phenolics as well as the individual concentration of phenolic acids and flavonoids, a response which has been linked to protection against oxidative stress. In fact, the primary function of phenolics (especially the dihydroxy-B-ring flavonoids) is to counter oxidative stress (Agati *et al*., 2020) via the scavenging of reactive oxygen species (ROS). Phenolics have a high total antioxidant capacity *in vitro* (Csepregi *et al*., 2016; Hernández *et al*., 2009) and are capable of neutralizing high efficiency ROS molecules (Csepregi & Hideg, 2018). Moreover, a positive correlation has been detected between phenolic content and antioxidant capacity in grapevine leaves (*Vitis vinifera*) (Csepregi *et al*., 2019).

Capsicum annuum, a member of the Solanaceae family is one of the most economically important crop plants in the world (Kim *et al*., 2014). The fruit is full of a source of nutrients in human diet containing carotenoids, ascorbic acids, flavonoids, and other phenolic compounds (Assefa *et al.,* 2021) with the leaves also being consumed (Kim *et al.,* 2014). Moreover, leaves also utilized for their antimutagenic and antimicrobial activity in the cosmeceutical (Kim *et al.,* 2014), pharmaceutical and medicinal industries (Rodríguez-Calzada *et al.,* 2019). Intriguingly, little is known about the UV absorption and antioxidant capacity of metabolites found in pepper leaves, and the protective role they play against individual ROS. In this experiment, we focused on the antioxidant and UV-absorbing properties of pepper leaf extracts (*Capsicum annuum*) grown under outdoor conditions. To have a more comprehensive knowledge, we applied three various total antioxidant capacity (TAC) methods, to account for discrepancies in results caused by differences in methods (Csepregi *et al*., 2016; Csepregi *et al*., 2019).

2. MATERIAL and METHODS

2.1. Chemicals

Test compounds for the calibrations were purchased from Merck (Merck Industrial and Laboratory chemicals, Darmstadt, Germany). All other chemicals were purchased from VWR (VWR International Kft., Debrecen, Hungary).

2.2. Experimental Design

Capsicum annuum cv. amy seeds were sown on garden soil in 12x12 cm pots and germinated outside. Research was started in the month of May at the Research Institute Viticulture and Oenology, University of Pécs (latitude: 46°04’ N, longitude: 18°11’ E). The experimental work included seven plants which were not exposed to UV radiation (covered by Rosco 3114 UV filter, Roscolab Ltd, London, UK) and seven plants that were exposed UV radiation (covered by cellulose-diacetate plastic foil, Courtauds Chemicals, Derby, UK) since seeding. The average global irradiances in May, June and July were 68.27 Wm^{-2}, 63.72 Wm^{-2} and 60.68 Wm^{-2} while relative humidity were 16.79 %, 21.18 % and 24.12 % respectively. Air temperatures were 18.03 °C, 23.12 °C and 26.65 °C. All environmental data was provided by the meteorological station on the premises of the Faculty of Natural Sciences of the University of Pécs (joido.ttk.pte.hu).

At the end of July, the leaves were collected. Two fully developed pepper leaves were collected from the 3rd node of each plant and pooled together. Plant leaves were frozen in liquid nitrogen and stored at -60 °C for further antioxidant capacity measurements. The frozen leaves were lyophilized (SCANVAC CoolSafe 110-4, LaboGene, Denmark) to make a more homogeneous extracts from the dried material.

The leaf samples extracted in methanol aqueous (70:30 v/v) and sonicated in a sonic bath for 15 minutes (RoHS JP-020, Shenzhen, China) and finally centrifuged (Thermo Fisher Scientifc Inc., Waltham, MA, USA) for 10 minutes at 15000xg. The supernatant was collected used to pipettes, and the process was repeated two more times. All supernatants were subsequently combined.

2.3. Folin-Ciocalteu Reactivity (FC)

The FC measurement was carried out with some modifications (Csepregi *et al*., 2016). 90 µL aqueous Folin-Ciocalteu (1:10 with distilled water) reagent is added to the 20 µL diluted sample. After 5 minutes at room temperature, another 90 µL of Na_2CO_3 solution was added. After 90 minutes of incubation at room temperature, the absorbance of the solution was measured (Multiskan FC plate reader, Thermo Fischer Scientific, Shanghai, China) at 651 nm. The calibration curve was prepared with gallic acid solution using the above method, then the results are given in µM gallic acid equivalent per mg leaf dry weight.

2.4. Trolox Equivalent Antioxidant Capacity (Teac)

The TEAC measurement was carried out according to Rice -Evans *et al.,* (1997). $ABTS^{\bullet+}$ (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic cation radical) solution was prepared by mixing 100 µL ABTS (0.1 mM), 100 µL H_2O_2 (0.1 mM) and 100 µL horse radish peroxidase (0.0125 µM) in a 9.7 mL 6.0 pH sodium phosphate buffer (50 mM). During the 15 minutes incubation at room temperature, the formation of $ABTS^{\bullet+}$ was indicated by its blue-green color. 190 µL of this solution was mixed with 10 µL diluted plant extract and the absorption at 651 nm was recorded. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used to prepare a calibration and the results are given as µM Trolox equivalent per mg leaf dry weight.

2.5. Ferric Reducing Antioxidant Power (Frap)

FRAP method was performed following (Csepregi *et al*., 2016). The reaction mixture contained 1.25 mL TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) (10 mM with 40 mM HCl) and 1.25 mL mM $FeCl_3$ (20 mM with distilled water) in 12.5 mL 3.6 pH acetate buffer (300 mM). 10 µL diluted leaf extract was added to 190 µL of reagent, and absorptions were measured at 620 nm, followed by an incubation at room temperature for 30 minutes. FRAP values are given as µM ascorbic acid equivalent per mg leaf dry weight.

2.6. Hydrogen Peroxide Scavenging Capacity

This assay is based on the oxidation of potassium iodide (KI) but instead of the iodine absorption maximum the reaction is evaluated at 405 nm as described earlier (Csepregi & Hideg, 2016). The KI solution (0.85 M) is prepared in 7.0 pH potassium-phosphate buffer (100 mM), to 140 µL of which added 10 µL diluted plant extract, finally added 50 µL of H_2O_2 during an orange color reaction can be observed, which was characterized by measuring the absorption two times (zero and three minutes) at 405 nm (Csepregi & Hideg, 2016). Thus, the extent of antioxidant activity can be deduced from the absorption decreasing. In this way, the 50% inhibitory concentration (IC_{50}) can be specified, which is given as Trolox equivalents.

2.7. UV Absorption Measurements

Leaf extracts were diluted with a mixture of acidified ethanol and their absorption spectra were measured between 280-400 nm (Shimadzu Corporation UV-1800, Kyoto, Japan). The area under the curve was identified with a spectrophotometer and separately determined in the UV-A (315-400 nm) and UV-B (280-315 nm) ranges (Csepregi & Hideg, 2018). The results are given in µM quercetin equivalents, based on the literature (Csepregi & Hideg, 2018).

2.8. Statistical Analyses

Statistical analyses of data were carried out using Excel (Version 2007, Microsoft Corporation, Redmond, WA, USA) while graphs were prepared using OriginPro (OriginLab Corporation, Northampton, Massachusetts, USA). The leaf samples were characterized by means and standard deviations of seven biological samples for the UV deprived and seven for the full sunlight exposed plants. The significance of differences was assessed using Student's t-tests where the level of significance was $p < 0.05$.

3. RESULTS

Pepper leaf extracts were measured using various total antioxidant (TAC) and UV absorbing capacity assays (Figures 1 and 2). In addition, specific ROS scavenging capacity were also measured (Figure 3). The three different TAC methods and UV absorbing capacity have been calibrated with the appropriate standards. Full sunlight exposed plants showed similar TAC values in case of TEAC (Figure 1A) and FC (Figure 1B), while only FRAP (Figure 1C) measurement displayed significantly higher (p<0.05) antioxidant capacity values (Figure 1). Phenolics, like flavonoids and phenolic acids are strong antioxidants, although there can be differences in their *in vitro* TAC reactivity because of intramolecular differences (Csepregi *et al*., 2016). The B ring hydroxylation pattern of flavonoids significantly affect TEAC, FC and FRAP results. Nevertheless, the presence of -OH group in the C ring (C3 position) is likely affect TEAC and FRAP, but not FC values in case of flavonoids. The hydroxylation pattern of phenolic acids significantly alters FRAP, but not TEAC and FC (Csepregi et al., 2016). Based on these, we can postulate that the higher FRAP value of full sunlight exposed pepper leaves is more likely due to the accumulation of phenolic acids and not flavonoids.

In another experiment, phenolic content of UV-B exposed pepper leaves was examined with HPLC. In this work, the flavonoid level did not increase significantly, moreover, the level of apigenin-8-C-hexoside even decreased. In contrast, the level of chlorogenic acid (a phenolic acid) increased, therefore the compound which is responsible for the higher FRAP value can be this compound (Rodrígez-Calzada *et al.,* 2019). In our experiment, a different pepper variety were chosen, but it is hypothesized that they may have the same or similar special metabolites in the leaves, only to a different extent.

This study shows that under outdoor conditions, the UV-A and UV-B absorbing properties of leaf extracts were unchanged (Figure 2). From previous works were well known that phenolic acids absorbing the UV radiation mostly more efficiently than flavonoid glycosides (Csepregi & Hideg, 2018). However, chlorogenic acid occurs in relatively low concentration in *Capsicum annuum* cv. Coronel pepper leaf, compared to the total phenolic content (Rodríguez-Calzada *et al.,* 2019).

Overall, leaf extracts displayed higher UV-B absorbing capacity when compared with UV-A absorbing capacity (Figure 2). This could be due to the phenomena that both phenolic acids and monohydroxy B-ring flavonoid glycosides have higher UV-B absorbing capacity (Csepregi & Hideg, 2018).

The specific ROS scavenging capacities were not changed significantly (Figure 3). Phenolic acids like chlorogenic acid have relatively low H_2O_2 neutralizing capacity compared to dihydroxy B-ring flavonoids and monohydroxy B-ring flavonoids with higher neutralizing activity (Csepregi & Hideg, 2018) Potentially the increase in phenolic acid level is not necessarily elevating the non-enzymatic H_2O_2 neutralizing capacity of outdoor grown pepper leaves.

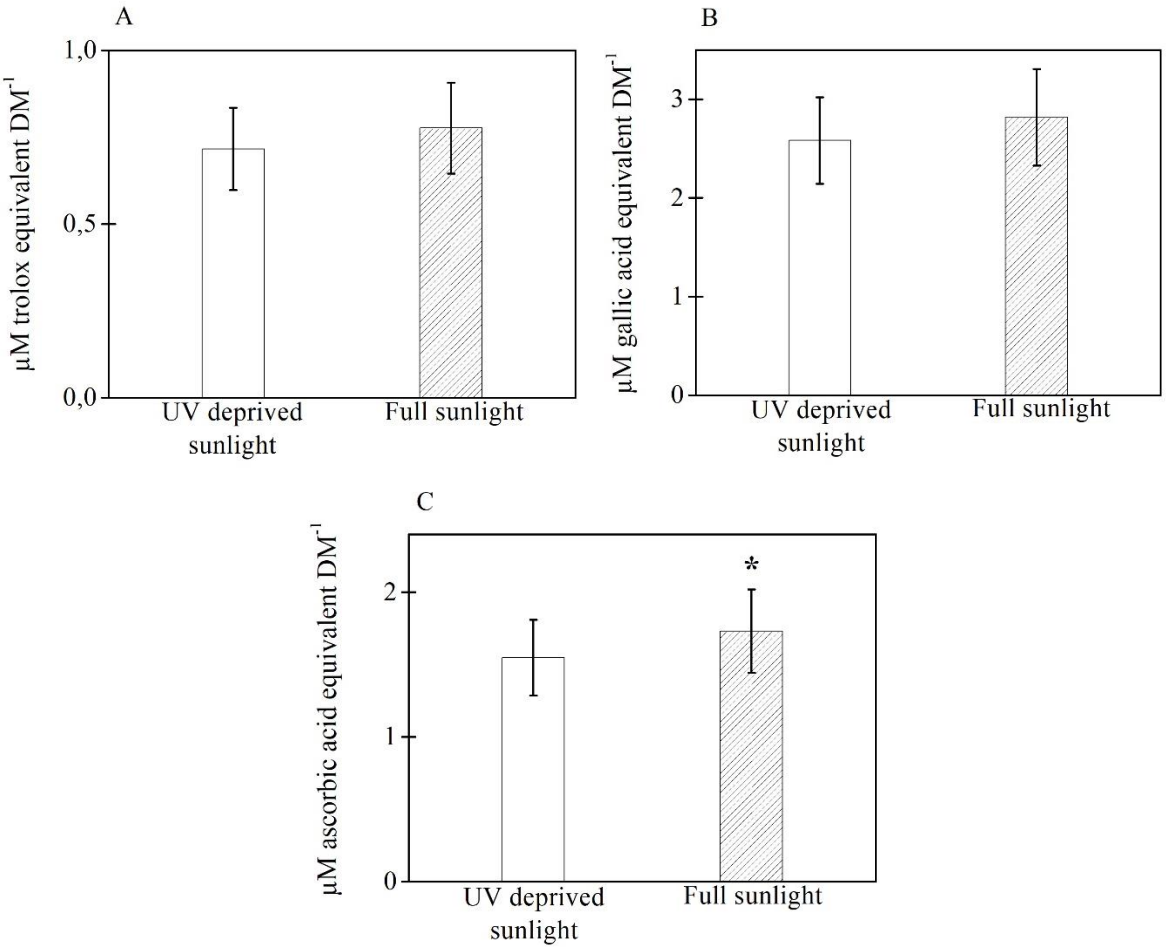

Figure 1. A) TEAC (Trolox Equivalent Antioxidant Capacity); B) Folin-Ciocalteu reactivity (FC); C) FRAP (Ferric Reducing Antioxidant Power). White columns: plants were deprived from UV radiation, dashed columns: plants were received the full sunlight.

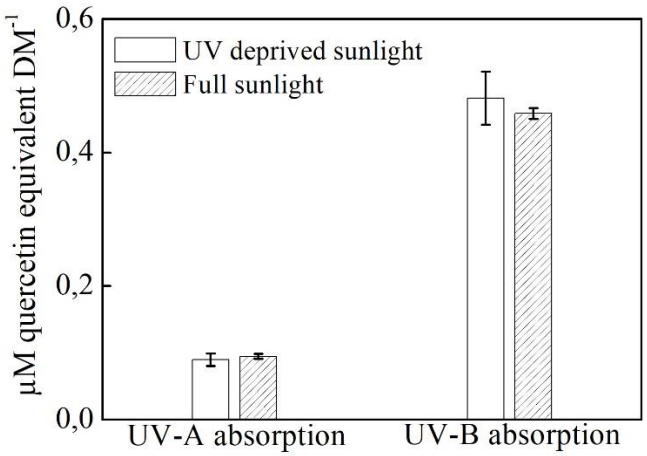

Figure 2. UV absorption measurements: UV-A absorption (315–400 nm) and UV-B absorption (280–315 nm) of the samples. White columns: plants were deprived from UV radiation, dashed columns: plants were received the full sunlight.

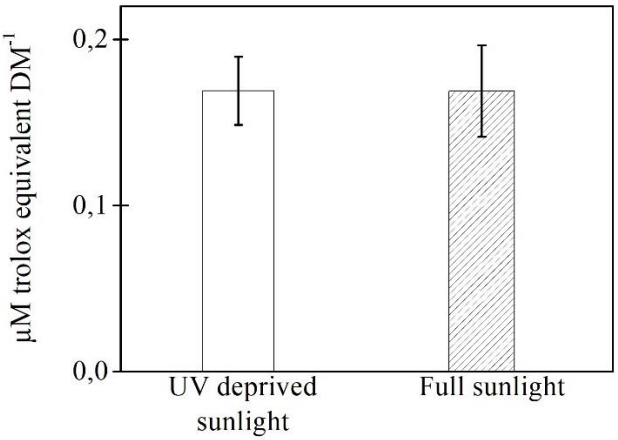

Figure 3. Non-enzymatic hydrogen peroxide scavenging capacity. White columns: plants were deprived from UV radiation, dashed columns: plants were received the full sunlight.

4. DISCUSSION and CONCLUSION

In outdoor experiments, there are several highly fluctuating environmental factors which may be stress inducing. Factors including temperature (Csepregi *et al*., 2019), drought (Rodríguez-Calzada *et al.,* 2019) and UV radiation can alter the phenolic profile of leaves. Indeed, both UV deprived, and full sunlight exposed plants were continuously exposed to potential stressors like high temperature or low air humidity. Therefore, we attribute the unchanged TEAC, FC, UV absorbing capacity and H_2O_2 scavenging capacity to the experimental conditions. These results suggests that UV might not the most important environmental stress factor considering the overall level of plant secondary metabolites. But nor negligible either, because the elevated level of FRAP suggesting a slightly modification in the phenolic content presumably the level of chlorogenic acid. This is corresponding to the most recent findings, that plants defend themselves firstly against other environmental stress factors rather than UV radiation (Agati *et al*., 2020). In accordance with the literature, our findings support the hypothesis that UV induces changes in the phenolic content of plant, elevating level of chlorogenic acid which is thought to be a key component in the specific defense against UV stress in case of *Capsicum annuum* L. cv. Amy pepper leaves.

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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. **Ethics Committee Number**: Department of Biology, Faculty of Sciences, University of Pécs, H-7624 Pécs, Ifjúság útja 6. Hungary

Authorship Contribution Statement

Valér Góra: Investigation, Visualization, Formal Analysis, and Writing. **Kristóf Csepregi**: Methodology, Supervision and Writing. Authors may edit this part based on their case.

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Research Article

Determination of essential oil components of *Ammi* L. genus in Türkiye and their effects on some storage pests

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Abstract: Effects of essential oil components obtained by hydrodistillation of *Ammi* genus members (*Ammi majus* L., *Ammi visnaga* L. (Lam.)), which have important chemical and active components were investigated against two important storage pests; fig borer *Cadra cautella* (Walker) (Lepidoptera: Pyralidae) and flour moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). A total of 23 and 14 components were detected in *A. majus* and *A. visnaga*, respectively, and the product yield was found to be 96.05%, 82.53%. Among them, the major components for *A. majus* are 2 heptadecanone, benzoic acid, 2 pentadecanone while for *A. visnaga* they are linalol, nonadecane, carvacrol. Essential oil of *A. visnaga* extended the adult emergence times in *E. kuehniella* and *C. cautella* while the increase in pupation time was found statistically significant only in *E. kuehniella*. *A. visnaga* essential oil reduced the adult life span in *E. kuehniella* at the highest dose while a decrease was detected in both doses applied in *C. cautella*. Adult weight and number of eggs decreased due to the application of *A. visnaga* in both insects. Also, alterations were observed in the adult emergence, pupation time, and pupal period. In *E. kuehniella* and *C. cautella*, adult life spans, weights and egg production of females showed statistically significant decreases depending on the application of *A. majus* essential oil. The findings obtained within the scope of the current study reveal that the essential oils of *A. majus* and *A. visnaga* species have the potential to be used in the control of storage pest insects.

1. INTRODUCTION

The widespread use of synthetic insecticides on Earth has led to numerous adverse consequences such as insecticide resistance, toxicity to mammals and non-target animals, residue issues, and environmental pollution, resulting in an increased interest in natural products (Isman, 2006). In recent years, research on the use of botanical oils as an alternative to synthetic chemical insecticides has become the focus of many researchers. These studies have played a significant role in the commercialization of plant-derived insecticides (Caballero, 2004). Essential oils are volatile, natural, complex secondary metabolites characterized by a strong aroma and generally have lower density than water (Tripathi *et al.*, 2009). In the control

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of stored product pests, volatile oils can have various effects, including fumigant activity, penetration into insect bodies as contact insecticides, acting as repellents or feeding deterrents, and influencing certain biological parameters such as growth rate (Natalia *et al.*, 2011). Medical and aromatic plants have attracted the attention of numerous researchers worldwide as a significant source of raw materials used in the pharmaceutical, cosmetic, flavor, and perfume industries. From ancient times to the present day, they have been utilized in various fields despite the advancements in synthetic drug research. Many drugs currently in use are derived from plants through the application of traditional practices combined with modern technologies (Canter *et al.*, 2005; Singh & Singh, 2001).

In the present day, the consumption of synthetic repellents worldwide has increased to protect grains, fruits, and other cellulose-based materials in storage facilities from various pests, many of which are arthropods. A similar situation is observed concerning human and animal health. In recent years, there have been significant concerns regarding the potential harm synthetic chemicals used in pest control may pose to the environment and human health. Therefore, the identification of natural products with good efficacy as alternatives to these chemicals is widely accepted among researchers. In recent years, researchers have conducted comprehensive studies aimed at testing the effect and insect-repellent properties of volatile oils obtained from various plants. Within the scope of this thesis, the effects of the *Ammi* genus, belonging to the Apiaceae family, which is rich in secondary metabolites and active components, on the development biology of the almond moth (*Cadra cautella* Walker) (Lepidoptera: Pyralidae) and the Mediterranean flour moth (*Ephestia kuehniella* Zeller) (Lepidoptera: Pyralidae), two storage pests, have been examined.

2. MATERIAL and METHODS

2.1. Collection of Plant Material

In this study, taxa belonging to the *Ammi* genus, namely *Ammi majus* and *Ammi visnaga* (Figure 1 and Figure 2), naturally occurring in the Balıkesir-Gömeç (Karaağaç) region, were collected during the flowering period, which spans from June to July. Following collection from their natural habitats, plant specimens were photographed in their respective natural habitats and then processed into herbarium material. Taxonomic identifications were carried out by Prof. Dr. Şükrü HAYTA at Balıkesir University Herbarium (BUH) using the Botanical guide and Flora of Turkey Volume 4, depending on the flowering periods of samples collected from localities.

Figure 1. *Ammi majus.*

Figure 2. *Ammi visnaga.*

2.2. Chemical Analyses

2.2.1. *Extraction of essential oils*

After collecting the taxa of the *Ammi* genus from their natural environments, they were dried in a cool, shaded, and sun-free environment. Following the drying process, above-ground organs were separated. For each taxon, approximately 100 g of dried plant samples were taken, and the Clevenger apparatus was used to obtain the essential oils. Water distillation method was preferred as the extraction method. The yield of essential oils was expressed as a percentage obtained by water distillation over 100 g of dried plant sample. The essential oils obtained through water distillation were transferred from the Clevenger apparatus system to vials and then sent to the Biology Department, Plant Products and Biotechnology Research Laboratory (BUBAL) at Firat University for chemical analyses. Chemical analyses were conducted using the GC-MS (Gas Chromatography-Mass Spectrometry) device in this laboratory with the aim of determining the qualitative and quantitative composition of the essential oils obtained through water distillation.

2.2.2. GC and GC-MS analyses

Chromatographic procedures were conducted using the Hewlett Packard system, HP-Agilent 5973 N GC-FID, and GC-MS (Gas Chromatography-Mass Spectrometry) 6890 GC system. The column used in the device was DB-5 MS (30m x 0.25 mm inner diameter), and helium was used as the carrier gas. The injector temperature was set at 250 °C, split flow rate at 1 ml/min., and the GC temperature was initially set at 60 °C for 2 minutes, followed by an increase of 10 °C/min. until it reached 150 °C. After 15 minutes, the temperature reached 240 °C and was held for 5 minutes. The most commonly used electronic libraries for characterizing the components of essential oils, such as WILEY, NIST, and the Essential Oil Library, were preferred. The data obtained from these analyses are presented in Table 1.

2.3. Rearing of Stored Grain Pests

2.3.1. *C. cautella walker (Lepidoptera: Pyralidae) (Almond moth)*

To establish laboratory stocks and successive cultures of *C. cautella*, a core insect culture containing larvae, pupae, and adult *C. cautella* individuals were reared in the laboratory (Figure 3). Male and female adult *C. cautella* individuals were collected daily and placed in jars of various sizes containing food. Cloth covers were used on the jars to ensure air circulation. A specific mixture of flour (%40 corn flour, %40 fine bran, %20 molasses) was used to feed the almond moths. Additional food was occasionally added to the cultures to meet the nutritional needs based on population density. The core, stock, and successive cultures were maintained in a laboratory at 25±1°C, %65±5 R.H., and a 12:12-hour (Dark: Light) photoperiod (Boz, 2013, Shakarami *et al.*, 2015, Usta, 2021).

Figure 3. *Cadra cautella* A. Larvae, B. Pupae, C. Adult

2.3.2. *E. kuehniella* Zell. (*Lepidoptera: Pyralidae) (Flour moth)*

To establish laboratory stocks and ongoing cultures of *E. kuehniella*, a core culture containing larvae, pupae, and adult *E. kuehniella* individuals were created in the laboratory (Figure 4). Male and female adult *E. kuehniella* individuals were collected daily and placed in glass jars of

various sizes containing food. A specific mixture of flour was used to feed the flour moths, consisting of %40 wheat flour, %20 corn flour, %20 barley flour, and %20 fine bran. The core, stock, and successive cultures were maintained in a laboratory at 25±1°C, %65±5 R.H., and a 12:12-hour (Light: Dark) photoperiod (Boz, 2013, Shakarami *et al.*, 2015, Usta, 2021).

Figure 4. *Ephestia kuehniella* A. Larvae, B. Pupae, C. Adult.

2.3.3. *Application of volatile oils*

To observe the effects of *A. majus* and *A. visnaga* volatile oils on the developmental biology of *C. cautella* and *E. kuehniella*, two different concentrations, stock and 50% (1:2 PBS (Phosphate Buffered Saline, Sigma), 1:2 Stock volatile oil), were determined in addition to the control group. These oils were topically applied to the dorsal side of the last larval stage (starting from the prothorax and along the dorsal side) using a micro-pipette at a volume of 5 µL (Luo *et al.*, 2017). The experiments were conducted with three replicates with 30 larvae in each replicate (n=90), and 96% ethanol was used in the control group.

2.3.4. *Effects of volatile oils on the developmental biology of stored grain pests*

To determine the effects of different concentrations of volatile oils on the pupation period of the last instar larvae, petri dishes including experimental larvae were monitored daily. The larval period was defined as the duration from when the larvae were introduced into the petri dish until the onset of pupation. To evaluate the impact of volatile oils with varying concentrations on the duration of the pupal stage, daily observations were conducted. The pupal period, denoting the time from pupation to the emergence of adult individuals, was thus assessed. To ascertain the influence of volatile oils on the timing of adult emergence, the duration from the application of volatile oil to the appearance of adult individuals was recorded.

2.3.5. *Adult longevity, weight, and total egg number*

To determine the longevity of individuals whose adult emergence period was determined, individuals were monitored daily until their death in an environment with 25±1°C, %65±5 R.H. The sex and date of death of each individual were recorded. The time elapsed from when the individuals emerged as adults to their death was calculated and the data were recorded as adult lifespan. Additionally, the effects of volatile oils on the weight of adult individuals were determined. The adults were individually measured using a motion-sensitive, precision analytical balance. After obtaining unmated adult female individuals following the application of volatile oils, they were encouraged to lay eggs by placing gauze between Petri dishes. Petri dishes were kept in incubators at 25±1°C, %65±5 R.H. No daily foods were provided to the insects. The eggs laid by adult females until their death were counted.

2.4. Statistical Analysis

All data obtained from the experiments were expressed as mean and standard error. The IBM SPSS statistics for Windows, Version 2018, was used for all statistical analyses. Experiment results expressed as percentages were normalized by taking the arcsine square root before statistical analysis, and the results were presented as percentages. The suitability of the data for normal distribution was determined using the Levene test. In cases where normal distribution was not observed, differences between means in terms of pupation duration were compared

using the Kruskal-Wallis and Mann-Whitney tests, while differences between means for other data, which showed normal distribution, were determined using one-way analysis of variance, and differences between means were identified using the Turkey HSD test. The results were considered significant at $p<0.05$.

3. RESULTS

Essential oil components extracted by water distillation method of taxa belonging to the genus *Ammi* L. has been given Table 1. A total of thirty two components were detected in plant samples. While nineteen of these components were found only in *Ammi majus*, nine of them were found only in *Ammi visnaga*, and the remaining five components were found to be common to both taxa. When the ratio of the components in the total oils were examined, it is seen that %96.05 in *Ammi majus* and %82.53 in *Ammi visnaga*.

Table 1. GC-MS Analysis Results of Essential Oils of *Ammi majus* and *Ammi visnaga*.

Components	RI	*Ammi majus (%)*	*Ammi visnaga (%)*
Monoterpenes			
α–Pinene	1022	1.25	-
α–Phellandrene	1077	0.3	-
α-Terpinene	1085	0.9	2.8
p-Cymene	1091	1.3	2.4
Limonene	1095	0.24	-
Oxygenated Monoterpenes			
1,8-Cineol	1097	0.8	1.3
Linalool	1148	-	**26.69**
cis-Sabinene hydrate	1156	-	1.7
Camphor	1186	0.9	2.7
Sesquiterpenes			
β-Bourbonene	1369	-	1.75
Aromadendrene	1424	1.56	-
Germacrene-D	1439	-	5.22
Oxygenated Sesquiterpenes			
Nerolidol	1484	-	3.38
Phenolic Components			
Carvacrol	1259	-	**9.75**
Thymol	1296	1.56	-
2-Methoxy-4-Vinyl phenol	1305	0.2	-
Hydrocarbons			
2-Heptanal	1037	1.47	-
Benzene, 1-Methyl-2 (2-Propenyl)	1087	-	2.94
Dodeconoic acide	1486	0.34	-
Butane acide	1584	-	2.51
Benzoic acide	1601	**20.04**	-
Cyclopentadecane	1636	**10.68**	-
2-Pentadecanone	1631	1.3	-
2-Heptadecanone	1665	**37.31**	-
N-Hexadeconoic acide	1696	9	-
Sesqui lavandulyl acetate	1800	1.04	-
Nonadecane	1803	-	**16.89**
Methyl lilonate	1809	1.29	-
Chrysanthenyl	1812	0.6	-
Octadecanoic acide	1833	0.43	2.50
1 H-Indene	1842	2.41	-
Tricosane	1852	1.13	-
Total		96.05	82.53

RI: Retention index

3.1. Effect of *A. visnaga* on the Developmental Biology of *E. kuehniella* and *C. cautella*

In *E. kuehniella*, among the experimental groups, the larval period is shortest in the control individuals and longest at the %50 dose. The increase in other doses compared to the control is statistically significant (F=14.840; sd=2.87; p=0.001) (Table 2). However, it was found that increases and decreases in other doses compared to the control in *C. cautella* are not statistically significant (F=1.004; sd=2.87; p=0.371) (Table 2). The impact of volatile oil application at two different concentrations on the pupal period is presented in Table 3 for *E. kuehniella* and *C. cautella*. In *E. kuehniella*, no statistically significant difference is observed among doses compared to the control (F=1.121; sd=2.87; p=0.331). However, the increase at the %50 dose compared to the control is statistically significant (F=21.544; sd=2.87; p=0.001) in *C. cautella*. The impact of volatile oil application at two different concentrations on the adult emergence time is presented in Table 4 for *E. kuehniella* and *C. cautella*. A statistically significant increase in *E. kuehniella* adult emergence time compared to the control is observed (F=6.654; sd=2.87; p=0.002). While there is no statistical difference was demonstrated in the adult emergence time of *C. cautella* compared to the control in stock concentration, a statistically significant increase is observed at the %50 dose compared to the control (F=11.592; sd=2.87; p=0.001).

Table 2. Influence of *A. visnaga* on larval period (day).

Concentration (mg/ml)	*E. kuehniella*		*C. cautella*	
	Min.-Max	±*SE**	Min.-Max	±*SE**
Control	5.44-6.29	5.86±0.20a	5.80-6.32	6.06±0.12a
%50	7.23-8.43	7.83±0.29b	5.68-6.25	5.96±0.76a
%100	6.71-7.88	7.30±0.28b	6.04-6.35	6.20±0.40a

* The difference between values with different letters in the same column is statistically significant ($p < 0.05$).
E. kuehniella: (F=14.840; sd=2.87; p= 0.001), *C. cautella:* (F=1.004; sd=2.87; p= 0.371).

Table 3. Influence of *A. visnaga* on pupal period (day).

Concentration (mg/ml)	*E. kuehniella*		*C. cautella*	
	Min.-Max	±*SE**	Min.-Max	±*SE**
Control	10.30-11.29	10.80±0.24a	9.45-10.14	9.80±0.16a
%50	9.68-11.11	10.40±0.34a	11.25-11.94	11.60±0.17b
%100	10.40-11.66	11.03±0.30a	9.34-10.52	9.93±0.28a

* The difference between values with different letters in the same column is statistically significant ($p < 0.05$).
E. kuehniella: (F=1.121; sd=2.87; p= 0.331), *C. cautella:* (F=21.544; sd=2.87; p= 0.001).

Table 4. Influence of *A. visnaga* on adult emergence time (day).

Concentration (mg/ml)	*E. kuehniella*		*C. cautella*	
	Min.-Max	±*SE**	Min.-Max	±*SE**
Control	16.07-17.25	16.66±0.28a	15.50-16.36	15.93±0.20a
%50	17.62-18.84	18.23±0.29b	17.15-17.97	17.56±0.20b
%100	17.37-19.29	18.33±0.47b	15.42-16.84	16.13±0.34a

* The difference between values with different letters in the same column is statistically significant ($p < 0.05$).
E. kuehniella: (F=6.654; sd=2.87; p= 0.002), *C. cautella*: (F=11.592; sd=2.87; p= 0.001

3.2. Effect of *A. visnaga* on Adult Longevity, Weight, and Total Egg Number in *E. kuehniella* and *C. cautella*

The impact of volatile oil application at two distinct concentrations on adult longevity and weight is presented in Table 5 for *E. kuehniella* and *C. cautella*. In *E. kuehniella*, a statistically significant reduction in adult longevity (F=13.419; sd=2.87; p=0.001) is observed at the stock concentration relative to the control. Additionally, decreases in adult weight (F=12.998; sd=2.87; p=0.001) are also found to be statistically significant in oil applied insects compared to the control. In *C. cautella* reductions in adult longevity among doses relative to the control are statistically significant (F=27.173; sd=2.87; p=0.001). Upon scrutiny of weight data in Table 6, a statistically significant decrease is observed in different concentrations compared to the control (F=17.682; sd=2.87; p=0.001). The volatile oil of *A. visnaga* has significantly affected the egg number in stored pest insects (Table 7). A substantial decrease in egg number is observed in both *E. kuehniella* (F=37.308; sd=2.42; p=0.001) and *C. cautella* (F=123.254; sd=2.42; p=0.001) relative to the control. In *E. kuehniella*, where the average egg number in control groups was 131.13, the total number of eggs decreased to 37.46 at the concentration of %100. In *C. cautella*, the average egg number in control groups, which was 109.46, decreased to 10.07 at the stock concentration.

Table 5. Influence of *A. visnaga* on adult longevity (day).

Concentration (mg/ml)	*E. kuehniella*		*C. cautella*	
	Min.-Max	±*SE**	Min.-Max	±*SE**
Control	13.03-15.03	14.03±0.48a	6.14-7.32	6.73±0.28a
%50	12.50-14.42	13.46±0.46a	4.31-5.81	5.06±0.36b
%100	9.54-11.72	10.633±0.53b	3.24-4.08	3.66±0.20c

* The difference between values with different letters in the same column is statistically significant ($p < 0.05$). *E. kuehniella*: (F=13.419; sd=2.87; p= 0.001), *C. cautella*: (F=27.173; sd=2.87; p= 0.001).

Table 6. Influence of *A. visnaga* on adult weight (day)

Concentration (mg/ml)	*E. kuehniella*		*C. cautella*	
	Min.-Max	±*SE**	Min.-Max	±*SE**
Control	10.98-12.33	11.66±0.32a	7.01-7.69	7.35±0.16a
%50	9.59-10.24	9.92±0.15b	5.87-6.36	6.12±0.12b
%100	9.83-10.82	10.33±0.24b	5.99-6.70	6.35±0.17b

* The difference between values with different letters in the same column is statistically significant ($p < 0.05$). *E. kuehniella*: (F=12.998; sd=2.87; p= 0.001), *C. cautella*: (F=17.682; sd=2.87; p= 0.001).

3.3. Effect of *A. majus* on the Developmental Biology of *E. kuehniella* and *C. cautella*

For *E. kuehniella*, within the experimental groups, the shortest larval period is observed in groups with the stock concentration, while the longest is seen at the 50% concentration (Table 7). No statistically significant changes are observed among doses compared to the control (F=2.165; sd=2.87; p=0.121). Upon examining Table 8, in *C. cautella*, the analysis reveals that increases and decreases among concentrations compared to the control are not statistically significant (F=4.991; sd=2.87; p=0.009). Due to the application of volatile oil at two different concentrations, a statistically significant increase in the pupal period is observed in *E. kuehniella* compared to the control (F=25.816; sd=2.87; p=0.001). However, in *C. cautella*, no statistically significant changes are observed among doses compared to the control (F=0.824; sd=2.87; p=0.442) (Table 9). The impact of volatile oil application at two different

concentrations on adult emergence time is presented in Table 10 for *E. kuehniella* and *C. cautella*. In *E. kuehniella*, a statistically significant increase is observed at the 50% dosage compared to the control (F=22.757; sd=2.87; p=0.001). No statistically significant changes are observed in the adult emergence time of *C. cautella* among doses compared to the control (F=0.207; sd=2.87; p=0.813).

Table 7. Influence of *A. visnaga* on total number of eggs.

Concentration (mg/ml)	*E. kuehniella*		*C. cautella*	
	Min.-Max	±*SE**	Min.-Max	±*SE**
Control	113.27-148.98	131.13±8.32a	93.19-125.74	109.46±7.58a
%50	60.47-96.86	78.66±8.48b	7.33-13.99	9.90±1.55b
%100	24.60-50.32	37.46±5.99c	9.80-25.79	10.07±3.72b

* The difference between values with different letters in the same column is statistically significant (p< 0.05).
E. kuehniella: (F=37.308; sd=2.42; p= 0.001), *C. cautella:* (F=123.254; sd=2.42; p= 0.001)

Table 8. Influence of *A. majus* on larval period (day).

Concentration (mg/ml)	*E.kuehniella*		*C.cautella*	
	Min.-Max	±*SE**	Min.-Max	±*SE**
Control	5.36-6.30	5.83±0.23a	6.29-6.83	6.56±0.13a
%50	5.82-6.24	6.03±0.10a	5.85-6.05	5.95±0.47b
%100	4.90-5.95	5.43±0.25a	6.08-6.71	6.40±0.15ab

* The difference between values with different letters in the same column is statistically significant (p < 0.05).
E. kuehniella: (F=2.165; sd=2.87; p= 0.121), *C. cautella*: (F=4.991; sd=2.87; p= 0.009).

Table 9. Influence of *A. majus* on pupal period (day).

Concentration (mg/ml)	*E. kuehniella*		*C. cautella*	
	Min.-Max	±SE*	Min.-Max	±SE*
Control	9.81-10.85	10.33±0.25a	9.16-9.77	9.46±0.14a
%50	12.41-13.71	13.06±0.31c	9.24-10.35	9.80±1.49a
%100	11.23-12.16	11.70±1.23b	8.85-9.94	9.40±1.45a

* The difference between values with different letters in the same column is statistically significant (p < 0.05).
E. kuehniella: (F=25.816; sd=2.87; p= 0.001), *C. cautella*: (F=0.824; sd=2.87; p= 0.442).

Table 10. Influence of *A. majus* on adult emergence time (day).

Concentration (mg/ml)	*E. kuehniella*		*C. cautella*	
	Min.-Max	±*SE**	Min.-Max	±*SE**
Control	15.65-16.69	16.17±0.25a	15.61-16.45	16.03±0.20a
%50	18.46-19.74	19.10±0.31b	15.36-16.70	16.03±0.32a
%100	16.43-17.91	17.17±0.36a	15.11-16.48	15.80±0.33a

* The difference between values with different letters in the same column is statistically significant (p < 0.05).
E. kuehniella: (F=22.757; sd=2.87; p= 0.001), *C. cautella*: (F=0.207; sd=2.87; p= 0.813)

3.4. Effect of *A. majus* on Adult Longevity, Weight, and Total Egg Number in *E. kuehniella* and *C. cautella*

The effects of volatile oil application at different concentrations on adult longevity and weight in *E. kuehniella* and *C. cautella* are presented in Table 11 and Table 12. In *E. kuehniella*, a statistically significant decrease in adult longevity (F=24.415; sd=2.87; p=0.001) and adult weight (F=10.775; sd=2.87; p=0.001) is observed compared to the control. In *C. cautella*, decreases in adult longevity among doses compared to the control are statistically significant

(*F*=24.234; *sd*=2.87; *p*=0.001). The application of *A. majus* volatile oil results in a statistically significant decrease in adult weight compared to the control (*F*=16.320; *sd*=2.87; *p*=0.001) (Table 12).

Table 13 illustrates changes in egg counts due to volatile oil application in *E. kuehniella* and *C. cautella*. A statistically significant decrease in egg counts is observed in *E. kuehniella* (*F*=40.498; *sd*=2.42; *p*=0.001) and *C. cautella* (F=54.542; *sd*=2.42; *p*=0.001) compared to the control. In *E. kuehniella*, average egg counts, which were 131.26 in the control group, decreased to 37.26 in the stock dosage group. In *C. cautella*, the average egg counts, starting at 115.60 in the control group, decreased to 28.26 in the stock concentration group.

Table 11. Influence of *A. majus* on adult longevity (day).

Concentration (mg/ml)	*E. kuehniella*		*C. cautella*	
	Min.-Max	±*SE**	Min.-Max	±*SE**
Control	15.43-16.64	16.03±0.29a	6.61-7.78	7.20±0.28a
%50	10.05-12.62	11.33±0.62b	3.63-5.22	4.43±0.38b
%100	9.35-12.32	10.83±0.72b	3.32-4.80	4.06±0.36b

* The difference between values with different letters in the same column is statistically significant ($p < 0.05$).
E. kuehniella: (*F*=24.415; *sd*=2.87; *p*= 0.001), *C. cautella*: (*F*=24.234; *sd*=2.87; *p*= 0.001).

Table 12. Influence of *A. majus* on adult weight.

Concentration (mg/ml)	*E. kuehniella*		*C. cautella*	
	Min.-Max	±*SE**	Min.-Max	±*SE**
Control	10.98-12.32	11.65±0.32a	6.90-7.64	7.27±0.18a
%50	9.10-10.45	9.78±0.32b	5.72-6.22	5.97±0.12b
%100	9.12-10.46	9.79±0.32b	5.69-6.35	6.02±0.89b

* The difference between values with different letters in the same column is statistically significant ($p < 0.05$).
E. kuehniella: (*F*=10.775; *sd*=2.87; *p*= 0.001), *C. cautella*: (*F*=16.320; *sd*=2.87; *p*= 0.001).

Table 13. Influence of *A. majus* on total number of eggs.

Concentrations (mg/ml)	*E. kuehniella*		*C. cautella*	
	Min.-Max	±*SE**	Min.-Max	±*SE**
Control	113.67-148.85	131.26±8.20a	96.22-134.97	115.60±9.03a
%50	36.22-67.64	51.93±7.32b	71.19-84.67	77.93±3.14b
%100	19.50-55.02	37.26±8.27b	20.21-36.31	28.26±3.75c

* The difference between values with different letters in the same column is statistically significant ($p < 0.05$).
E. kuehniella: (*F*=40.498; *sd*=2.42; *p*= 0.001), *C. cautella*: (*F*=54.542; *sd*=2.42; *p*= 0.001).

4. DISCUSSION and CONCLUSION

In this study, water distillation method has been used to obtain essential oils from the above-ground parts of taxa belonging to *Ammi* genus. Of these taxa, *Amni visnaga* has a productivity of 100 g per each dry sample of 0.2 ml. Whereas *Ammi majus* has 0.4 ml. per each. Method of Gas Chromotography and Mass Spectrometry (GC-MS) have been used for the identification of chemical components existing in the essential oils. The essential oils of two *Ammi* species were studied and thirty two components, in all, were identified representing (82.53%) and (96.05%) of the oils respectively. While eighteen of these thirty two different components

detected in plant samples have been found only in *Ammi majus*, nine components have been found in *Ammi visnaga*, and the five components that remain have been detected in common in both taxa. As a result of the analysis, the 2-heptadecane (%37.31), benzoic acid (%20.04) and cyclopentadecane (%10.68) were major components for *Ammi majus* whereas linalool (%26.69), carvacrol (%9.75) for *Ammi visnaga*. According to their chemical structures, components are classified in seven different groups: Hydrocarbons and their derivatives, monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, fenolic components and fatty acids (Table 1).

Chemical components of essential oils in *Ammi visnaga* are ranged mainly between monoterpene and nonterpene groups. In addition to these, diterpene and sesquiterpene groups are found in low amounts (Abdul-Jalil *et al.*, 2010; Sellami *et al.*, 2011). Monoterpenes that make a large number of volatile components detected in *Ammi visnaga* are oxygenated or hydrocarbon monoterpenes. The mainly defined components in *A. visnaga* are linalool and thymol. These two components make up the highest percentage among the other monoterpenes. The other identified monoterpenes are α-thujen, α-pinene, β-pinene and β-myrcene (Khalfallah *et al.*, 2011; Khadhri *et al.*, 2011). In the literature, the content of *Ammi visnaga* essential oils is the most high amounts of compounds; isoamyl 2-methylbutyrate, isoamyl isobutyrate, iso-butyl-2-methylbutyrate, 2-methylbutyrate 2-methylbutyrate, 2-methylbutyl isobutyrate and isoamyl isovalerate (Zrira *et al.*, 2008; Khalfallah *et al.*, 2011); butanoic acid, 2-methyl-, pentyl ester,(Z)-β-ocimene, D- limonene, linalool, pulegone and lavandulyl-butyrate (Kamal *et al.*, 2022); linalool, isoamyl 2-methylbutyrate and isopentyl isovalerate (Khadhri *et al.*, 2011) it was reported. As a result of our analyses of the essential oils belonging to *Ammi visnaga*, it was found that the chemical compounds in the essential oil contents contained a high amount of monoterpene groups in accordance with the literature, and among the identified monoterpenes, linalool and carvacrol were found in quite high amounts. Determination of linalool as the main component and characterization of essential oil content of *Ammi visnaga* with monoterpenes by Khadhri *et al.* (2011), and Kamal *et al.* (2022) it is in line with the 2022 studies, but in line with other studies it shows some differences. It is thought that these differences are related to stress and changes in the environment, solar radiation, type of soil, and also different biotypes and geographical origins. It is claimed that these factors may lead to upward and downward regulations of biosynthetic pathways by causing activation or inactivation of certain enzimatic groups (Hashim *et al.*, 2014). Linalool is a monoterpene component that is extensively found as the main component of several aromatic essential oils and is used as a tranquilizer traditionally. More than 200 plants such as citrus and lavender contain linalool (Elisabetsky, 2002). Linalool is a monoterpene that is mostly extracted from lavender (*Lavandula* spp.), rose (*Rosa* spp.), basil (*Ocimum basilicum*), and neroli oil (*Citrus aurantium*) (Russo & Marcu, 2017). It has also been reported in many studies that linalool has insecticidal activity against insects in stored products (Tripathi & Mishra, 2016). No general research into the detection of essential oil components belonging to *Ammi majus* has been encountered in the literature so far. It is also observed that only a few studies on essential oil components in fruits has been reported. In one of these studies by Nayebi and friends, (2013) it has been proven that *Ammi majus* components of essential oils contain n-alkanes, carboxylic acid, terpenoids, cycloalkanalcohol, ketone, aldehyde, alkene and alkylhalides. It has been found that terpenoids are the dominant groups and major components are toluene (3.766%), thymol (12.81%), and carvacrol (37.81%). In another study on the contents of essential oils in fruits (Akhtar *et al.*, 2010). It has been detected that they contain thirteen monoterpene (40.3%) and fifteen sesquiterpen (34.2%) and there is a large amount of carvone (13.4%), 1.8-cineol (6.9%), α-terpinyl acetate (5.9%), trans-pinocarveol (3.2%) and citronellal (3.2%) it has been identified. It has been informed that fruits involve some derivatives of furanocoumarin and these components in the structure of coumarin are umbelliferon, psoralen, imperatorin, 5- methoxypsoralen and 8- methoxypsoralen and

contain fixed oil and bitter substances (Baytop, 1999; Nayebi et *al.*, 2013; Harsahay *et al.*, 2014). It is also, *Ammi majus* fruits contain flavonoids such as quercetin, campherol, as well as luteolin glycosides (Harborne & Williams, 1972; Abdul-jalil *et al.*, 2010) in terms of fatty acids, it has also been shown that fatty acids such as petroselinic acid and oleic acid are highly present (Kleiman & Spencer 1982). As a result of analyzes during our studies, it is thought that the main reason why high-rate components belonging to *Ammi majus* is not exactly compatible with the ones in the literature is that these studies recorded in the literature were carried out with only fruits and the plant samples we had collected were of the late-vegetation period.

The use of pesticides for combating detrimental insects is widely acknowledged as hazardous to both the environment and human health. Consequently, there is a growing interest in plant-derived substances as potential alternatives to pesticides. Plant compounds, owing to their non-toxicity to non-target organisms and lack of persistence in nature, are increasingly recognized as viable alternatives. To protect crops and stored products from pests, various methods are employed, and one such method involves the use of plant extracts and essential oils. In a study conducted by Kanat & Alma (2004), nine different essential oils, including those derived from *P. brutia*, *Laurus nobilis*, *Liquidambar orientalis*, *Juniperus communis* L. subsp *nana*, *Cupressus sempervirens*, *Lavandula stoechas*, *Lavandula angustifolia*, *Eucalyptus camadulensis*, and *Thymus vulgaris*, were applied to larvae in three different doses (25%, 50%, and 100%). All essential oils exhibited insecticidal activity at all three applied doses. Aromatic plants belonging to the Apiaceae and Lamiaceae families have been known since ancient times for their antiseptic and medicinal properties (Hussain *et al.*, 2011). Currently, significant research is underway to evaluate the repellent and insecticidal effects of volatile oils and their components against harmful insects (Adorjan & Buchbauer, 2010). In our study, we utilized essential oils derived from *A. visnaga* and *A. majus*, both belonging to the Apiaceae family, to assess their impact on stored-product pests.

The application of *A. visnaga* essential oil to late-stage larvae in our study resulted in an increase in the pre-adult development period compared to the control. Following *A. visnaga* application to *E. kuehniella* larvae, a dose-dependent increase in pupation was observed compared to the control. For *C. cautella* larvae treated with *A. visnaga*, a dose-dependent increase in the pupal period was noted compared to the control. Various concentrations of *A. visnaga* resulted in significant decreases in the adult longevity, weight, and egg numbers of both *C. cautella* and *E. kuehniella*. Several plant species from the Apiaceae family are known for their significant acaricidal and insecticidal effects against numerous insects (Papachristos & Stamopoulos, 2002). It is noteworthy that there are limited studies in the literature on the insecticidal effects of *A. visnaga* essential oil or various extracts. Previous research has indicated its inhibitory effects on the growth and development of the desert locust *S. gregaria* (Ghoneim, 2014). Additionally, *A. visnaga* extract has demonstrated efficacy in protecting stored grains against the granary weevil *Sitophilus granarius* and the rice weevil *Sitophilus oryzae* (Abdel Latif, 2004). Previous studies have explored *A. visnaga's* larvicidal and insecticidal properties, reporting effectiveness against *Oncopeltus fasciatus* (Hemiptera) and *Aedes aegypti* (Diptera) larvae (Maleck *et al.*, 2013). Therefore, khellin and visnagin from *A. visnaga* have been suggested to produce new botanical acaricides (Maleck *et al.*, 2013). The ethanol extract of *A. visnaga* fruit has been shown to inhibit lipid content in nymphs and adults' hemolymphs, while the n-butanol extract of *A. visnaga* has inhibited the activity of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) in nymphs and adults' hemolymphs (Ghoneim, 2014).

In the case of *A. majus* essential oil application, an increase in immature stages development and pupation periods were observed in *E. kuehniella*. Moreover, different concentrations of *A. majus* essential oil resulted in decreased adult lifespan and weight in *C. cautella* and *E. kuehniella*. *A. majus* caused significant reductions in egg numbers for both stored-product pests.

Sub-lethal doses of botanical control agents can alter mortality rates, reproductive capabilities, and the genetic makeup of new generations in pests, affecting their egg-laying and egg-hatching rates (Moriarty, 1969). The obtained results suggest that various concentrations of *A. majus* and *A. visnaga* essential oils have inhibitory effects on the development and egg production of *E. kuehniella* and *C. cautella*. Additionally, the application of essential oils in this study proved to be significantly effective during the targeted period and considering the lack of environmental and human harm associated with volatile oils, they are anticipated to be an effective method in biological pest control applications.

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

Şükrü Hayta: Investigation, Visualization, Software, Formal Analysis, and Writing -original draft. **Aysel Manyas**: Resources, Supervision, and Validation. **Aylin Er:** Methodology, Visualization and Writing -original draft.

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Research Article

Antioxidant activity of polyphenol compounds extracted from *Nypa fruticans* Wurmb. (Nipa palm) fruit husk with different ethanol concentration

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Abstract: Oxidative stress is a condition characterized by a higher content of free radicals than the potential antioxidants in the body. Exogenous antioxidants are needed to resolve this condition. The *Nypa fruticans* (Nipa palm) fruit husk is a source of polyphenol potential and can be used as a natural antioxidant agent. Therefore, this study aimed to determine the effect of ethanol concentration on polyphenol and tannin contents and their antioxidant activities. The polyphenol substances were extracted using several ethanol concentrations, whereas the antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl method. The results show that the ethanol concentration has no effect on the yield of extraction. However, it affects the total polyphenol and tannin contents with high levels in the 50% and 70% ethanol concentrations. Fifty percent ethanol exhibits more effective antioxidant activity when compared to other ethanol concentrations. Therefore, a 50% ethanol concentration is a suitable solvent to extract polyphenol and tannin substances from nipa palm fruit husk and can be used as an alternative natural antioxidant.

1. INTRODUCTION

Oxidative stress is a condition caused by an imbalance between free radicals and antioxidant potential in the body (Rad *et al*., 2020). A free radical is a substance with an unpaired electron in the outer orbital that is highly reactive with other molecules such as lipids and proteins, including deoxyribonucleic acid (Lobo *et al*., 2010). When the free radical level is higher than the potential endogenous antioxidant, the body needs an external source of antioxidant (exogenous antioxidant), which can be obtained from natural sources through functional foods or dietary supplements (Xu *et al*., 2017).This antioxidant is generally composed of some

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bioactive compounds, such as polyphenols, vitamins, pigments, and polysaccharides (Lourenço *et al*., 2019).

A polyphenol compound is a type of secondary metabolite that is generally found in plants. Polyphenol substances include phenolic acids, flavonoids, stilbenes, and lignans. Tannins are a class of polyphenol compounds that belong to the flavonoid subgroup. Polyphenol groups are known as antioxidant agents because they inhibit free radical formation. It can act as a radical scavenger by donating hydrogen atoms or electron transfer mechanisms. Polyphenols can be extracted from terrestrial and aquatic plants, such as seaweeds (Ismail *et al*., 2023), water lettuce (*Pistia stratiotes*) (Herpandi *et al*., 2021), and yellow velvetleaf (*Limnocharis flava*) (Serang & Laili, 2021). A previous study reported that tannin compounds were successfully extracted from coconut (Family Arecaceae) husk using an ethanol solvent (Buamard & Benjakul, 2017). The family Arecaceae, also known as the Palmae family, contains species of tropical shrubs, climbers, and trees commonly known as palm trees. *Nypa fruticans* Wurmb. is also a family of Arecaceae widely found in tropical countries. The empirical observation indicated that coconut husks show morphological similarity with the nipa palm fruit husk. Additionally, nipa palm endosperm and leaves have been reported to contain polyphenol compounds (Gazali *et al*., 2019; Prasad *et al*., 2013). Therefore, we hypothesized that the nipa palm fruit husk is also composed of polyphenols, including tannins.

In general, polyphenolic compounds can be extracted from plants using an ethanol solvent or its mixture with water (Mojzer *et al*., 2016). Ethanol was chosen as a solvent due to its relatively low toxicity and classification as a safe food additive (Plaskova & Mlcek, 2023). A previous study reported that different ethanol concentrations have a significant effect on the total phenolic and tannin contents of *Centella asiatica* (Chew *et al*., 2011). Also, different ethanol concentrations show different effects on the total polyphenol and tannin contents of *Andrographis paniculata* (Thoo *et al*., 2013). These conditions indicated that different ethanol concentrations have different effects on the total polyphenol and tannin content. Therefore, we hypothesized that different ethanol concentrations would also have different effects on the polyphenol and tannin contents of the nipa palm fruit husk extract. Thus, this study aimed to investigate the effects of the ethanol concentration on the total polyphenol and tannin contents of the nipa palm fruit husk extract as well as its antioxidant activity.

2. MATERIAL and METHODS

2.1. Materials

The *Nypa fruticans* fruit husk was collected from Sung Sang Village, South Sumatra. The ethanol absolute, Folin-Ciocalteu'sphenol reagent, natrium carbonate (Na_2CO_3), tannic acid, gallic acid, and *2,2*-diphenyl-*1*-picrylhydrazyl (DPPH) powder were purchased from Sigma Aldrich.

2.2. Preparation and Extraction

The small pieces of fruit husk were oven-dried (Memmert Universal Oven UN55) at 45°C for 24 hours and ground to obtain dried fruit husk powder using a grinding machine (Microphyte disintegrator B-One DM-120M). It was extracted using several concentrations of ethanol solvent with the maceration method according to previous methods (Chew *et al*., 2011; Złotek *et al*., 2016). Briefly, 20 g of extract was mixed with 200 mL of each ethanol concentration (50%, 60%, 70%, and 80%; ethanol mixed with distilled water) in the Erlenmeyer flask. The extraction was performed at 30°C for 3 hours on the hot plate and stirred with a magnetic stirrer (IKA-C MAG HS 7) at 120 rpm. After the extraction time, the filtrate and residue were separated using filter paper (Whatman No. 42). The filtrate was collected in a collection tube, whereas the residue was reextracted in the same condition as the first extraction, and five extractions were performed in total. After that, all the filtrates were collected in a new

collection. The filtrate was evaporated at 45°C using a rotary vacuum evaporator (Biobase RE-301) to remove the solvent and was completely dried using a freeze dryer (Biobase BK-FD10S) to obtain a dried extract. The percentage yield of extraction (%) was calculated as dried fruit husk powder (g) divided by dried extract (g) and multiplied by 100%.

2.3. Total Polyphenol and Tannin Contents Analysis

The polyphenol content of the nipa palm fruit husk ethanol extract was analyzed according to the previous method (Chandra *et al*., 2014). Briefly, 100 mg of the extract was dissolved in 10 mL of distilled water in an Erlenmeyer flask. Then, 0.2 mL of the extract solution was mixed with Folin-Ciocalteu's reagent (1:1, v/v) in the reaction tube and allowed to react at room temperature for 5 minutes. After the reaction time, 1 mL of 8% natrium carbonate solution was pipetted into the reaction tube, and the volume was increased to 3 mL with distilled water. The mixture was allowed to react at room temperature for 30 minutes. After that, it was centrifuged (Oregon LC-04S Centrifuge) at 3,000 rpm for 30 minutes. The supernatant was collected and absorbance was measured using a UV-Vis spectrophotometer at 765 nm immediately. Gallic acid was used as a standard; therefore, the total polyphenol content was calculated as mg gallic acid equivalent per g of dried sample (mg GAE/g).

The total tannin of the nipa palm fruit husk ethanol extract was measured according to the previous study (Rajkumar *et al*., 2022). Briefly, 2 mg of extract was dissolved in 2 mL of ethanol solvent in a tube. Then, 0.1 mL of the extract solution was pipetted into a reaction tube and added to 7.5 mL of distilled water. The mixture was then added to 0.5 mL of Folin-Ciocalteu's reagent and 1 mL of 35% natrium carbonate, bringing the volume up to 10 mL with distilled water. After 30 minutes of reaction at room temperature, the absorbance was immediately measured using a UV-Vis spectrophotometer at 700 nm. Tannic acid was used as a standard; therefore, the total tannin content was calculated as mg tannic acid equivalent per g of dried sample (mg TAE/g).

2.4. Antioxidant Activity Assay

The antioxidant activity of nipa palm fruit husk ethanol extract was analyzed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Sudirman *et al*., 2022). Briefly, the extract was dissolved in ethanol to make a serial concentration (0 – 1,000 µg/mL). Then, 1 mL of each sample concentration was mixed with 0.2 mM DPPH solution (1:1, v/v) and incubated at 37°C for 30 minutes. The absorbance was immediately measured using a UV-Vis spectrophotometer (Genesys 150 ThermoScientific) at 517 nm. The antioxidant activity was calculated as the inhibition of the extract on the DPPH radical according to this formula:

$$\text{Percentage (\%) of inhibition} = \frac{\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{blank}}} \times 100\%$$

Whereas: Abs_{blank}, the absorbance at 517 nm without sample; Abs_{sample}, the absorbance at 517 nm with sample.

2.5. Data Analysis

All data were expressed as the mean ± standard deviation (SD). The total polyphenol, tannin, and antioxidant activity were analyzed by one-way analysis of variance and Duncan's post-hoc test at $p<0.05$. All graphics were produced using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, United States).

3. RESULTS

3.1. Yields of Extraction

The yield of crude extract from *N. fruticans* fruit husk is shown in Table 1. The different ethanol concentrations also have different effects on the extraction yield. The higher-yield extract is at the 50% ethanol concentration (17.74%±5.85) and lower at the 70% ethanol concentration (13.66±4.78%).

Table1. Yield of crude extract from *N. fruticans* husk with different ethanol concentrations.

Parameter	Ethanol concentrations (%)			
	50	60	70	80
Yield* (%)	17.74±5.85	14.25±0.91	13.66±4.78	14.83±2.34

*The data are shown as the mean ± SD (*n*=3).

3.2. Total Polyphenol and Tannin Contents

The total polyphenol content of the *N. fruticans* fruit husk ethanol extract is shown in Figure 1. The ethanol concentrations of 70% (16.63±2.25 mg GAE/g), 50% (15.48±1.48 mg GAE/g), and 80% (14.71±1.62 mg GAE/g) are significantly higher on the total polyphenol contents when compared to 60% (8.08±1.27 mg GAE/g). Whereas, the tannin content of *N. fruticans* fruit husk ethanol extract is shown in Figure 2. The ethanol concentrations of 70% (257.34±0.66 mg TAE/g) and 50% (255.54±11.45 mg TAE/g) are significantly higher on the total tannin contents when compared to 80% (232.87±2.86 mg TAE/g) and 60% (228.73±2.90 mg TAE/g).

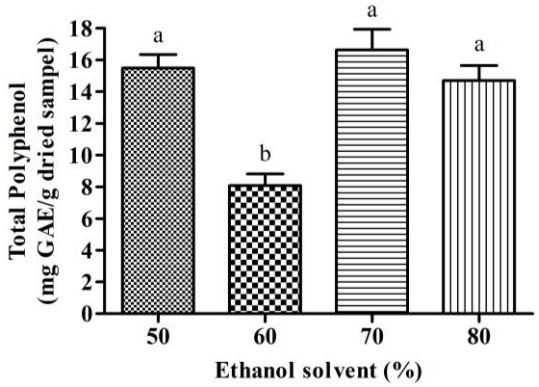

Figure 1. Total polyphenol content of the extract from *N. fruticans* fruit husk with different ethanol concentrations. The data are shown as the mean ± SD (*n*=3). Different letters above the bars indicate a statistically significant difference at $p<0.05$.

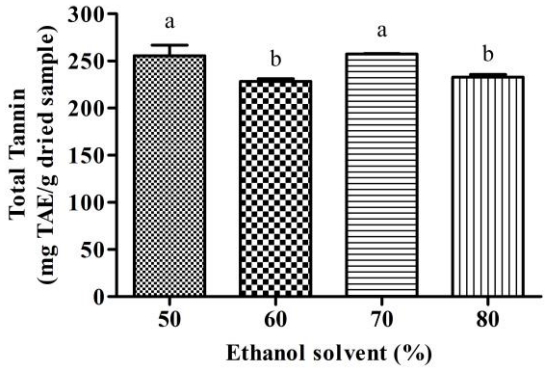

Figure 2. Total tannin content of the extract from *N. fruticans* fruit husk with different ethanol concentrations. The data are shown as the mean ± SD (*n*=3). Different letters above the bars indicate statistically a significant difference at $p<0.05$.

3.3. Antioxidant Activity

The antioxidant activity of the *N. fruticans* fruit husk ethanol extract is shown in Figure 3. Different ethanol concentrations significantly affected the antioxidant activities of the extract. The ethanol concentration of 50% showed the highest antioxidant activity, with the half-maximum inhibitory concentration (IC_{50}) of about 208.96±1.58 µg/mL and the lower in the 80% (429.98±50.11 µg/mL).

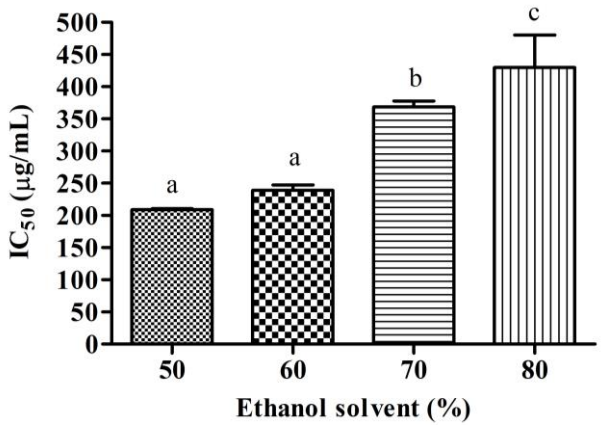

Figure 3. Antioxidant activity of the polyphenol extract from *N. fruticans* husk with different ethanol concentrations. The data are shown as the mean ± SD (n=3). Different letters above the bars indicate a statistically significant difference at p<0.05.

4. DISCUSSION and CONCLUSION

In this study, we successfully extracted polyphenol and tannin compounds from *Nypa fruticans* fruit husk. The different ethanol concentrations also have different effects on the extraction yield (Table 1). A previous study reported that the highest yield of ethanol extracts from *Sauropus androgynus* leaf is 50% ethanol (37.77±0,93%) and the lowest is 96% (33.55±2.77%) (Hikmawanti *et al*., 2021). A previous study reported that the yield of 70% ethanol crude extract from *Pistia stratiotes* leaf was about 16.80% (Sudirman *et al*., 2022). Additionally, the yield of extraction from peanuts using 80% ethanol is 13.01% and that using 20% ethanol is 8.67% (Jitrangsri *et al*., 2020). Different extraction yields are due to different ethanol concentrations caused by the different solvent polarities of each ethanol solvent. Solvents with high polarity also had the ability to extract a wide range of compounds (Do *et al*., 2014). Several factors can affect the extraction process, including the solvent type (Sulaiman *et al*., 2017). A previous study reported that different ethanol concentrations have been used for bioactive compound extraction from plants (Sun *et al*., 2015). Ethanol was chosen as a solvent due to its relatively low toxicity and classification as a safe food additive (Plaskova & Mlcek, 2023); therefore, the extract can be used as a functional food ingredient (Chemat *et al*., 2019).

Different ethanol concentrations show a different effect on the total polyphenol content of nipa palm fruit husk (Figure 1). The highest total phenolic content of grape stem was also extracted with 50% ethanol (Moreno *et al*., 2019).Additionally, 70% ethanol also shows the highest concentration of polyphenol content in some medical plants (Haq *et al*., 2019; Lezoul *et al*., 2020). The different ethanol concentrations used in the extraction also indicate different effects on the tannin contents (Figure 2). A previous study also reported that 50% ethanol solvent is the optimum concentration for tannin extraction from *Areca catechu* nut (Jakfar & Azwar, 2023). These results indicated that different ethanol concentrations also have different effects on the total polyphenol and tannin content. A previous study also reported that different ethanol concentrations have a significant effect on the total phenolic and tannin contents of *Centella asiatica* (Chew *et al*., 2011). Additionally, different ethanol concentrations also show

different effects on the total polyphenol and tannin contents of *Andrographis paniculata*(Thoo *et al*., 2013). In the solvent extraction method, the solvent only extracts those phytochemical or bioactive substances that a have similar polarity to the solvent, according to the "like dissolves like" principle. The ethanol concentration is the factor that has a significant effect on the bioactive compound extraction (Zhang *et al*., 2007).

The ethanol extracts from nipa palm fruit husk exhibit antioxidant activity (Figure 3). The low IC_{50} value indicated that the extract exhibits high potential antioxidant activity and *vice versa* (Goutzourelas *et al*., 2023). This condition is due to its high level of polyphenol and tannin content. A previous study reported that the ethanol extract of *Piper crocatum* exhibits antioxidant activity (Safithri *et al*., 2022). Polyphenol compounds are known as a source of natural antioxidant agents by single electron transfer (SET) or hydrogen atom transfer (HAT) mechanisms that inhibit free radicals and result in the reduction of the adverse effects of free radicals (Lee *et al*., 2015). In a previous study, tannin also exhibited antioxidant and antiradical properties (Maisetta *et al*., 2019).

Overall, in this present study, the bioactive compounds were successfully extracted from *N. fruticans* fruit husks using an ethanol solvent. Different ethanol solvents have different effects on the yield of extraction. However, it shows different effects on the total polyphenol and tannin contents, with a high content at 50% and 70% ethanol concentrations. Filthy percent ethanol also shows the highest antioxidant activity. Therefore, 50% ethanol solvent is a suitable solvent to extract polyphenol and tannin substances from nipa palm fruit husk and can be used as an alternative natural antioxidant.

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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

Sabri Sudirman: Supervision and Writing – editing and final approval. **Aprilia Kusuma Wardana**: Methodology and Formal Analysis. **Herpandi:** Supervision. **Indah Widiastuti**: Writing -original draft. **Dwi Indah Sari**: Formal Analysis and Writing -original draft. **Miftahul Janna**: Methodology and Formal Analysis.

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Review Article

Investigating medicinal plants for antimicrobial benefits in a changing climate

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Abstract: As the world's climate changes, there is growing concern about how it is affecting human health, including the rise of antimicrobial resistance. Medicinal plants have been used for centuries and their antimicrobial properties have been recognized by many cultures. This article focuses on exploring the potential of medicinal plants for antimicrobial activity in the face of climate change challenges. The article discusses the challenges and opportunities associated with using medicinal plants as a source of new antimicrobial agents, including issues related to the changes in plant chemistry caused by climate change, and the need for sustainable and ethical sourcing practices. The article also examines the importance of traditional knowledge and cultural practices in the development and conservation of medicinal plants. Finally, the article highlights the importance of interdisciplinary research and collaboration in harnessing the potential of medicinal plants for combating antimicrobial resistance in the context of a changing climate.

1. INTRODUCTION

Antimicrobial activity is the ability of a substance to kill or inhibit the growth of microorganisms, such as bacteria, viruses, fungi, and parasites. This is an important property of medicinal plants because many infectious diseases are caused by these microorganisms. Antimicrobial compounds found in medicinal plants have been used for centuries to treat a variety of illnesses, ranging from common colds to life-threatening infections. In recent years, antimicrobial resistance has become a major public health concern, as more and more microorganisms are becoming resistant to the antibiotics and other antimicrobial drugs that are currently available. The World Health Organization has identified antimicrobial resistance as one of the top 10 global public health threats facing humanity (World Health Organization, 2021). In this context, the search for new and effective antimicrobial compounds is of utmost importance.

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Medicinal plants offer a diverse and potentially rich source of antimicrobial compounds. Plants produce a wide range of secondary metabolites, many of which have been shown to possess antimicrobial properties (Jain *et al*., 2019). These compounds may act by disrupting the cell membranes or cell walls of microorganisms, or by interfering with their metabolic processes. The importance of antimicrobial activity in medicinal plants lies in the potential to discover new and effective compounds for the treatment of infectious diseases. As the threat of antimicrobial resistance continues to grow, the development of new antimicrobial agents is essential. By exploring the antimicrobial activity of medicinal plants, we can identify new compounds that may help to combat resistant microorganisms and contribute to the development of more effective treatments.

In addition to the potential for discovering new antimicrobial compounds, medicinal plants also offer other advantages as a source of antimicrobial agents (Farzaneh & Carvalho, 2015). Many of these plants are easily accessible and affordable, particularly in low-income and rural communities where access to modern healthcare may be limited. This makes them an attractive option for traditional medicine, which is still widely practiced in many parts of the world. Medicinal plants are often used in combination with other plants or natural remedies, which may enhance their therapeutic effects or reduce their side effects. This approach, known as synergistic or complementary therapy, has been shown to be effective in many cases and may help to reduce the reliance on synthetic drugs.

The use of medicinal plants for antimicrobial activity can contribute to the conservation and sustainable management of plant species. Many medicinal plants are harvested from the wild, and overexploitation can lead to the depletion of plant populations and the loss of biodiversity. By promoting the sustainable use of medicinal plants, we can help to ensure their long-term availability and protect the ecosystems in which they grow (Chen *et al*., 2016). The importance of antimicrobial activity in medicinal plants lies in their potential to provide new and effective compounds for the treatment of infectious diseases, particularly in the context of antimicrobial resistance. Additionally, medicinal plants offer advantages such as affordability, synergy with other natural remedies, and contribution to conservation and sustainable management. As such, continued research into the antimicrobial properties of medicinal plants is crucial for the development of effective and sustainable treatments for infectious diseases.

Climate change can have significant impacts on the growth, distribution, and chemical composition of medicinal plants, which can affect their antimicrobial properties (Kumar & Sharma, 2018). These impacts can be direct or indirect and can vary depending on the specific plant species and the region in which they grow. One of the most direct ways in which climate change can affect medicinal plants is through changes in temperature and precipitation patterns. For example, droughts, floods, and extreme weather events can lead to decreased plant growth and productivity and may alter the chemical composition of plant tissues. This can result in changes in the concentrations of secondary metabolites, including those with antimicrobial properties.

In addition to these direct impacts, climate change can also affect the interactions between medicinal plants and other organisms, such as pollinators and pests. Changes in temperature and precipitation patterns can alter the timing of flowering and pollination, which can affect the reproductive success of medicinal plants (Kudo & Hirao, 2006). This can, in turn, affect the availability of medicinal plant material and the diversity of secondary metabolites produced. Furthermore, climate change can lead to the spread of new pests and diseases, which can have indirect effects on the antimicrobial properties of medicinal plants. For example, pest infestations can alter the metabolic pathways of plants, leading to changes in the production of secondary metabolites. Additionally, pests and diseases can reduce the growth and productivity of medicinal plants, further reducing their availability and potentially affecting the composition

of secondary metabolites. Climate change can have significant impacts on the antimicrobial properties of medicinal plants through direct and indirect effects on plant growth, chemical composition, and interactions with other organisms. As such, it is important to consider the potential impacts of climate change when studying the antimicrobial properties of medicinal plants, and to work towards sustainable management practices that take these impacts into account.

One of the most important effects of climate change on medicinal plants is the alteration of the distribution and abundance of plant species. As temperature and precipitation patterns change, the suitable habitat for medicinal plants may shift, leading to changes in their geographic distribution. This can affect the availability and diversity of medicinal plants, which in turn can have implications for their antimicrobial properties. Moreover, the adaptation of medicinal plants to changing environmental conditions can also affect their antimicrobial properties (Ncube *et al*., 2012). Plants may respond to changes in climate by altering their physiology and metabolism, leading to changes in the production and concentration of secondary metabolites. This can lead to variations in the antimicrobial properties of medicinal plants, making it difficult to predict their therapeutic effects.

Another way in which climate change can affect the antimicrobial properties of medicinal plants is through the introduction of new plant pathogens and pests (Borges *et al*., 2017). Climate change can create conditions that are favorable for the spread of invasive species, which can outcompete native medicinal plants or introduce new diseases to plant populations. This can lead to changes in the chemical composition of medicinal plants and a loss of biodiversity, which can ultimately affect their antimicrobial properties. The impacts of climate change on human health can also indirectly affect the antimicrobial properties of medicinal plants. Changes in temperature and precipitation patterns can alter the distribution of disease vectors, such as mosquitoes and ticks, which can affect the prevalence of infectious diseases (Lafferty, 2009; Patz *et al*., 2003). This can increase the demand for effective antimicrobial treatments, placing additional pressure on medicinal plant populations. The impacts of climate change on medicinal plants can have complex and varied effects on their antimicrobial properties. The adaptation of medicinal plants to changing environmental conditions, changes in plant distribution and abundance, the introduction of new pests and diseases, and indirect effects on human health can all play a role in shaping the therapeutic properties of medicinal plants. As such, it is important to consider these impacts when studying the antimicrobial properties of medicinal plants and to develop sustainable management practices that take these impacts into account.

2. THE RELATIONSHIP BETWEEN MEDICINAL PLANTS AND ANTIMICROBIAL ACTIVITY

Medicinal plants are known to produce a wide range of secondary metabolites that exhibit antimicrobial activity against various microorganisms. These compounds can be classified into different groups based on their chemical structures and mechanisms of action. Here are some examples of the different types of antimicrobial compounds found in medicinal plants:

1. Alkaloids: These are nitrogen-containing compounds that exhibit a wide range of pharmacological activities, including antimicrobial properties. Examples of alkaloids found in medicinal plants include berberine from barberry (*Berberis vulgaris*), quinine from cinchona (*Cinchona* spp.), and nicotine from tobacco (*Nicotiana tabacum*) (Kukula-Koch & Widelski, 2017).

2. Flavonoids: These are polyphenolic compounds that are widely distributed in the plant kingdom and exhibit a broad range of biological activities, including antimicrobial properties. Examples of flavonoids found in medicinal plants include quercetin from onions (*Allium cepa*),

epicatechin from green tea (*Camellia sinensis*), and kaempferol from ginkgo (*Ginkgo biloba*) (Bahmani *et al*., 2014).

3. Terpenoids: These are compounds derived from the isoprene unit and are widely distributed in the plant kingdom. They exhibit a diverse range of biological activities, including antimicrobial properties. Examples of terpenoids found in medicinal plants include menthol from peppermint (*Mentha piperita*), thymol from thyme (*Thymus vulgaris*), and artemisinin from sweet wormwood (*Artemisia annua*) (Ludwiczuk *et al*., 2017).

4. Tannins: These are polyphenolic compounds that are widely distributed in the plant kingdom and exhibit a broad range of biological activities, including antimicrobial properties. Examples of tannins found in medicinal plants include ellagitannins from oak bark (*Quercus robur*), catechins from green tea (*Camellia sinensis*), and condensed tannins from grape seeds (*Vitis vinifera*) (Sieniawska & Baj, 2017).

5. Essential oils: These are volatile compounds that are present in small amounts in various plant parts, including leaves, flowers, and fruits. They exhibit a broad range of biological activities, including antimicrobial properties. Examples of essential oils found in medicinal plants include eucalyptus oil from eucalyptus (*Eucalyptus globulus*), peppermint oil from peppermint (*Mentha piperita*), and tea tree oil from tea tree (*Melaleuca alternifolia*) (Talbert & Wall, 2012).

It is important to note that the antimicrobial activity of medicinal plants can be attributed to a combination of different compounds working synergistically to enhance their effectiveness. For example, some medicinal plants contain a mixture of flavonoids and alkaloids, which work together to inhibit the growth of microorganisms. Furthermore, the antimicrobial properties of these compounds can vary depending on the microorganism being targeted. Some compounds may be more effective against bacteria, while others may be more effective against fungi or viruses (Chao *et al*., 2000). As such, it is important to identify the specific microorganisms that a particular compound is effective against in order to optimize its use as an antimicrobial agent.

The mechanisms of action of these compounds can also vary. For example, some compounds disrupt the cell membrane of microorganisms, while others interfere with their DNA replication or protein synthesis (Etebu & Arikekpar, 2016). Understanding the mechanisms of action of these compounds can provide insights into their effectiveness and potential side effects. In addition to their antimicrobial properties, many of these compounds also exhibit other pharmacological activities, such as anti-inflammatory, antioxidant, and anticancer effects. This makes medicinal plants a promising source of natural compounds for the development of new drugs and therapeutic agents. However, it is important to note that the use of medicinal plants for antimicrobial purposes requires careful consideration and regulation. The overuse or misuse of these compounds can lead to the development of drug-resistant microorganisms, which can have serious implications for public health (Serwecińska, 2020). As such, sustainable harvesting practices and appropriate dosage and administration protocols are necessary to ensure the long-term viability and effectiveness of these natural antimicrobial agents.

The use of medicinal plants for the treatment of microbial infections has been practiced for thousands of years across many different cultures. Traditional healers and practitioners have used various parts of plants, including leaves, flowers, stems, and roots, to prepare remedies for a range of illnesses caused by bacteria, viruses, and fungi. For example, in traditional Chinese medicine, herbal remedies have been used for centuries to treat a range of bacterial and viral infections, including respiratory infections, gastrointestinal infections, and skin infections. Some commonly used medicinal plants in Chinese medicine include honeysuckle (*Lonicera japonica*), Forsythia (*Forsythia suspensa*), and Andrographis (*Andrographis paniculata*) (Huang *et al*., 2019).

Similarly, in Ayurvedic medicine, a traditional system of medicine practiced in India, medicinal plants have been used to treat a range of infectious diseases, such as tuberculosis, malaria, and typhoid fever (Seth & Sharma, 2004). Some commonly used medicinal plants in Ayurvedic medicine include neem (*Azadirachta indica*), tulsi (*Ocimum tenuiflorum*), and turmeric (*Curcuma longa*) (Rupani & Chavez, 2018). In many African cultures, traditional healers have used a range of medicinal plants to treat infectious diseases, including malaria, HIV/AIDS, and respiratory infections. For example, the bark of the cinchona tree (*Cinchona ledgeriana*) has been used in traditional medicine in Africa to treat malaria for centuries (Meshnick & Dobson, 2001). Many of the traditional uses of medicinal plants for the treatment of microbial infections have been validated by scientific research, with many studies confirming the antimicrobial properties of various plant extracts and compounds. However, it is important to note that traditional uses of medicinal plants may not always be safe or effective, and the use of these plants for medicinal purposes should be carefully regulated and evaluated to ensure their safety and effectiveness.

It is also worth noting that many of the traditional uses of medicinal plants for the treatment of microbial infections have been based on empirical evidence and observation rather than scientific research. Traditional knowledge of medicinal plants has often been passed down through generations and has been refined and improved over time based on experience. In recent years, there has been increasing interest in traditional medicinal plants as a source of natural antimicrobial agents, particularly in developing countries where access to modern medicines may be limited. Many scientists are exploring the potential of medicinal plants to develop new antimicrobial agents to combat the growing problem of drug-resistant microorganisms (Alvin *et al*., 2014; Mulat *et al*., 2019).

In addition, the use of medicinal plants for the treatment of microbial infections is often more affordable and accessible than conventional medicines, particularly in rural and remote areas where modern medicines may be scarce or expensive. This has important implications for global health and the fight against infectious diseases, particularly in low- and middle-income countries. The traditional use of medicinal plants for the treatment of microbial infections highlights the potential of natural compounds to combat infectious diseases (Anand *et al*., 2019). As scientific research continues to explore the antimicrobial properties of these compounds, there is the potential to develop new and effective treatments for a range of microbial infections, while also promoting sustainable harvesting practices and protecting traditional knowledge and cultural practices.

3. CLIMATE CHANGE AND ITS IMPACT ON MEDICINAL PLANTS

Climate change is expected to have significant impacts on the growth and distribution of medicinal plants, which could have implications for the availability and effectiveness of natural antimicrobial agents. Changes in temperature and rainfall patterns can affect the phenology (timing of life cycle events) of medicinal plants, such as the timing of flowering, fruiting, and seed germination. This can impact the production and availability of plant parts used for medicinal purposes. For example, the production of active compounds in some plants may be affected by changes in temperature and moisture levels, which could lead to fluctuations in the quality and quantity of medicinal compounds available (Figueiredo *et al.*, 2008).

Changes in temperature and rainfall can also affect the distribution of medicinal plants. For example, some plant species may be forced to migrate to new areas as their preferred climate zones shift. This can lead to changes in the genetic diversity of these plant species, which can impact their ability to adapt to changing environmental conditions and affect their medicinal properties. Additionally, climate change can also increase the prevalence and spread of pests and diseases that can affect the growth and health of medicinal plants (Rosenzweig *et al*., 2001). This can lead to reduced yields, lower quality of plant parts used for medicinal purposes, and

increased use of pesticides and other chemicals to control these pests and diseases, which could have negative impacts on human health and the environment. The impacts of climate change on medicinal plants are complex and can have significant implications for the availability and quality of natural antimicrobial agents (Ncube *et al*., 2012). As such, it is important to monitor and assess these impacts and take measures to protect and promote the growth of medicinal plants to ensure their continued availability and effectiveness as sources of natural compounds for the treatment of microbial infections.

In addition to the direct impacts of climate change on medicinal plants, there are also indirect impacts that can affect their growth and distribution. For example, changes in land use patterns and habitat loss due to deforestation and urbanization can reduce the availability of suitable habitats for medicinal plants. This can lead to declines in the populations of certain plant species and the loss of traditional knowledge associated with their use. Moreover, changes in the frequency and severity of extreme weather events such as droughts and floods can also affect the growth and survival of medicinal plants. Droughts, for example, can lead to water stress and reduced growth, while floods can cause soil erosion and damage to plant roots (Henry *et al*., 2007). These events can also have cascading effects on the ecosystem as a whole, affecting the availability of pollinators and other plant-animal interactions that are essential for the growth and reproduction of medicinal plants.

To mitigate the impacts of climate change on medicinal plants, it is important to promote sustainable harvesting practices and the conservation of natural habitats (Das *et al*., 2016). This can involve the establishment of protected areas, the development of sustainable cultivation practices, and the promotion of agroforestry and other land use practices that support the growth and diversity of medicinal plants. Additionally, efforts should be made to integrate traditional knowledge and practices into conservation and management strategies, recognizing the important role that local communities play in the preservation and promotion of medicinal plants. The impacts of climate change on medicinal plants are complex and multifaceted, and require a holistic approach that integrates scientific research, conservation, and community engagement. By promoting the sustainable use and conservation of medicinal plants, we can ensure the continued availability and effectiveness of natural compounds for the treatment of microbial infections, while also supporting the resilience of ecosystems and the well-being of local communities.

Changes in temperature, precipitation, and other environmental factors can impact the concentration of antimicrobial compounds in medicinal plants. For example, variations in temperature and moisture can affect the rate of photosynthesis and other biochemical processes in the plant, which can in turn affect the production and accumulation of active compounds. Studies have shown that increased temperature can lead to changes in the concentration of antimicrobial compounds in some plant species. Higher temperatures can cause stress on the plant and trigger the production of stress-related compounds that may compete with the production of other secondary metabolites, including antimicrobial compounds (Jan *et al*., 2021). In contrast, cooler temperatures may promote the accumulation of certain compounds in some plants, potentially increasing their antimicrobial activity.

Changes in precipitation can also have an impact on the concentration of antimicrobial compounds in medicinal plants. Drought stress, for example, can lead to the production of stress-related compounds that may interfere with the production of other secondary metabolites, such as antimicrobial compounds. Conversely, plants may increase the production of certain compounds in response to water stress as a mechanism for survival, potentially increasing their antimicrobial activity. Other environmental factors, such as soil pH, nutrient availability, and light intensity, can also affect the production and accumulation of antimicrobial compounds in medicinal plants (Ncube *et al*., 2012). For example, plants grown in nutrient-poor soils may

allocate more resources towards the production of secondary metabolites, including antimicrobial compounds, as a mechanism for survival.

It is worth noting that while changes in environmental factors can impact the concentration of antimicrobial compounds in medicinal plants, the extent of this impact can vary depending on the plant species and the specific compounds in question. Furthermore, the interaction of multiple environmental factors may have complex and sometimes unpredictable effects on the production and accumulation of active compounds in plants. Understanding how changes in temperature, precipitation, and other factors can impact the concentration of antimicrobial compounds in medicinal plants is important for ensuring the availability and effectiveness of natural compounds for the treatment of microbial infections, particularly in the face of climate change (Prinsloo & Nogemane, 2018). Further research in this area can help identify strategies for promoting the growth and cultivation of medicinal plants with high levels of antimicrobial activity, even in changing environmental conditions.

In addition to understanding how environmental factors can impact the concentration of antimicrobial compounds in medicinal plants, it is also important to consider how other factors, such as genetic variation and harvesting practices can affect the quality and quantity of active compounds. Genetic variation among plant populations can result in differences in the concentration and composition of secondary metabolites, including antimicrobial compounds (Dumas *et al*., 2021). For example, some plant populations may have evolved to produce higher levels of certain compounds in response to local environmental conditions or pressure from herbivores and pathogens. Identifying and conserving genetic variants with high levels of antimicrobial activity can be an important strategy for maintaining the availability and effectiveness of natural compounds in the face of climate change.

Harvesting practices can also have an impact on the quality and quantity of active compounds in medicinal plants (Pandey & Savita, 2017). Over-harvesting and unsustainable harvesting practices can lead to the decline or even extinction of certain plant populations, reducing the availability of important compounds for medicinal use. In contrast, sustainable harvesting practices, such as selective harvesting and cultivation, can promote the growth and survival of medicinal plants, while also ensuring the continued availability of active compounds for therapeutic use.

While changes in temperature, precipitation, and other environmental factors can impact the concentration of antimicrobial compounds in medicinal plants, a holistic approach is needed to ensure the availability and effectiveness of natural compounds for the treatment of microbial infections (Borges *et al*., 2017). This includes consideration of genetic variation, sustainable harvesting practices, and the conservation and cultivation of medicinal plant species in the face of changing environmental conditions. Through continued research and collaboration among scientists, conservationists, and local communities, we can work towards a more sustainable and effective approach to utilizing the antimicrobial properties of medicinal plants.

4. CHALLENGES IN EXPLORING THE POTENTIAL OF MEDICINAL PLANTS FOR ANTIMICROBIAL ACTIVITY

Identifying and isolating antimicrobial compounds from medicinal plants can be a challenging and complex process. This is due to the large number of secondary metabolites produced by plants, the complexity of their chemical structures, and the often low concentrations of these compounds in plant tissues (Croteau *et al*., 2000). One of the main challenges in identifying and isolating antimicrobial compounds from medicinal plants is the need for specialized techniques and equipment for extraction, purification, and characterization of the compounds. These techniques can involve complex procedures such as solvent extraction, chromatography,

and spectroscopy. Moreover, the use of these techniques requires skilled researchers who have specialized knowledge in natural product chemistry.

Another challenge is the potential for variability in the composition of active compounds in plants due to factors such as environmental conditions and genetic variation. This means that a particular compound may be present in some samples, but not in others, or may be present in different concentrations, making it difficult to obtain consistent results. Despite these challenges, the identification and isolation of active compounds from medicinal plants are crucial for the development of new antimicrobial agents. With the help of advanced techniques and interdisciplinary research, scientists have been able to identify and isolate a range of compounds from medicinal plants with potent antimicrobial activity. These compounds have been used to develop new drugs for the treatment of infectious diseases and have the potential to offer an alternative to conventional antibiotics. While identifying and isolating antimicrobial compounds from medicinal plants can be challenging, it is an important step in the development of new and effective therapies for microbial infections. Further research is needed to identify and characterize new compounds from medicinal plants, as well as to optimize extraction and purification methods to increase the yield and potency of these compounds (Brusotti *et al*., 2014). Through continued efforts to explore the potential of natural compounds from medicinal plants, we can work towards developing new and sustainable solutions for the treatment of infectious diseases in the face of emerging antimicrobial resistance and climate change.

Additionally, there are several other factors that can complicate the process of identifying and isolating antimicrobial compounds from medicinal plants. For example, some plant species may contain multiple compounds with antimicrobial activity, making it difficult to isolate a specific compound for further study. Moreover, the antimicrobial activity of a compound may be influenced by the presence of other compounds in the plant, which can affect the bioavailability and pharmacokinetics of the active compound (Vaou *et al*., 2021). Furthermore, some active compounds may only be present in specific plant tissues or at specific growth stages, which can add an additional layer of complexity to the isolation process. For instance, some compounds may only be present in the roots, leaves, or flowers of a plant, or may only be produced during specific developmental stages.

Another challenge is the potential for interactions between active compounds and other medications or supplements that a patient may be taking (Alissa, 2014). Some natural compounds may interact with prescription medications, altering their effectiveness or causing unwanted side effects. Therefore, it is important to thoroughly investigate the safety and efficacy of natural compounds before they are used for therapeutic purposes. Despite these challenges, the potential benefits of identifying and isolating antimicrobial compounds from medicinal plants are significant. By harnessing the natural antimicrobial properties of these compounds, we can develop new and effective treatments for microbial infections, including those caused by antibiotic-resistant pathogens (Nascimento *et al*., 2000). Moreover, the use of natural compounds can provide a sustainable alternative to conventional antibiotics, which are becoming increasingly ineffective due to emerging resistance. Overall, the identification and isolation of antimicrobial compounds from medicinal plants is an important area of research that holds great promise for the future of healthcare.

Testing the efficacy of medicinal plant extracts against microbial infections presents a number of challenges, which can complicate the development and evaluation of new natural antimicrobial agents. One of the main challenges in testing the efficacy of medicinal plant extracts is the variability in the composition and activity of plant extracts. Plant extracts can contain a complex mixture of secondary metabolites, and the concentrations of these compounds can vary depending on the species, geographic location, and growth conditions of the plant. Moreover, the activity of plant extracts can be influenced by the extraction method

used, the solvent used, and the preparation of the extract (Sultana *et al*., 2009). This variability can make it difficult to compare the activity of different plant extracts, and to identify the most effective extracts for further study.

Another challenge is the need to evaluate the antimicrobial activity of plant extracts against a wide range of microbial pathogens. Different pathogens can have different susceptibilities to natural compounds, and the activity of a compound against one type of microbe may not necessarily translate to other types of microbes. Therefore, a comprehensive evaluation of the antimicrobial activity of plant extracts requires testing against a wide range of microorganisms, including bacteria, fungi, viruses, and parasites (Joshi *et al*., 2020). Additionally, the complexity of the interactions between plant extracts and microorganisms can make it difficult to determine the mode of action of natural compounds. The activity of plant extracts can be influenced by factors such as the pH of the environment, the presence of other compounds, and the metabolic state of the microbe. Moreover, the activity of natural compounds can be influenced by the presence of biofilms, which can protect microorganisms from antimicrobial agents. Testing the efficacy of medicinal plant extracts against microbial infections requires careful consideration of the variability in composition and activity of plant extracts, the range of microorganisms that need to be tested, and the complexity of the interactions between plant extracts and microorganisms. Through continued research and development, we can overcome these challenges and harness the natural antimicrobial properties of medicinal plants to develop new and effective therapies for infectious diseases.

Another challenge in testing the efficacy of medicinal plant extracts against microbial infections is the lack of standardized protocols for evaluating the activity of natural compounds. There is currently no consensus on the best methods for testing the antimicrobial activity of natural compounds, which can lead to variability in the results obtained from different studies. This can make it difficult to compare the activity of different plant extracts and to determine the most effective extracts for further study. Furthermore, the lack of standardization can make it difficult to assess the safety and efficacy of natural compounds. The variability in the activity of plant extracts can result in differing levels of toxicity and adverse effects, which can be difficult to predict. Therefore, it is important to establish standardized protocols for evaluating the safety and efficacy of natural compounds in order to ensure that they are safe and effective for use in humans.

Another challenge in testing the efficacy of medicinal plant extracts against microbial infections is the need to evaluate the activity of natural compounds in vivo in order to determine their effectiveness in living organisms. In vitro studies can provide valuable insights into the activity of natural compounds, but they do not necessarily reflect the complex interactions that occur in living organisms. Therefore, it is important to conduct animal studies and clinical trials to evaluate the safety and efficacy of natural compounds in vivo, and to determine the optimal dosing and administration strategies. Despite these challenges, the evaluation of the antimicrobial activity of medicinal plant extracts holds great promise for the development of new and effective therapies for infectious diseases. By leveraging the natural antimicrobial properties of these compounds, we can develop sustainable alternatives to conventional antibiotics, which are becoming increasingly ineffective due to the emergence of antibiotic-resistant pathogens. With continued research and development, we can overcome the challenges in testing the efficacy of medicinal plant extracts and unlock the full potential of these natural compounds as therapeutic agents.

5. OPPORTUNITIES FOR UTILIZING MEDICINAL PLANTS FOR ANTIMICROBIAL ACTIVITY IN THE FACE OF CLIMATE CHANGE

The use of medicinal plants for antimicrobial activity holds great potential for addressing the challenges posed by climate change. As we have discussed earlier, climate change can have

significant impacts on the growth, distribution, and concentration of antimicrobial compounds in medicinal plants (Chandra *et al*., 2022). However, by harnessing the antimicrobial properties of these plants, we can develop sustainable and effective therapies for infectious diseases, which are expected to become more prevalent as a result of climate change. One of the key benefits of using medicinal plants for antimicrobial activity is the potential for developing new and effective therapies that are not reliant on conventional antibiotics. As the effectiveness of antibiotics diminishes, there is an urgent need to develop alternative therapies that can treat infectious diseases caused by resistant pathogens. Medicinal plants offer a rich source of natural compounds with potent antimicrobial activity, which can be developed into new therapies with broad-spectrum activity against a wide range of pathogens (Srinivasan *et al*., 2001).

In addition, the use of medicinal plants for antimicrobial activity can also promote sustainability in healthcare. Unlike conventional antibiotics, which are often derived from synthetic compounds and are subject to limitations in their production and distribution, medicinal plants can be grown and harvested sustainably, without the use of harmful chemicals or intensive agricultural practices. This makes them a more environmentally friendly and socially responsible option for healthcare. The use of medicinal plants for antimicrobial activity can also contribute to the conservation of biodiversity. Many medicinal plants are endemic to specific regions and are threatened by climate change and other environmental pressures (Sharma *et al*., 2020). By promoting the sustainable use of these plants for healthcare, we can help to protect and preserve biodiversity, and ensure that future generations have access to the natural resources they need for health and wellbeing. The use of medicinal plants for antimicrobial activity holds great promise for addressing the challenges posed by climate change and the emergence of antibiotic-resistant pathogens. By harnessing the natural antimicrobial properties of these plants, we can develop new and effective therapies that are sustainable, socially responsible, and environmentally friendly, while also contributing to the conservation of biodiversity.

It is important to note, however, that the use of medicinal plants for antimicrobial activity is not without its challenges. As we have discussed earlier, there are difficulties in identifying and isolating the active compounds in these plants, as well as in testing their efficacy against specific pathogens. In addition, there are also concerns around the quality, safety, and standardization of herbal medicines, which can vary widely depending on factors such as cultivation, harvesting, processing, and storage. To address these challenges, there is a need for greater investment in research and development of medicinal plants for antimicrobial activity. This includes efforts to better understand the mechanisms of action of these plants, to optimize their cultivation and processing techniques, and to develop robust quality control and regulatory frameworks to ensure the safety and efficacy of herbal medicines.

Furthermore, it is important to acknowledge the role of traditional knowledge and practices in the use of medicinal plants for healthcare (Payyappallimana, 2010). Indigenous and local communities have long relied on medicinal plants for their health and wellbeing, and their knowledge and expertise can provide valuable insights into the properties and uses of these plants. As we move towards a more sustainable and equitable approach to healthcare, it is important to respect and support the traditional knowledge and practices of these communities, and to ensure that they are fairly compensated for their contributions to the development of herbal medicines. The use of medicinal plants for antimicrobial activity represents a promising avenue for addressing the challenges posed by climate change and the emergence of antibiotic-resistant pathogens. While there are challenges and limitations to this approach, there is also great potential for developing new and effective therapies that are sustainable, socially responsible, and environmentally friendly. By investing in research and development, promoting traditional knowledge and practices, and ensuring robust quality control and

regulatory frameworks, we can harness the full potential of medicinal plants for the benefit of global health and biodiversity.

The use of medicinal plants for antimicrobial activity can be a sustainable and environmentally friendly approach to combating microbial infections for several reasons. Unlike synthetic antibiotics which are often produced using fossil fuels and chemicals, medicinal plants can be grown and harvested in a more sustainable manner. Many medicinal plants can be cultivated using organic farming methods, which rely on natural inputs such as compost, cover crops, and beneficial insects, and do not use synthetic pesticides or fertilizers that can harm the environment. The use of medicinal plants for antimicrobial activity can help to preserve biodiversity and protect natural ecosystems (Sen & Samanta, 2015). Many medicinal plants are native to specific regions and are adapted to local environmental conditions. By cultivating and using these plants, we can help to preserve these unique ecosystems and the biodiversity they support.

The use of medicinal plants for antimicrobial activity can promote social and economic sustainability. Many communities around the world rely on medicinal plants for their health and wellbeing, and the cultivation and trade of these plants can provide important sources of income and employment. By supporting the sustainable cultivation and trade of medicinal plants, we can help to promote social and economic development while also preserving natural resources and protecting the environment. The use of medicinal plants for antimicrobial activity can provide a sustainable and environmentally friendly approach to combating microbial infections. By promoting the cultivation and use of these plants, we can harness the power of nature to address one of the greatest challenges facing global health today, while also promoting sustainability and protecting the environment for future generations.

It is also worth noting that the use of medicinal plants for antimicrobial activity can help to address the growing problem of antibiotic resistance. Antibiotic-resistant bacteria are a major public health threat and can cause serious and sometimes life-threatening infections (Tomasz, 1994). The overuse and misuse of synthetic antibiotics have contributed to the emergence and spread of antibiotic-resistant bacteria. By developing and promoting the use of medicinal plants for antimicrobial activity, we can provide new and effective therapies that are less likely to contribute to the development of antibiotic resistance. Furthermore, the use of medicinal plants for antimicrobial activity can be part of a broader approach to promoting health and preventing disease. Many medicinal plants have been used for thousands of years in traditional medicine systems to promote health and prevent disease (Shakya, 2016). By incorporating these plants into our diets and lifestyles, we can support our immune systems and reduce the risk of infections and other diseases.

In addition to their antimicrobial properties, medicinal plants have many other potential health benefits, such as anti-inflammatory, antioxidant, and immune-stimulating effects. By promoting the use of these plants, we can support a holistic and integrated approach to healthcare that focuses on promoting health and preventing disease, rather than just treating symptoms and illnesses after they occur. The use of medicinal plants for antimicrobial activity represents a promising and sustainable approach to addressing the challenges posed by climate change and the emergence of antibiotic-resistant pathogens. By investing in research and development, promoting sustainable cultivation and trade, and respecting traditional knowledge and practices, we can harness the full potential of medicinal plants for the benefit of global health and biodiversity.

6. CONCLUSION

The exploration of medicinal plants for antimicrobial activity in the face of climate change presents both challenges and opportunities. On one hand, the changing climate can affect the growth and availability of medicinal plants, potentially limiting their use for medicinal

purposes. On the other hand, the vast biodiversity of plants offers a wealth of opportunities to discover new antimicrobial compounds that could combat emerging infectious diseases. Furthermore, the use of medicinal plants for antimicrobial activity has the potential to address the growing problem of antibiotic resistance, which poses a major threat to public health. It is essential that efforts are made to sustainably manage and protect medicinal plant species, as well as to conduct further research to identify new antimicrobial compounds. The potential of medicinal plants to provide effective and sustainable solutions to antimicrobial resistance and infectious diseases is promising. By taking advantage of the opportunities presented by medicinal plants, while also addressing the challenges of climate change and sustainability, we can work towards a healthier and more resilient future.

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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Review Article

Star fruit (*Averrhoa carambola* L.): Exploring the wonders of Indian folklore and the miracles of traditional healing

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Abstract: Herbal medicines have been used for centuries to treat various ailments and have now gained global importance for their therapeutic and economic implications. One such plant is *Averrhoa carambola* commonly known as star fruit, a tropical plant belonging to the Oxalidaceae family. With a rich history of traditional medicinal uses in India and Southeast Asia, it has been employed to treat various ailments, including urinary infections, hypertension, diabetes, and gastrointestinal issues. The fruit is popular for its sweet and sour taste and finds culinary use in salads, beverages, and garnishing. The therapeutic potential of *A. carambola* is attributed to its abundance of natural antioxidants and phenolic compounds such as gallic acid in gallotannin form, catechins, and epicatechins. These bioactive constituents contribute to its medicinal properties and health benefits. However, scientific investigations have revealed potential health risks associated with *A. carambola* consumption. High oxalic acid content in the fruit poses a risk of toxicity, particularly for individuals with renal impairments who consume excessive fruit juices. Additionally, the presence of caramboxin, a neurotoxin, can interfere with the GABAergic system, leading to neurotoxic effects. This review offers a comprehensive and well-organized examination of the current progress regarding the benefits, phytochemistry, pharmacology, and toxicity of *A. carambola*. The insights presented in this review have the potential to drive advancements and novel treatments involving star fruit in modern times. As interest in herbal medicines continues to rise, further research on *A. carambola* holds promise for exploring new therapeutic applications in the realm of natural remedies.

1. INTRODUCTION

In recent decades, there has been a significant shift in people's attitudes towards the modern medicinal system. Many individuals have become increasingly interested in exploring ancient healing systems such as Ayurveda, Siddha, and Unani. This shift is primarily driven by concerns about the adverse effects associated with synthetic drugs used in modern medicine. As a result, herbal drugs are gaining prominence and are seen as vital components of health care programs, especially in developing countries (Kamboj, 2000). Alternative medicine is not

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widely accepted in developed nations because of poor documentation and strict quality control. Properly documenting research on traditional medicines is crucial to build trust and integrate these practices into mainstream healthcare (Kirtikar & Basu, 2005). For traditional medicines India is one of the ancient heritages, and for folklore customs and conventional characteristics of medicinally significant secondary metabolites. India provides Materia medica with full of information. Materia medica of India provides approximately 2000 natural origin drugs list which are the derivatives of folklore and traditional systems. For the discovery of new pharmaceuticals historically natural products and plants that originated from higher plants played a pivotal role (Gupta *et al.*, 2001; Mukherjee, 2002). According to WHO (World health organization) eighty percent people of developing countries depend on traditional remedial system for their primary healthcare system (Chopra & Nayar, 1956; Gupta & Gupta, 2020). Around the world, 21000 plants species are used medicinally and in India around 2500 plant species are used indigenously (Yadav *et al*., 2006). About 400 families of flowering plant, out of which 315 are represented by India (Jain *et al*., 2006).

The plant *Averrhoa carambola* L., commonly known as star fruit or "Kamarakh," belongs to the Oxalidaceae family and is native to Malaysia. Its distinct star-shaped appearance is matched by its delightful sweet and sour flavor (Warrier, 1993). The Sanskrit term "Karmaranga" is derived from "*carambola*," signifying a culinary appetizer. *A. carambola* is a hardy, drought-resistant tree known for its longevity (Manda *et al*., 2012). It holds significant commercial value in India due to its versatile applications and the delectable nature of its fruit (Warrier, 1993). While the unripe fruit is employed as a vegetable, ripened fruits find their way into various forms such as jams, jellies, fermented and non-fermented beverages, and desserts (Patil *et al*., 2010).

Considering the potential of ethnobotany in future medicinal applications, *A. carambola* is gaining substantial attention. Various parts of the tree have been utilized in traditional folk medicine. Additionally, it serves as a rich source of essential nutrients including copper, potassium, folate, and pantothenic acid. The level of ascorbic acid in a ripe fruit is a determining factor for its taste, whether sweet or tart (Manda *et al*., 2012).

2. BOTANICAL STUDY

2.1. *Averrhoa carambola* (Oxalidaceae): Herbal Plant

The fruit scientifically known as *Averrhoa carambola* goes by various names in different languages. In English, it is commonly referred to as Star fruit or simply carambola. In Hindi, it is known as Kamrakh or Karmal, in Myanmar known as mak-hpung, zaung-ya while in Malay and Indonesia, it is called Belimbing. In Sanskrit, it is referred to as Karmaranga, in Bengali as Kamranga, and in Gujarati as Kamrakh. In Marathi, it is known as Karambal, and in Tamil, it goes by Thambaratham or Tamarattai (Nandkarni, 1976; Orwa *et al*., 2009).

Star fruit, scientifically known as *Averrhoa carambola*, belongs to the Plantae kingdom, which encompasses all plants. It falls under the category of vascular plants (Tracheobionta), indicating that it has specialized tissues for conducting water and nutrients. Further classification places it in the Spermatophyta division, denoting plants that produce seeds. Within this division, it is categorized as an Angiospermae, signifying that it produces flowers and encloses its seeds in fruits.

In terms of class, star fruit is classified as Magnoliopsida, which refers to a class of flowering plants. This class includes a wide range of familiar plants like roses, peas, and sunflowers. Within the order Oxalidales, star fruit finds its place. This order includes various families of plants, one of which is the Oxalidaceae family to which star fruit belongs. Oxalidales is a diverse order that encompasses several different plant families, each with its own unique characteristics.

Finally, within the genus *Averrhoa*, star fruit is identified by the species name *carambola* L., specifically referring to the *carambola* variety (Avinash *et al*. in 2012).

2.2. Botanical Description with Habitat and Distribution

A. carambola is a tiny, slow-growing, evergreen tree that grows to a height of 3–16 m. The leaves of *A. carambola* are alternating, spirally organised, and range in length from 15 to 25 cm.The under side of leaves is quite pale in colour, while the upper side is smooth and greenish. Leaves at night incline and curl but most active in daylight, so delicate to sudden jolt. Surface of leaves are glabrous, acute type of apex and base is oblique with characteristic taste and odor (Kirtikar & Basu ,1987; Dasgupta *et al*., 2013). Clusters of small, six-millimetre-wide flowers, crimson in color with purple-red hues, often reveal a delicate hint of pink or white on their backside. These blooms form tight clusters along the leaf axis, creating a visually stunning display. (Avinash *et al*., 2012). The versatile *A. carambola* fruits are both edible and multifunctional. These fruits are fleshy and start off as light green when unripe, transitioning to an orangish-yellow hue upon maturity. Their brown seeds are contained within a 5-6 edged, star-shaped cross-section, spanning 5-15 cm in length. With a crisp, waxy texture, their taste spectrum ranges from sour to sweet, emitting a fragrance reminiscent of oxalic acid shown in Figure 1 (Dasgupta *et al*., 2013). For centuries, *A. carambola* has been cultivated in Malaysian and various Southeast Asian nations. Originating from Ceylon and the Moluccas, this fruit has thrived and spread across these regions (Morton, 1987). This annual herbaceous plant is generally full-fledged in the warmer parts of India, Bangladesh, Brazil, Philippines, China, Malaysia, Indonesia, Israel, Florida, Thailand, Taiwan, Australia, and other divisions of world with similar climate (Ghani, 1998). It can tolerate wider range of climate and survive in both the subtropical countries and hot tropical areas together with Israel and Egypt and freezing temperatures as low as −3° C can tolerate. Ideally, they grow in humus rich soil with acidic pH range but also withstand with pH range of 8.5 also (Bircher & Bircher, 2000).

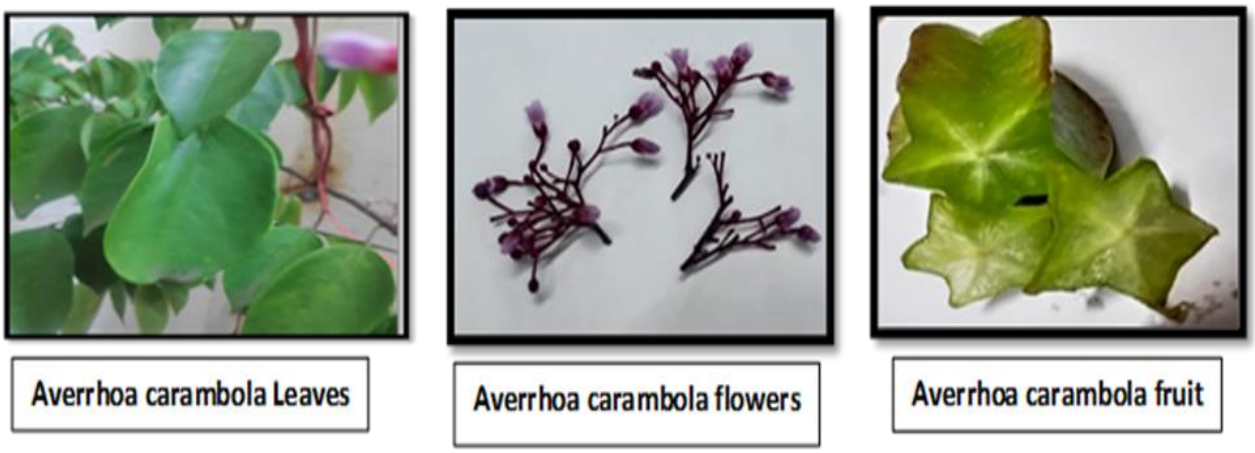

Figure 1. Pictorial representation of *Averrhoa carambola* plants parts.

3. TRADITIONAL AND POTENTIAL BENEFITS

3.1. Conventional Practices of *A. carambola*

For millennia, *A. carambola* has been employed in traditional medicine for a diverse range of ailments. This medicinal plant encompasses various components—roots, leaves, stems, fruits, flowers, and seeds—each offering distinct therapeutic properties in different regions such as Malaysia, Brazil, India, and China.

In India, the juice and ripe fruits of *A. carambola* have been used to address scurvy, boost appetite, act as an astringent, stimulate saliva production, alleviate fever, treat hemorrhoids, relieve thirst, and act as a purgative (Vasant & Narasimhacharya, 2014; Sheth, 2005).

Meanwhile, in Brazil, the ripe fruit is employed for urinary concerns, serving as a diuretic for bladder and kidney-related issues, and proving useful in managing hypertension and diabetes (Soncini *et al*., 2011). Sri Lanka recognizes *A. carambola* for its remarkable hypoglycemic effects, employing it in diabetes prevention (Abeysekera *et al*., 2015; Herbal Medicine Research Centre, 2002). In Malaysian traditional medicine, the fruit serves as a febrifuge, addresses recurrent aphthous ulcers, acts as an emetic, and aids in chest pain relief (Yang *et al*., 2020). Additionally, its leaves are utilized for treating ringworm, chickenpox, and headaches (Pang *et al*., 2017).

In Chinese medicine, ripe *A. carambola* fruits are employed for countering food poisoning from flesh consumption and for addressing an enlarged spleen (Carolino *et al*., 2005). They are also used to manage conditions like jaundice, diarrhea, throat infections, inflammation, toothaches, rashes, and strokes. Moreover, in women, it is utilized to stimulate milk secretion and, when the dosage is increased, to promote menstrual discharge. It is also known to enhance sexual desire in both males and females (Sung *et al*., 1998). Simultaneously, star fruit leaves find application in improving diabetes mellitus, alleviating emesis, addressing coughs, countering hangovers, and managing headaches (Ferreira *et al*., 2008; Thomas *et al*., 2008). Regarding the use of seeds, crushed seed decoctions are utilized to stimulate milk production, acting as a galactagogue. For colic pain and asthma, powdered seeds are applied. In Southeast Asia, *A. carambola* flowers are beneficial for skin inflammation and are also used as febrifuges, vermifuges, and for addressing malaria (Sheth, 2005; Sung *et al*., 1998).

3.2. Star Fruit Benefits as Food

There are numerous advantages and uses of *A. carambola* as a food. In different countries it has different uses from various parts of plant like flowers and leaves having sour and delicate taste unless used as salads. Due to its exotic flavor and unique shape, *A. carambola* finds application in crafting beverages and enhancing pastry presentations. Ripe, fresh fruit serves as a base for alcoholic drinks, achieved by fermenting it with *Saccharomyces cerevisiae* yeast. This yeast naturally converts the fruit sugars into alcohol and CO2 during a five-day fermentation process at 25°C, resulting in the creation of fruit wine (Napahde *et al*., 2010; Valim *et al*., 2016). Asian peoples used these fruits in different ways in cooking as sausages to cakes and jams to sauces while population of Malaysia generally prepare a sauce by boiling the fruit with sugar, cloves, and apples. Similarly Thai people used unripe fruit boiled with shrimp but the Chinese's people cooked the fish with the fruit. Also, in Taiwan and China star fruit is cut into strips and exported in boxes (Roopa *et al*., 2014). Even star fruit juice is used as energizing drinks or for its sour taste and also as condiments in dishes of fish. From the juice of star fruit an excellent frozen dessert can also be prepared by mixing the gelatin, sugar in the juice and kept its mixtures at low temperature for few hours (Ferrara, 2018). The unripe fruit serves as a key ingredient in jam-making, undergoing a process of marination with salt and vinegar, followed by maceration in honey overnight. After this, it is briefly cooked, transforming into jam, ready to be preserved in sterilized, sealed jars (Prati *et al*., 2002).

4. CHEMICAL COMPOSITION AND PHARMACOLOGICAL ACTIVITIES

4.1. Significant Phytochemical Constituents of *A. carambola*

When *A. carambola* analyzed for their phytochemical screening it showed the presence of alkaloid, phenylpropanoids, terpenes, saponins, phenols and flavonoids too. (Thomas *et al*., 2008). In the fruit major sterols found are, lupeol (a), Isofucosterol (b), β-sitosterol (c), and campesterol (d) apart of these sterols four major plant fatty acids are found – linolenic acid (e), linoleic, oleic and palmitic acid (f). (Gupta & Gupta, 2020; Nordby & Hall, 1979) The fruit edible portion is found to be rich source of dietary fibers, pectin, cellulose, reducing and non-reducing sugars, minerals, calcium, phosphorous, hemicellulose and carotenoid compositions

(Tiwari *et al*., 1979). The *A. carambola* fruit also includes gallic acid in gallotannin form, epicatechin (g), proanthocyanidins and L-ascorbic acid. Till now approximately 132 compounds are extracted and identified from *A. carambola* (Shui & Leong, 2004). Some of the extracted compound structures from *A. carambola* are shown in Figure 2.

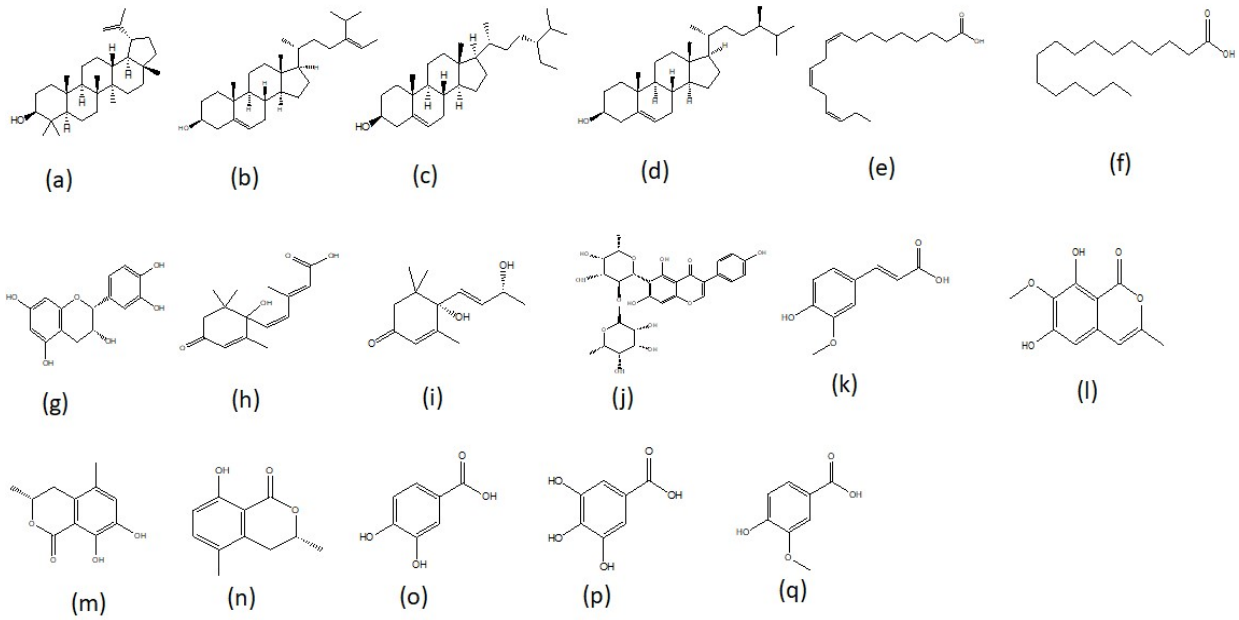

Figure 2. Chemical structures of some isolated compounds from *A. carambola.*

4.1.1. *Terpenes*

Terpenes are also called isoprenoids as isoprene is the building blocks of terpenes and it is found as the secondary metabolites in plants and specifically used for fragrances and pigmentation for plants, vegetables and fruits but apart from this, having lots of medicinal properties (Bahramsoltani *et al*., 2020; de Paulo Farias *et al*., 2020). About 29 terpenes have been identified and separated from *A. carambola* fruits using various spectroscopic techniques. The major terpenes found were cis and trans-abscisic acid (h), cis and trans-abscisic alcohol β-D-glucopyranoside, cis-abscisic alcohol β-D-glucopyranoside (6S,9R)-roseoside, and vomifoliol (i) (Gunawardena *et al*., 2015). Apart from this two components C_{13}- and C_{15}-norisoprenoids derived from *A. carambola* of terpenes massively intensify the flavor of fruits (Jia *et al*., 2019).

4.1.2. *Flavonoids*

Flavonoids have its place in class of natural products specifically as plant secondary metabolite with polyphenolic structures. Maqsood *et al*. (2020) identified a flavonoid in *A. carambola* that exhibits excellent pharmacological action, primarily in the area of radical scavenging and antioxidant activity, making it effective for the treatment or prevention of heart problems. From different organs of *A. carambola* plants like roots, leaves, fruits and stems around 51 flavonoids were isolated and characterized via various spectroscopic techniques viz Mass spectrometry (MS) and Nuclear magnetic resonance (NMR). Among these 51 compounds some are dihydrochalcone C-glycosides- Carambolaside R1-R3, Carambolaside A-H, Carambolaside I, Ia, Carambolaside J, Ja, Carambolaside M-Q, Carambolaside T1-T3, 3-Hydroxycarambolaside T1, Carambolaside S1-S2, 3-Hydroxycarambolaside P (Abeysekera *et al*., 2015; Jia *et al*., 2019; Yang *et al*., 2015) and other agents which exhibited radical scavenging activity against ABTS

and DPPH are flavan-3-ols- Epicatechin-(5,6-bc)-4β-(p-hydroxyphenyl)-dihydro-2(3H)-pyranone,Epicatechin-(7,8-bc)-4α-(p-hydroxyphenyl)-dihydro-2(3H)-pyranone, Epicatechin-(7,8-bc)-4β-(p-hydroxyphenyl)-dihydro-2(3H)-pyranone, (–)-Epicatechin, 6-(R-2-pyrrolidinone-5-yl)-epicatechin, 6-(S-2-Pyrrolidinone-5-yl)-epicatechin, (+)-Epicatechin, 8-carboxymethyl-(+)-epicatechin methyl ester.(28, 38, 39) Other compounds are also found with multiple structures like Pinobanksin 3-O-β-D-glucoside, Helicioside A, Aromadendrin 3-O-β-D-glucoside, (+)-Catechin, Norathyriol, Taxifolin 3′-O-β-D-glucoside, Isovitexin, sovitexin 2″-O-α-L-rhamnopyranoside, Hovertichoside C, Cyanidin-3-O-β-D-glucoside, Cyanidin-3, 5-O-β-D-diglucoside, Apigenin 6-C-(2″-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside, also Carambolaflavone A and B (j) with outstanding antihyperglycemic activity in animal model (Cazarolli *et al*., 2009; Cazarolli *et al*., 2012).

4.1.3. *Phenylpropanoids*

With a three-carbon propene tail of coumaric acid and an aromatic ring, phenylpropanoids are a class of chemicals that are produced by plants from the amino acids phenylalanine and tyrosine. It is an abundant supply of secondary metabolites from plants and is used in the manufacture of various other molecules, including lignans and flavonoids (Yao *et al*., 2020). During the spectroscopic analyses by ^{13}C- NMR and ^{1}H- NMR found around 18 phenylpropanoids compounds are productively isolated from fruits and roots of *A. carambola* plant. Out of total phenylpropanoids 12 lignans compounds are isolated from roots- (+)-isolariciresinol 3α-O-β-D-glucopyranoside, (-)-isolariciresinol 3α-O-β-D-glucopyranoside, Tarennanosides A, Fernandoside, (+) and (-)-lyoniresinol 3α-O-β-D-glucopyranoside, (+) and (−)-5′-methoxy-isolariciresinol 3α-O-β-D-glucopyranoside, 7α-[(β-glucopyranosyl) oxy]-lyoniresinol, and four compounds are simple phenylpropanoids- 1-O-feruloyl-β-D-glucose, (+) and (-)-lyoniresinol 9-O-β-D-glucoside, Ferulic acid (k) derived from fruits of *A. carambola* plant . Moreover, from fruits of *A. carambola* four coumarins compounds are isolated- reticulol that showed modest antioxidant capacity and 6-O-methyl-reticulol (l) that represented as iso-coumarin, 7-hydroxy-5- methylmellein (m) and 5- methylmellein (n) are Dihydroisocoumarins structurally (Sritharan *et al*., 2019).

4.1.4. *Polyphenols*

Phenolic compounds, derived from secondary pathways in plants, encompass tannins, flavonoids, lignans, phenolic acids, and coumarins. Within star fruit, these compounds are widely distributed across its fruits and roots, as highlighted by various studies (Wen *et al*., 2012; Jia *et al*., 2017; Yang *et al*., 2014; Liao *et al*., 2019; Gupta *et al*., 2021). Through techniques such as 1H-NMR, 13C-NMR, and FT-IR, researchers have isolated and characterized 16 phenolic compounds from the roots and fruits of *A. carambola*. These compounds exhibit remarkable radical scavenging capabilities. Notably, among these compounds are 8,9,10-trihydroxythymol, 3,4,5-trimethoxyphenol-1-O-β-D-glucopyranoside, Protocatechuic acid (o), Gallic acid (p), Vanillic acid (q), Methoxyhydroquinone-4-β-D-glucopyranoside. Within this array, alkylphenols such as 2,5-dimethoxy-3-undecylphenol, Carambolaside K, L, and 5-methoxy-3-undecylphenol stand out structurally (Pang *et al*., 2016).

4.1.5. *Additional compounds*

In addition to the previously mentioned compounds, various other chemical constituents have been investigated within *A. carambola*. Notably, quinone compounds such as 2-methoxy-6-nonyl-cyclohexa-2,5-diene-1,4-dione, 2-dehydroxy-5-O-methylembelin, 2-dodecyl-6-methoxycyclohexa-2,5-diene-1,4-dione, (+)-cryptosporin, and 5-O-methylembelin have been identified, alongside Heptyl vicianoside, Octyl vicianoside, cis-3-hexenyl rutinoside, and Methyl 2-O-β-D-fucopyranosyl-α-L-arabinofuranoside classified as alkyl glycosides. Furthermore, tetrahydroisoquinoline alkaloids like (1R*,3S*)-1-(5-hydroxymethylfuran-2-yl)-

3-carboxy-6-hydroxy-8-methoxyl-1,2,3,4-tetrahydroisoquinoline and (1S*,3S*)-1-methyl-3-carboxy-6-hydroxy-8-methyoxyl-1,2,3,4-tetrahydroisoquinoline have been identified.

Of particular interest is a compound derived from the roots of *A. carambola* plants, namely 2-dodecyl-6-methoxycyclohexa-2,5-diene-1,4-dione, exhibiting diverse bioactive properties. This compound has shown anti-inflammatory properties (Xie *et al*., 2016), anticancer effects (Gao *et al*., 2015; Chen *et al*., 2017; Gupta *et al*., 2022), neuroprotective activity (Wei *et al*., 2018), anti-obesity potential (Li *et al*., 2016), and antidiabetic properties (Lu *et al*., 2019).

4.2. Potential Medicinal Benefits of *A. carambola*

The *A. carambola* plant holds immense medicinal potential, showcasing a wide array of benefits such as anti-inflammatory, hepatoprotective, antimicrobial, antioxidant, neuroprotective, and antitumor activities, among others, as illustrated in Figure 3. In both Ayurvedic and Traditional Chinese Medicine (TCM), *A.carambola* is valued for its diverse medical advantages, addressing conditions such as coughs, dermal fungal infections, eczema, severe headaches, and diarrhea (Wang *et al*., 2016; Patel *et al*., 2015).

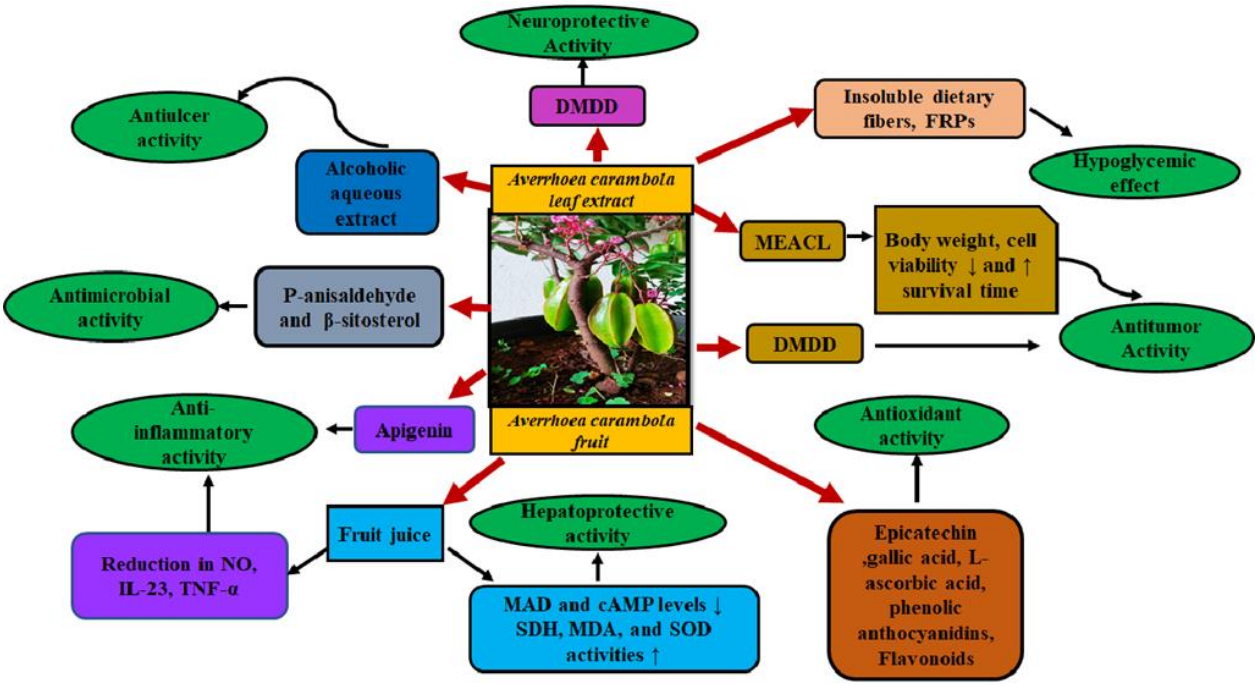

Figure 3. Diagrammatical representations of potential medicinal benefits of *Averrhoa carambola*.

4.2.1. *Antitumor activity*

Various studies have shown that an alcoholic extract from the stems of A. carambola was used against brain tumour cells, while an extract from the leaves was more effective against liver cancer cells (Tadros & Sleem, 2004). Methanol extract of *A. carambola* leaves (MEACL) at the dose of 25 and 50 mg/kg, i.p. once a day for 5 days substantially decreases the viability of cell and weight of the body, progressive change in hematologic estimations (Hgb, White Blood Cells, Red Blood Cells) and prolongs survival time in Ehrlich ascites carcinoma (EAC) cell-bearing mice (Siddika *et al*., 2020).

4.2.2. *Anti-inflammatory activity*

One study stated that *A. carambola* leaf ethanolic extract and butanol, ethyl acetate, and hexane fractions showed beneficial effects to diminishing the induced ear edema by croton oil and cellular migration in animal (Cabrini *et al*., 2011; Soncini *et al*., 2011). In another investigation,

aqueous extract of *A. carambola* inhibited carrageenan-induced rat paw inflammation when given intraperitoneally. At the same time stem extract of *A. carambola* stated the bactericidal activity against *Streptococcus aureus* (Sripanidkulchai *et al*., 2002).

4.2.3. *Hypoglycemic effect*

A. carambola has demonstrated the potential to lower blood sugar levels. This effect was attributed to specific types of dietary fiber found in the fruit, including those that are not soluble in alcohol or water. These fiber-rich fractions were isolated from the leftover pulp of *A. carambola* (Chau *et al*., 2004).

In a different study, it was found that the ripe fruit pulp of *A. carambola* had a hypoglycemic effect in healthy male Sprague Dawley rats. This effect was observed after an 8-week treatment period, specifically in comparison to the control group of normal rats (Dasgupta *et al*., 2013). This suggests that regular consumption of ripe star fruit may help regulate blood sugar levels.

4.2.4. *Antimicrobial activity*

In a study using the disc diffusion method, researchers investigated the antimicrobial properties of the *A. carambola* plant. They found that various extracts from the bark of *A. carambola* including petroleum ether, carbon tetrachloride, chloroform, aqueous soluble fractions, and methanolic extract were effective in inhibiting the growth of both gram-positive and gram-negative bacteria as well as fungi. The potency of these extracts varied depending on their concentration, with the lowest concentration (0.78125 µg/ml) showing the highest effectiveness and the highest concentration (400 µg/ml) exhibiting the lowest. Notably, the carbon tetrachloride soluble fraction of the methanolic extract demonstrated the most promising antimicrobial activity. This suggests that *A. carambola* extracts, particularly from the methanolic fraction, have potential as natural antimicrobial agents against a range of microorganisms (Mia *et al*., 2007).

4.2.5. *Antioxidant activity*

In a study assessing antioxidant capabilities through various tests like ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH), vitamin C content, total phenolic content, and total flavonoid content, *A. carambola* ranked as the third most potent antioxidant among 20 commonly available fruits in Sri Lanka (Silva & Sirasa, 2018). Furthermore, when the methanol extract of *A. carambola* leaves (MEACL) was tested at concentrations ranging from 50 to 375 µg/mL, it displayed a dose-dependent moderate antioxidant effect in both DPPH and ABTS+ assays. The IC50 values, which indicate the concentration required to achieve a 50% reduction in activity, were measured at 62.0 µg/mL for DPPH and 6.0 µg/mL for ABTS+. This signifies that the leaf extract of *A. carambola* holds promising antioxidant properties (Siddika *et al*., 2020).

4.2.6. *Hepatoprotective activity*

Azeem *et al*. (2010), in their study, investigated the impact of *A. carambola* fruit extract on liver damage induced by carbon tetrachloride. The results showed a significant decrease in serum levels of enzymes ALT, AST, and ALP, indicating improved liver function. Additionally, the levels of liver-reduced glutathione significantly increased 24 hours after administering carbon tetrachloride, indicating enhanced antioxidant activity.

In another research conducted by Huang *et al*. (2019), mice with acute liver injury were treated with *A. carambola* root extract for seven days. The study found that the treatment led to lower levels of enzymes associated with liver damage (AST and ALT), reduced levels of inflammatory markers (IL-1 and IL-6), and decreased levels of malondialdehyde (MDA) in the liver. Conversely, levels of antioxidants like superoxide dismutase (SOD), Glutathione (GSH),

and plasma glutathione peroxidase (GSH-Px) were elevated. At the molecular level, proteins involved in inflammation and cell death were downregulated, suggesting a protective effect.

In their study Pang *et al*. (2017) investigated the impact of *A. carambola* free phenolic extract (ACF) on hepatic steatosis in mice deficient in the leptin receptor (db/db mice). After eight weeks of ACF administration, the content of liver triglycerides (TG) significantly decreased compared to the control group. The treatment worked in part by reducing the activity of key enzymes involved in lipogenesis (SREBP-1c, SCD1, and FAS) and increasing the activation of AMP-activated protein kinase α. Additionally, certain microRNAs, specifically mircoRNA-34a and mircoRNA-33, played a crucial role in modifying this signaling pathway. This indicates a potential therapeutic effect of ACF on liver steatosis.

4.2.7. *Neuroprotective activity*

In a study by Lu *et al*. (2019), they investigated the effects of a compound called DMDD (2-dodecyl-6-methoxycyclohexa-2,5-diene-1,4-dione) on memory and brain cell loss in mice with Alzheimer's disease (AD), specifically those with a genetic predisposition (APP/PS1 transgenic mice).

During the study, the mice were administered at varying doses of DMDD for a period of 21 days. The results showed that DMDD had a neuroprotective effect, meaning it helped protect the brain. It led to a reduction in cell death (apoptosis) in the hippocampal tissues of the APP/PS1 mice. This resulted in improved memory and spatial learning abilities, and also prevented the loss of neurons. This suggests that DMDD may have potential as a treatment for memory problems and brain cell loss in Alzheimer's disease.

4.2.8. *Antiulcer activity*

In their study Goncalves *et al*. (2006) investigated the potential gastroprotective effects of an alcoholic aqueous extract derived from the leaves of *A. carambola* in rats. They tested this extract in various ulcer models. When it came to an acidified-ethanol-induced model, they observed a notable anti-ulcer activity. However, in models involving indomethacin and acute-stress ulcer formation in mice, the extract did not demonstrate any protective benefits. This led the researchers to conclude that star fruit possesses a relatively lower level of antiulcer activity.

5. TOXICITY PROFILE OF STAR FRUIT

5.1. *A. carambola* Really Toxic or Fable

There's ongoing debate regarding the toxicity of star fruit, with conflicting findings. High intake, especially when the fruit contains elevated levels of oxalic acid, has been linked to nephrotoxicity. In cases of excessive consumption or concentrated fruit juice intake, nephropathic patients have exhibited symptoms like abdominal pain, vomiting, nausea, and kidney blockage (Neto *et al*., 2003; Chen *et al*., 2005). Studies suggest that individuals experiencing symptoms due to oxalate deposition can recover within about four weeks with standard treatment. However, in severe cases, obstruction caused by oxalate crystals may lead to renal failure. This isn't the sole reason; programmed cell death of renal epithelial cells, as depicted in Figure 3, might also contribute to this condition. Moreover, separate research has highlighted additional neurotoxic symptoms associated with star fruit toxicity. These symptoms include back pain, mental confusion, intractable hiccups, epileptic seizures, vomiting, and insomnia, which have proven lethal for some patients (Martin *et al*., 1993; Tsai *et al*., 2005).

Until the 1980s, neurotoxic symptoms were attributed to oxalic acid present in fruit juice, as indicated by Chen *et al*. in 2001. Research showed that injecting mice with a peritoneal dose of 8 g/kg resulted in tremors (Muir & Lam, 1980). However, recent studies have shed new light on the neurotoxic effects observed in the central nervous system, such as confusion, hiccups, convulsions, and even fatalities. These effects are linked to the inhibition of the GABAergic

system and a substance called caramboxin. Patients with fatal renal conditions exhibited severe symptoms in a dose-dependent manner, particularly those who consumed over 2 liters of juice or around eight carambola fruits. Intriguingly, a study challenged the notion of attributing toxicity solely to the carambola fruit. It revealed that avoiding this fruit for individuals with kidney disease eliminates the triggering of detrimental effects, as both oxalic acid and caramboxin are eliminated, as depicted in Figure 4 (Garcia-Cairasco *et al*., 2013).

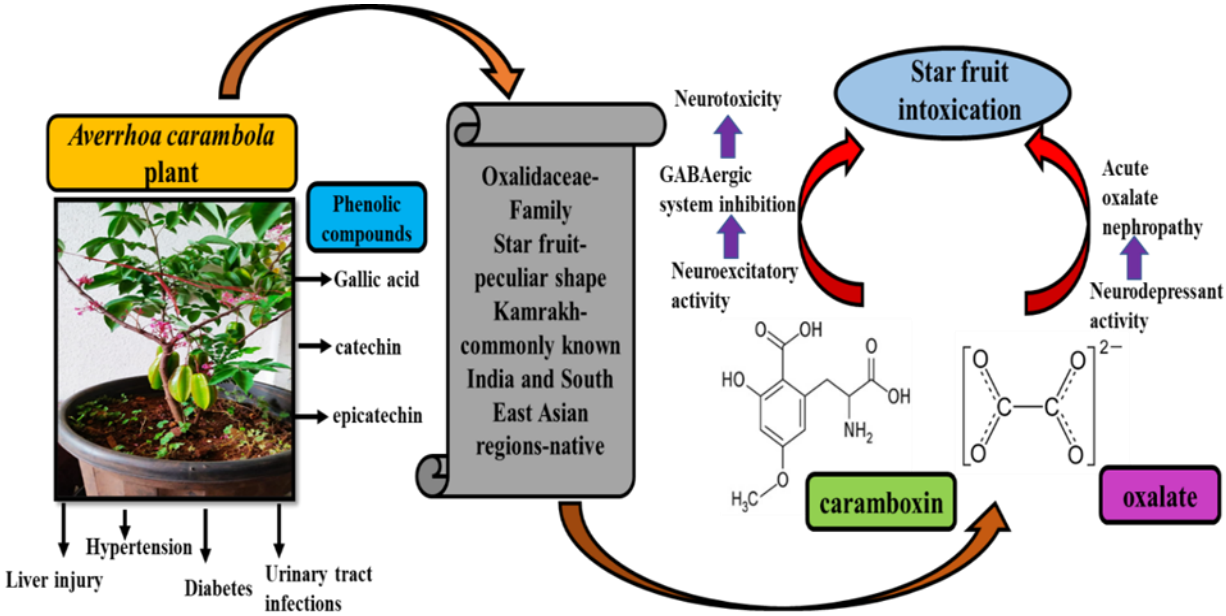

Figure 4. Schematic representation of toxicity, benefits, and description about *A. carambola.*

6. CONCLUSION

The current analysis provided an overview of Averrohoa carambola's traditional usage, therapeutic benefits, phytochemical, pharmacological, and intoxication profile. These days, the culinary and pharmaceutical industries are very interested in star fruit. *A. carambola* is a top pick for the pharmaceutical and health industries because of its superior nutritional, medicinal, and pharmacological qualities. The *A. carambola* plant is widely spread worldwide and possesses a variety of pharmacological properties, including anti-inflammatory, hepatoprotective, hypoglycemic, analgesic, antioxidant, and antibacterial properties. Star fruit contains several phytoconstituents with specific pharmacological activity i.e., gallotannin, catechin, epicatechin, flavonoids, saponins, alkaloids. There have been few investigations on the toxicity of consuming large amounts of star fruit juice, and the substances caramboxin and oxalates have been linked to nephrotoxicity and neurotoxicity. Further study is needed to close this knowledge gap and determine the precise mechanism underlying star fruit intoxication. Until there are a number of studies on starfruit that address the phytoconstituent and its bioactive components, additional work and studies need to be concentrated.Certainly, this plant has many opportunities with countless beneficial effects, but we must not forget the challenges. This review offers a balanced assessment, setting it apart from other studies.

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

Suchita Gupta: as the first author, contributed to the literature review and manuscript preparation. **Reena Gupta**: as the corresponding author, provided invaluable guidance, oversight, and expertise throughout the entire process of writing and finalizing this review paper.

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Research Article

Chemical composition and biological activities of essential oils and extract of *Eucalyptus citriodora* Hook

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Abstract: In this study, the leaves of *Eucalyptus citriodora* Hook (Lemon-Scented Eucalyptus) were harvested and collected from Üzümlü neighborhood of Fethiye district of Muğla in 2021. Chemical content analysis of steam distillation and hydrodistillation of essential oils were determined by GC-MS, while phenolic content of methanol extract was determined by HPLC-DAD. Antioxidant activities of essential oils and methanol extracts were determined by DPPH radical removal, ABTS cation removal, *β*-carotene linoleic acid, and CUPRAC activity methods; Anticholinesterase activity against AChE and BChE enzymes was determined by Ellman method; and tyrosinase inhibition associated with melanin hyperpigmentation, *α*-amylase inhibition, and *α*-glucosidase inhibition activities associated with diabetes were determined as an *in vitro*. The bioactivities and chemical contents of *E. citriodora* species, a great value of, Türkiye, were determined, bringing new natural products to organic chemistry. As a result of the study, new bioactive extracts would be obtained and thus, they can effectively reveal the potential of new business opportunities. Since methanol extract is effective against incurable diseases such as Alzheimer's and diabetes, it will also be possible to develop therapeutics of such diseases by investigating the advanced chemistry and *in vivo* activities of the extracts with new projects.

1. INTRODUCTION

Eucalyptus is a large genus of the Myrtaceae family, comprising approximately 900 species and subspecies (Brooker & Kleinig, 2004). *Eucalyptus citriodor*a species of *Eucalyptus,* is widely used in perfumery and in cleaning the air as an important ingredient in cosmetics and air fresheners. Previous studies showed that the essential oil obtained from *E. citriodora* has antibacterial, antifungal, anticandidal, insecticidal, acaricidal, antitrypanosomal, and herbicidal activities (Ramezani *et al*., 2002; Singh, *et al*., 2005; Batish *et al*., 2008; Habila *et al*., 2010; Singh, *et al*., 2012).

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The essential oil obtained from *Eucalyptus* species has been used as an antiseptic, antipyretic, and analgesic since ancient times (Brooker & Kleinig, 2004). Known for its weed inhibition and insecticidal properties, *E. citriodora* (Kohli *et al*., 1998; İşman, 2000) has been reported to have a wide variety of biological activities, including antimicrobial, fungicidal, insect repellent, fumigant, pesticide, and acaricidal activity (Seyoum *et al*., 2003; Batish *et al*., 2008). In addition, *E. citriodora* essential oil has been reported to exert analgesic and anti-inflammatory effects for colds, flu, and sinus congestion (Silva *et al*., 2003; Singh *et al*., 2012).

Eucalyptus species are also known for their important volatile fatty acids such as cineole, citronellal, and citronelle (Ansari *et al*., 2021). The antifungal effects of the major component Citronellalin (*Rhizoctonia solani* and *Helminthosporium oryzae*) obtained from the essential oil of *E. citriodora* against two known rice pathogen species were investigated by Ramezani *et al*. (2002) and in their study it was observed that Citronellal obtained as the major component by hydrodistillation method showed more activity than *E. citriodora* oil (Ramezani *et al*., 2002).

Nowadays, there is an emerging need for new and natural therapeutic agents specifically since synthetic drugs used in the treatment of such diseases as Alzheimer's, diabetes, and ulcer, which are on the increase, and which do not have curative solutions yet have toxic or side effects.

Since *Eucalyptus* species have various medicinal activities, the expectation to find natural, antioxidant, anticholinesterase, tyrosinase inhibitor, urease inhibitor, α-amylase, and α-glucosidase inhibitors from *E. citriodora* species is increasing. In a study, α-amylase and α-glucosidase enzyme inhibition activity was evaluated on *Eucalyptus obliqua* L'Hér ethanol extract and it was found that both α-amylase and α-glucosidase enzyme inhibition activity of the ethanol extract studied showed very good activity compared to acarbose, which is used as a standard reference substance (Sabiu & Ashafa, 2016). In another study, the inhibition activities of α-amylase and α-glucosidase of the ethanol extracts of *Eucalyptus globulus* Labill. leaves, which were previously degreased and undegreased, were compared with acarbose IC_{50}: 23.6±1.2 μg/mL, IC_{50}: 14.8±1.2 μg/mL, IC_{50}: 5.2±1.3 μg/mL, respectively and were found to have a good antidiabetic effect (Bello *et al*., 2021).

In recent years, the search for new agents to be used in the treatment of diseases such as Alzheimer's, Type II diabetes and Melanoma has been increasing. The increase in skin diseases, Alzheimer's disease, duodenal ulcer diseases, and diabetes in Türkiye and worldwide spurred an increase in such research. In the related literature, it was determined that 15 compounds, one of which was a new compound, were active against tyrosinase enzyme inhibition in isolation studies performed on the extract obtained from *Eucalyptus globulus* leaves (Lin *et al*., 2019). In the study conducted by Ansari *et al*., (2021), the insulinotropic and antidiabetic properties of *E. citriodora* leaves were investigated and bioactive phytomolecules were isolated. In their study the isolated phytocompounds responsible for β-cell effects were quercitrin, isocercitrin, and rhodomirtosone E while *E. citriodora* was found to favor glycemic control through multiple mechanisms.

In the literature studies carried out to date, the main non-volatile compounds abundant in *Eucalyptus* were determined to be phenolic compounds that contributed significantly to the antioxidant activities of the extracts. Epicatechin and catechin, among the phenolic compounds, in many experimental systems, are known to have anti-carcinogenic effects in many organs including the lung, liver, pancreas, esophagus, small intestine, colon, stomach, prostate, and mammary gland. Likewise, epicatechin and catechin provide protection against neurodegenerative diseases by protecting neurons from excessive oxidative stress (Almeida *et al*., 2009; Al-Sayed *et al*., 2012; Santos *et al*., 2012; Vázquez *et al*., 2012).

Herein, we report the phenolic constituents, chemical composition, antioxidant, anticholinesterase, α-amylase inhibition, and α-glucosidase inhibition activities of essential oils and methanolic extract of *Eucalyptus citriodora*.

2. MATERIAL and METHODS

2.1. Chemical Reagent for Biological Studies

The optical densities for bioassays were measured by using SpectraMax340PC384 (Microplate reader by Molecular Devices, Silicon Valley, USA). The phenolic profiling of the sample was done using Shimadzu 20AT series (HPLC-DAD) (Shimadzu Corporation, Japan). Ethylenediaminetetraacetic acid (EDTA), sodiumchloride, ferrous chloride, and copper (II) chloride dihydrate ($CuCl_2.2H_2O$) were acquired from Merck (Darmstadt, Germany). DPPH (1,1-diphenyl-2-picrylhydrazyl), butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), β-carotene, α-tocopherol, neocuproine, polyoxyethylene sorbitan mono palmitate (Tween-40), ferrene, ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonicacid) diammoniumsalt), linoleic acid, kojic acid, BChE (butyrylcholinesterase) from horse serum (EC 3.1.1.8, 11.4 U/mg) and AChE (acetylcholinesterase) from electric eel (Type-VI-S,EC 3.1.1.7, 425.84 U/mg), mushroom tyrosinase (EC 232-653-4, 250 KU, ≥1,000 U/mg), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), galantamine, butyryl-thiocholine chloride, acetylthiocholine iodide, L-DOPA (3,4-dihydroxy-*D*-phenylalanine), the certificated reference compounds used to screen the phenolic ingredients were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Solvents and chemicals were of analytical grade.

2.2. Collection and Extraction of Plant Materials

In this study, the leaves of *E. citriodora* species were harvested and collected from Üzümlü neighborhood of Fethiye district of Muğla in 2021. *E. citriodora* was compared with herbarium samples and *E. citriodora* leaves were divided into 3 parts. Some of the leaves were extracted with the Clevenger apparatus according to the American Pharmacopoeia, and some of the essential oils were extracted by the steam distillation method. The remaining part of the leaves was extracted with methanol at room temperature.

2.3. Determination of Chemical Contents

2.3.1. GC-MS analysis

The investigation of the volatile components was carried out using a GC-MS equipped with MS detector (Varian Saturn 2100T). Rx_i-5sil capillary column (30 m x 0.25mm, 0.25µm) was used for the analysis of essential oils (ECB and ECH). The injection temperature was set at 250^{o}C injection mode: split ratio was set to 1:20 and the injected volume was 0.2 μL of oil dissolved in hexane. It was prepared to rise from 60°C to 300°C. Pressure was set to 15.0 psi and helium gas was used as carrier gas. Analysis time for the chemical content of essential oils was determined as 76 minutes. For compounds in the resulting chromatogram, NIST-Wiley library was used (compared with literature).

2.3.2. HPLC-DAD analysis

The phenolic component analysis of methanol extract (ECM) of the leaves of *E. citriodora* was carried out using a modified method of Tokul-Ölmez *et al.* (2020). In this study, 41 substances (fumaric acid, gallic acid, protocatechic 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, kaempherol, apigenin, chrysin, 4-hydroxy resorcinol, 1,4-dichlorobenzene, pyrocatechol, 4-hydroxybenzaldehyde, epicatechin, 2,4-dihydroxybenzaldehyde, hesperedin, oleuropein, naringenin, hesperetin, genistein, curcumin) were investigated in ECM extract

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 ºC. The chromatographic separation was performed on a C_{18} (5 µm, 4.6 mm x 250 mm) reverse phase column and Inertsil C_{18} guard column (Tokul-Ölmez *et al*., 2020).

2.4. Biological Activities

2.4.1. Evaluation of antioxidant activity by β-carotene bleaching test

The antioxidant activity of the essential oils and methanol extract was evaluated using the *β*-carotene-linoleic acid system (Grina *et al*., 2020). The bleaching rate (R) of *β*-carotene was determined from the following equation: R=lna/b/t, where *ln* is the natural log, a is the absorbance at zero-time, and b is the absorbance at time t (120 min). We calculated the antioxidant activity as inhibition percent by the following equation:

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

BHA, BHT, and *α*-tocopherol antioxidant standards have been used for the comparison.

2.4.2. Free radical-scavenging activity (DPPH assay)

The antiradical activity of the methanol extract and essential oils was tested by the DPPH free radical (Kozłowska *et al*., 2016). DPPH is a colored radical that has a maximum absorbance at 517 nm, and upon reduction, its absorption decreases. Briefly, 0.1 mM DPPH (160 µL) was mixed with 40µL of the sample solution of various concentrations and incubated for 30 minutes in the dark, and the absorbance was measured at the same wavelength. The antioxidant activity of extract tested was compared with the known standards. The DPPH radical scavenging was calculated using the following equation:

$$DPPH\ Free\ radical\ scavenging\ ativity\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times\ 100$$

From the inhibitory activity versus concentration graph, the IC_{50} (µg/mL) values were calculated.

2.4.3. ABTS cation radical scavenging activity

$ABTS^{\bullet+}$ scavenging activity assay presents some advantages over DPPH scavenging test which is not convenient with water insoluble or bulky structured compounds. Therefore, the $ABTS^{\bullet+}$ scavenging activity of the extracts was also verified (Gupta *et al*., 2016). Briefly, 7 mM of ABTS and 2.45 mM of potassium persulfate were dissolved in water and kept for 16 hours in the dark to provide $ABTS^{\bullet+}$ solution. The tested $ABTS^{\bullet+}$ solution was prepared by diluting it with ethanol to get an absorbance of 0.700±0.025 at 734 nm in a one cm pathway. To each well containing 40 µL of the extract in methanol of various concentrations, 160 µL of diluted $ABTS^{\bullet+}$ solution was added and incubated for ten minutes, and then the absorbance was measured at 734 nm. For comparison, BHA, BHT, and *α*-tocopherol were used and each assay was performed in triplicate. The sample's capability to scavenge $ABTS^{\bullet+}$ was calculated using the formula given for the DPPH assay. The results of $ABTS^{\bullet+}$ scavenging activity were presented as IC_{50}.

2.4.4. Cupric reducing antioxidant capacity (CUPRAC)

The standard CUPRAC method with slight modifications was adopted (Maryam *et al*., 2016) and the absorbance was recorded using an Eliza reader. The aqueous solution including 50 µL of $CuCl_2.2H_20$ (10 mM), neocuproine (7.5 mM in absolute ethanol), and NH_4Ac buffer (100 mM, pH 7.0) was added to 50 µL of sample extract at various concentrations to make 200 µL final volume and then incubated for one hour at room temperature. The absorbance was recorded at 450 nm. The blank contains the same reactants except the plant methanol extract

and essential oils. The antioxidant standards were used for comparison. The results were expressed as $A_{0.5}$. The antioxidant standards such as BHA, BHT and *α*-tocopherol were used to compare the activity.

2.4.5. Determination of anticholinesterase activities

The inhibition of acetylcholinesterase (AChE; 5.32×10^{-3U}) and butyrylcholinesterase (BChE; 6.85×10^{-3U}) of the methanol extract and essential oils were tested using Elman's method (Öztürk *et al*., 2014). In a 96 well plate, each concentration (25-200 µg/mL) of the samples in ethanol (10 µL) was incubated at 25°C for 15 min with 20 µL of enzyme solution and 150 µL of sodium phosphate buffer (100 mM, pH=8). After incubation, Ellman's reagent, DTNB (0.5 mM, 10 µL), and substrates (10 µL) were added to each well to make 200 µL final volume. Then measurement was performed at 412 nm for 10 minutes and galantamine was used as a standard. The percent of both enzymes' inhibition was calculated using the following formula:

$$AChE/BChE\ inhibiton\ activity\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} x100$$

Where $A_{control}$ is the enzyme inhibitory activity of blank and A_{sample} is the enzyme inhibitory activity of the sample. Each test was conducted in triplicate. The results are presented as inhibition (%) at an extract concentration of 200 µg/mL.

2.4.6. Determination of tyrosinase inhibitory activity

In vitro tyrosinase inhibitory potential of methanol extract and essential oils were assessed using mushroom tyrosinase by following the Hearing method (Benso *et al*., 2018). The L-DOPA was employed as a tyrosinase substrate. Kojic acid was used as a standard to compare the activity. The tyrosinase inhibition (%) at each sample concentration (µg/mL) was calculated as used in AChE and BChE assays.

2.4.7. Determination of α-amylase inhibitory activity

α-Amylase inhibitory activity of methanol extract and essential oils was tested by using the method previously reported by Quan *et al.* (2019) with slight modifications in the use of incubation time, reagents, and amount of the reagents used and samples. 25 mL sample solution and 50 mL *α*-amylase solution (0.1 units/mL) in phosphate buffer (20 mM pH=6.9 phosphate buffer prepared with 6 mM NaCl) were mixed in a 96-well microplate. The mixture was pre-incubated for 10 minutes at 37°C. After pre-incubation, 50 mL starch solution (0.05 %) was added and incubated for 10 minutes at 37°C. The reaction was completed by adding 25 mL HCl (0.1 M) and 100 mL Lugol solutions. 96-well microplate reader was used to measure absorbance at 565 nm. Acarbose was used as standard. The sample concentration providing 50% inhibition activity (IC_{50}) was calculated from the graph of *α*-amylase inhibitory activity against sample concentrations.

2.4.8. Determination of α-glucosidase inhibitory activity

α-Glucosidase inhibitory activity of the methanol extract and essential oils was determined using the method previously reported by Kim *et al.* (2000) with slight modifications in the use of incubation time, reagents and amount of the reagents used and samples. 50 mL phosphate buffer (0.01 M pH=6.9), 25 mL PNPG (4-*N*-nitrophenyl-*α*-*D*-glucopyranoside) in phosphate buffer (0.01 M pH=6.9), 10 mL sample solution, and 25 mL *α*-glucosidase (0.1 units/mL) in phosphate buffer (0.01 M pH=6.0) were mixed in a 96-well microplate. The mixture was incubated for 20 minutes at 37°C. 90 mL sodium carbonate (0.1 M) was added into the microplate to end the reaction. A 96-well microplate reader was used to measure absorbance at 400 nm. Acarbose was used as standard. The sample concentration providing 50% inhibition

activity (IC_{50}) was calculated from the graph of α-glucosidase inhibitory activity against sample concentrations.

3. RESULTS

3.1. Gas Chromatography-Mass Spectrometer (GC-MS) Analysis Results of Essential Oils

The essential oil components of the ECB obtained by steam distillation method were determined with the help of GC-MS instrument. The results obtained are given in Table 1.

Table 1. GC-MS analysis results of ECB essential oil obtained by steam distillation method (%).

No	Retention time	Compound name	Amount %
1	8.251	Sabinene	2.44
2	10.652	Cineole	0.78
3	16.217	Isopulegol	0.02
4	16.899	Citronellal	47.05
5	16.969	β-Citronellal	1.02
6	17.442	(-)- Isopulegol	0.08
7	17.823	4-Terpineol	0.02
8	18.475	α-Terpineol	1.43
9	20.435	Citronellyl acetate	12.5
10	21.795	Linalool	0.06
11	25.279	2-Isoprophenyl-5-methylhex-4-enal	0.01
12	26.043	(+) 4-Carene	0.04
13	26.255	2,6-Octadien,2,6-dimethyl-$C_{10}H_{18}$	0.02
14	26.432	Eugenol	6.91
15	28.611	Methyleugenol	1.01
16	29.134	Caryophyllene	0.08
17	36.107	β- Caryophyllene	0.05

The data in Table 1 of the ECB extract was obtained by the steam distillation method. 17 components were scanned. Similar to literature studies the compound citronellal (47.05%) was detected as the major compound. Following the citronellal compound, citronellyl acetate (12.5%), eugenol (6.91%), sabinene (2.44%), α-terpineol (1.43%), β-citronellal (1.02%), methyleugenol (1.01%), cineole (0.78%), caryophyllene (0.08%), (-)- isopulegol (0.06%), linalool (0.06%), β-caryophyllene (0.05%), (+) 4-carene(0.04%), 2,6-octadiene,2,6-dimethyl-$C_{10}H_{18}$ (0.02%), isopulegol (0.02%), 2-isoprophenyl-5-methylhex-4-enal (0.01%) were determined and its concentrations were calculated as % component.

The essential oil components of the ECH obtained by hydrodistillation method were determined with the help of GC-MS instrument. The results obtained are given in Table 2. According to the data in Table 2 of the ECH obtained by the hydrodistillation method, 13 compounds were screened. Similar to literature studies the compound citronellal (38.01%) was detected as the major compound. Following the citronellal compound, β-citronellol (15.76%), citronellyl acetate (15.05%), (-)-isopulegol (9.42%), jasmone (3.48%), β-pinene (1.55%), β-caryophyllene (1.05%), caryophyllene oxide (0.88%), cineole (0.32%), L-isopulegol (0.20%), cedrene (0.05%), isopulegol (0.03%), and methoglycol (0.03%) were determined and their concentrations were calculated as % component.

Table 2. GC-MS analysis results of ECH essential oil obtained by hydrodistillation method (%).

No	Retention time	Compound name	Amount %
1	8.251	*β*- Pinene	1.55
2	8.881	Caryophyllene oxide	0.88
3	10.694	Cineole	0.32
4	16.187	Isopulegol	0.03
5	16.287	L-Isopulegol	0.20
6	16.670	Citronellal	38.01
7	16.990	*β*- Citronellol	15.76
8	17.369	(-)- Isopulegol	9.42
9	20.368	Citronellyl acetate	15.05
10	25.314	Methoglycol	0.03
11	26.043	*β*- Caryophyllene	1.05
12	28.327	Jasmone	3.48
13	31.842	Cedrene	0.05

3.2. High-Performance Liquid Chromatography (HPLC) Analysis

The results of the phenolic compounds of *E. citriodora* methanol extract are given in Table 3. When the HPLC-DAD results obtained were examined, *E. citriodora* was scanned at a wavelength of 254 nm in methanol extract, resulting in gallic acid, theobromine, theophylline, catechin, 6,7-dihydroxycoumarin, 4-hydroxy benzaldehyde, caffeic acid, epicatechin, taxifolin, ferrulic acid, coumarin, while 15 phenolic compounds were determined, including rutin, ellagic acid, myricetin, and chrysin.

Table 3. Phenolic component analysis results of *E. citriodora* methanol extract with the HPLC-DAD (mg/g extract).

No	Compounds	RT [a] (min)	Calibration equation	R^{2b}	ECM
1	Gallic acid	15.588	y = 45540x - 84708	0.9950	3.0676
2	Theobromine	26.227	y = 3942.7x + 81451	0.9983	3.8750
3	Theophylline	29.039	y = 36694x + 68674	0.9998	11.8226
4	Catechin	30.377	y = 2611.2x + 74392	0.9972	25.1016
5	6,7-dihydroxycoumarin	33.163	y = 27607x + 208788	0.9963	N.D.
6	4-hydroxybenzaldehid	33.560	y = 34376x + 4239.6	0.9996	2.3663
7	Caffeic acid	34.900	y = 49533x + 213471	0.9957	N.D.
8	Epicatechin	35.503	y = 2097.6x + 7998.2	0.9980	37.6590
9	Taxifolin	41.091	y = 29227x + 95.458	0.9993	1.8090
10	Ferrulic acid	42.868	y = 42245x + 110701	0.9992	7.9520
11	Coumarin	44.940	y = 81802x + 153471	0.9968	0.4170
12	Rutin	47.632	y = 47899x + 56096	0.9997	2.5286
13	Ellagic acid	50.208	y = 235073x - 7E+06	0.9808	4.8289
14	Myricetin	50.717	y = 136859x + 71185	0.9950	3.1280
15	Chyrsin	71.884	y = 26957x + 286396	0.9994	0.7277

*N.D.: Could not be calculated as quantity. RT[a]: Retention time of the compound in minutes, R^{2b}: linearity of the calibration graph

As a result of the concentration calculations made with the help of area, epicatechin (37.6590 mg/g) was found to have the highest concentration, followed by. catechin (25.1016 mg/g), theophylline (11.8226 mg/g), ferulic acid (7.9520 mg/g), and ellagic acid (4.8289 mg/g),

respectively. 6,7-dihydroxycoumarin and caffeic acid were detected, but in incalculable amounts. The HPLC-DAD chromatogram of *E. citriodora* methanol extract is given in Figure 1.

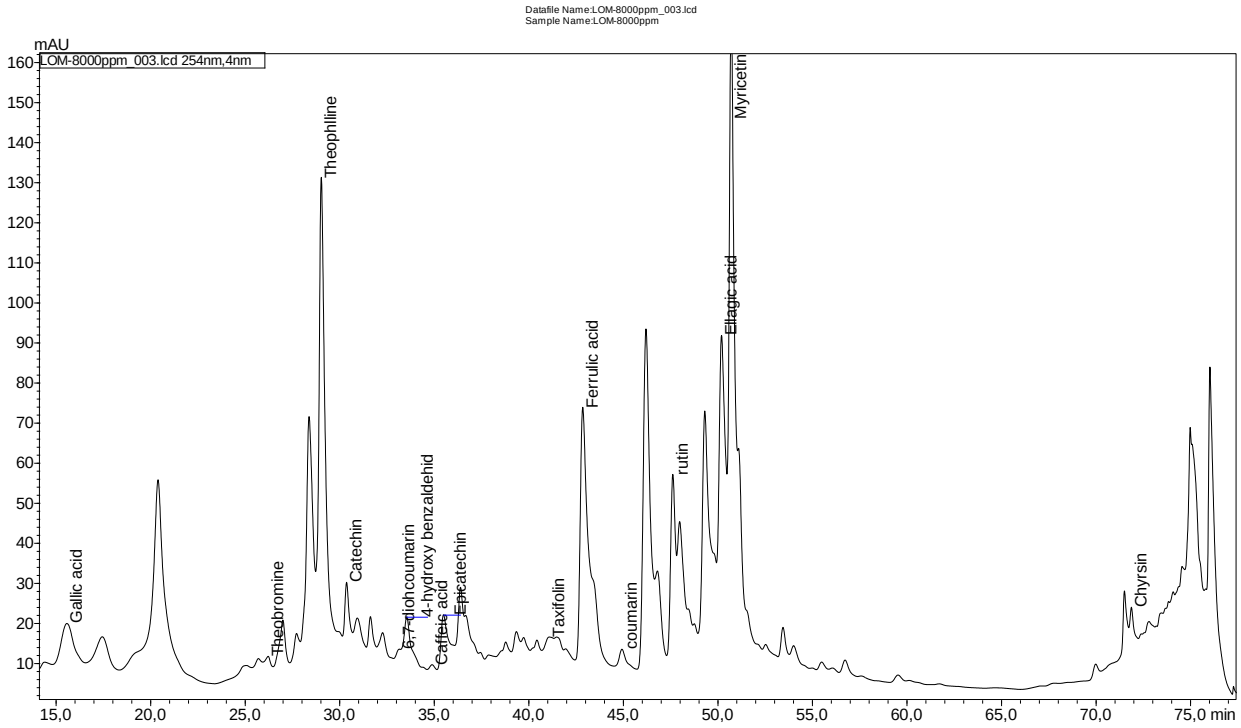

Figure 1. HPLC–DAD chromatogram of ECM extract at 254 nm.

3.3. Antioxidant Activities

The antioxidant activities of *E. citriodora* methanol extract (ECM) and essential oils (ECB and ECH) were carried out with 4 different methods. The results obtained are given in Table 4.

Table 4. Antioxidant activity results of *E. citriodora* essential oils and methanol extract[a].

Extract & Standard		Antioxidant Activity			
		$ABTS^{\bullet+}$ assay	$DPPH^{\bullet}$ assay	CUPRAC assay	*β*-carotene-linoleic acid assay
Code	Extract	IC_{50} (µg/mL)	IC_{50} (µg/mL)	$A_{0.5}$ (µg/mL)	IC_{50} (µg/mL)
ECM	Methanol	7.38 ± 0.47	11.51 ± 0.13	22.95 ± 0.84	32.26 ± 7.83
ECB (*Steam distillation*)	Essential oil	>200	>200	>200	>200
ECH (*Hydrodistillation*)	Essential oil	>200	>200	>200	>200
α-TOC[b]	Std	21.63 ± 0.45	26.61 ± 0.21	85.48 ± 8.64	1.63 ± 0.45
BHT[b]	Std	12.64 ± 0.21	9.02 ± 0.11	17.84 ± 0.31	3.42 ± 0.06
BHA[b]	Std	3.42 ± 0.06	8.28 ± 0.17	11.96 ± 0.27	1.64 ± 0.21

BHT, butyrylated hydroxy toluene; BHA, 2-tert-butyl-4-hydroxyanisole and 3-tert-butyl-4-hydroxyanisole; *α*-TOC, Alpha tocopherol..[a] The values expressed are the mean ± standard deviation of three parallel measurements. $p<0.05$. [b] Standard compounds.

$ABTS^{\bullet+}$ radical decolorization antioxidant activity results of *E. citriodora* methanol extract (ECM) and 2 different essential oils (ECB and ECH) were calculated. Methanol extract (ECM) (IC_{50}: 7.38±0.47 µg/mL) was found to have better activity when compared to BHT standard (IC_{50}: 12.64±0.21 µg/mL) and essential oil extracts (ECH and ECB) were used as reference compound.

When DPPH free radical decolorization activity results were examined, it was seen that the ECM extract (IC_{50}: 11.51±0.13 µg/mL) had an activity close to the BHT (IC_{50}: 9.02±0.11 µg/mL) used as a reference substance. It was then determined that the ECM extract (IC50: 11.51±0.13 µg/mL) showed better activity than the standard substance when compared to *α*-tocopherol (IC_{50}: 26.61±0.21 µg/mL) used as the standard reference substance.

Considering the Cu (II) reducing power activity results of *E. citriodora* essential oil and extract, ECM extract ($A_{0.5}$: 22.95±0.84 µg/mL) showed the best activity. It was found to show good activity when compared with standard reference compounds (BHA, $A_{0.5}$: 11.96 ± 0.27; BHT, $A_{0.5}$: 17.84 ± 0.31).

An examination of the results obtained in the *β*-carotene/linoleic acid bleaching activity test shows ECM extract of the *E. citriodora* (IC_{50}: 32.26±7.83 µg/mL), BHT (IC_{50}: 3.42±0.06 µg/mL) as essential oils. In comparison with (ECH and ECB), it was found that the best activity among the studied extracts belonged to the ECM extract.

3.4. Enzyme Inhibitory Activities

In addition to the antioxidant ability, we aimed to evaluate the enzyme inhibitory activities of *E. citriodora* essential oils and methanol extract on acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase and *α*-amylase, and *α*-glucosidase.

3.4.1. Anticholinesterase inhibition activity

The results of the enzyme inhibitory activities of *E. citriodora* essential oils and methanol extract on acetylcholinesterase (AChE), and butyrylcholinesterase are given in Table 5. Galantamine was the standard compound for comparison. The essential oils ECH and ECB showed a mild inhibitory activity against AChE and BChE (IC_{50}>200 µg/mL), while methanol extract exhibited a good inhibitory activity against BChE with IC_{50}: 20.84±0.74 µg/mL.

Table 5. Acetylcholinesterase, butyrylcholinesterase and tyrosinase inhibition activities of essential oils and methanol extract of *E. citriodora*[a]

Extract & Standard		Anticholinesterase activity		Tyrosinase activity
		AChE assay	BChE assay	
Code	Extract	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)
ECM	Methanol	>200	20.84 ± 0.74	23.03 ± 0.75
ECB (*Steam*)	Essential oil	>200	>200	NA
ECH (*Hydrodistillation*)	Essential oil	>200	>200	NA
Galantamin[b]	Std	5.65 ± 0.30	50.82 ± 0.16	ND
Kojic acid[b]	Std	ND	ND	0.71±0.54

[a]Values expressed herein are mean ± SEM of three parallel measurements. p<0.05. **NA:** not active. [b]Reference compounds.

3.4.2. Tyrosinase inhibition activity

The results of the tyrosinase inhibition activity of essential oils and methanol extract of the *E. citriodora* (Table 5) show that while the tyrosinase enzyme inhibition activity of the essential oil extracts ECB and ECH was not observed, the ECM extract showed tyrosinase enzyme inhibition activity. ECM extract (IC_{50}: 23.03±0.75 µg/mL) showed a mild inhibitory activity against kojic acid (IC_{50}:0.71±0.54 µg/mL) used as a reference substance.

3.4.3. Antidiabetic inhibition activity

The antidiabetic inhibition activity results of *E. citriodora* essential oils and methanol extract on enzymes of *α*-amylase and *α*-glucosidase are given in Table 6. Based on the results, it was determined that the best *α*-amylase inhibition activity belonged to the ECB extract (IC_{50}: 7.58 ± 0.78 µg/mL). The *α*-amylase inhibition activity of the ECM extract was calculated as IC_{50}:

13.46 ± 1.02 µg/mL whereas the α-amylase inhibition activity of the ECH obtained by steam distillation could not be calculated. When compared to acarbose used as a reference substance, it was determined that ECM and ECB extracts showed better α-amylase inhibition activity.

Table 6. Antidiabetic inhibitory activities of essential oils and methanol extract of *E. citriodora*[a]

Extract & Standard		Antidiabetic activity	
		α-Amylase inhibition	α-Glucosidase inhibition
Code	Extract	IC_{50} (µg/mL)	IC_{50} (µg/mL)
ECM	Methanol	13.46 ± 1.02	4.46 ± 1.38
ECB	Essential oil	7.58 ± 0.78	>200
ECH	Essential oil	NA	NA
Acarbose[b]	Std	25.14 ± 0.60	65.74 ± 7.29

[a]Values expressed herein are mean ± SEM of three parallel measurements. $p<0.05$. NA: not active. [b]Reference compound.

According to the results, it was determined that the best α-glucosidase inhibition activity belonged to the ECM extract (IC_{50}: 4.46 ± 1.38 µg/mL). The α- glucosidase inhibition activity of the ECB was calculated to be mild as acarbose (IC_{50}: 65.74 ± 7.29 µg/mL) whereas the α-amylase inhibition activity of the ECH obtained by steam distillation could not be calculated, which shows that the method of obtaining it is not suitable for antidiabetic activity.

4. DISCUSSION and CONCLUSION

Eucalyptus citriodora leaves, which grow naturally in Türkiye, were collected and blended from the Üzümlü neighborhood of Muğla's Fethiye district and were divided into 3 parts. Some of the leaves were extracted with the Clevenger apparatus according to the American Pharmacopoeia (ECH), while the other part was extracted by the steam distillation (ECB) method. The remaining part of the leaves was extracted with methanol at room temperature. The chemical content analysis of essential oils was determined by a GC-MS instrument, and phenolic content of methanol extract (ECM) was determined by an HPLC-DAD instrument. Antioxidant activities of essential oils and extracts were determined by DPPH free radical removal, ABTS cation radical removal, β-carotene linoleic acid and CUPRAC activity methods, while anticholinesterase activity against AChE and BChE enzymes was determined by Ellman method, and tyrosinase inhibition, α-amylase inhibition, and α-inhibition glucosidase activities were determined *in vitro*.

When the GC-MS results were examined, it was found that the ECH contained fewer bioactive components. The major citronellal compound was detected in both ECB (47.05%) and ECH (38.01%) essential oils. Citronellal was detected in both essential oils (ECB and ECH). The major component of the essential oil of *Eucalyptus citriodora* is reported as Citronellal in the literature (Ramezani *et al*., 2002; Lee *et al*., 2008; Ak Sakallı *et al*., 2022). When the effects of isopulegol and eugenol active substances detected in both essential oils are examined against *Eimeria oocysts in vitro*, it is known that essential acids are 90% effective against parasitic oocysts even at low doses (Remmal *et al*., 2013).

The phenolic content of *Eucalyptus citriodora* ECM extract shows a positive correlation with its antioxidant activities. Considering the results of antioxidant tests, it was found that ECM extract showed the best antioxidant activity in 4 different antioxidant experiments. It is known that epicatechin and catechin, which were determined as the main components in the phenolic content analysis of the ECM extract, are very important bioactive components. Epicatechin and catechin prevent tumor formation, bacteria and virus formation, and cell growth and are also bioactive molecules with anti-inflammatory and antioxidative effects. The hydroxy (-OH) group in the structure of catechins binds one electron to a free radical with a

single electron, reducing the number of free radicals and providing stability. It has been reported that high intakes of polyphenols such as catechin and gallic acid prevent and/or alleviate various chronic pathological conditions such as cardiovascular diseases, neurodegenerative diseases, diabetes, and cancer (Truong & Jeong, 2022; Dubey, 2023).

In the acetylcholinesterase enzyme activity experiment, the activity could not be calculated for 3 different extracts, two of which were essential oils. On the other hand, the butyrylcholinesterase activity results of ECB, ECH, and ECM extracts were examined and for both ECB and ECH essential oils, activities were calculated as IC_{50}: >200 µg/mL. Its comparison to the standard reference compound (Galantamin, IC_{50}:50.82±0.16 µg/mL) showed a very good butyrylcholinesterase enzyme activity compared to the ECM extract (IC_{50}: 20.84 ± 0.74 µg/mL). α-amylase and α-glucosidase inhibition activities of ECM and ECB extracts showed a very good activity compared to ECH essential oil. It can therefore be seen that the hydrodistillation method is not suitable for the antidiabetic activity method.

We believe that the extracts we obtained in our study are effective against diseases such as Alzheimer's and diabetes, for which there is no clear treatment yet, and that the extracts obtained can contribute to the development of therapeutics for such diseases by investigating their advanced chemistry and *in vivo* activities with new 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

Bihter Şahin: Investigation, Extraction, Interpretation of Results, Writing. **Cansel Çakır:** Activity experiments. **Yusuf Sıcak:** Finding Materials, Extraction, Activity Experiments. **Mehmet Öztürk:** Supervision, Interpretation of Results.

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