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Editor Prof. Dr. Selami SELVİ
Address Balikesir University, Altinoluk Vocational School, Edremit/Balıkesir, Türkiye
Phone +90 266 396 1552
E-mail sselvi@balikesir.edu.tr

Publisher Prof. Dr. Izzet KARA
Address Pamukkale University, Education Faculty, Kinikli Campus, 20070 Denizli, Türkiye
Phone +90 258 296 1036
Fax +90 258 296 1200
E-mail ikara@pau.edu.tr
ijsm.editor@gmail.com

Journal Contact Prof. Dr. Gurkan SEMIZ
Address Pamukkale University, Faculty of Science, Department of Biology, Kinikli Campus, Denizli, 20070, Türkiye
Phone +90 258 296 3582
Fax +90 258 296 1200
E-mail gsemiz@pau.edu.tr

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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:

1. Research articles: Original research in various fields of plant and allied organisms (algae, fungi, and lichens) will be evaluated as research articles.

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

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Research Article

Phytochemical profiling of the different organs of *Cupressus sempervirens* L. by LC-HR/MS

Ebru Erol [1,*]

[1]Bezmialem Vakif University, Faculty of Pharmacy, Department of Analytical Chemistry, İstanbul, Türkiye

Abstract: *Cupressus sempervirens* L. which is largely used in traditional medicine was collected from the Florya Atatürk Forest (İstanbul, Türkiye) to investigate the phytochemical profiling and antioxidant capacity of the seeds and cones. The antioxidant activities of hexane and methanol extracts of *C. sempervirens* L. were assessed *in vitro* using five complementary methods, including the *β*-carotene-linoleic acid assay for lipid peroxidation activity, the DPPH•, ABTS•+ assays for radical-scavenging activity, the CUPRAC method, and metal chelating methods. In addition, the phenolic profiling of the methanol extracts of the seeds and cones was analyzed using LC-HR/MS, for the first time. According to the findings, the antioxidant activity of the methanol extract obtained from seeds appears to be higher than that of cones in all assays. The methanol extracts of the seeds showed higher activity with an IC_{50}: 24.08±1.06, IC_{50}: 6.08±0.19, and $A_{0.5}$: 18.60±0.63 µg/mL in the DPPH•, ABTS•+, and CUPRAC assays, respectively than the BHA, and *α*-TOC. Also, the methanol extract of the cones showed strong activity with an IC_{50}: 38.87±0.03 and $A_{0.5}$:103.53±4.33 in ABTS•+ scavenging and CUPRAC assays. Moreover, twenty-eight phenolics were determined in the seeds while twenty-one phenolics were determined in the cones of the *C. sempervirens* using LC-HR/MS. The amounts of fumaric acid, vanilic acid, (-)-epicatechin, quercetin, hispidulin 7-glucoside, hyperoside, and quercitrin in the seeds are higher than those in the cones. Therefore, the results suggested that there was a strong relationship between the antioxidant activities of the extracts and their phenolic ingredients.

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1. INTRODUCTION

Cupressus sempervirens L., commonly known as the Mediterranean cypress or Italian cypress, is an evergreen tree species in the Cupressaceae family. It is native to the Mediterranean region and is widely cultivated in many parts of the world for its attractive, narrow, columnar shape and its ability to grow well in warm, dry climates. The other hand, *C. sempervirens* is found throughout Northern America, North Africa, Asia and Europe (Hassan Javed Chaudhary, 2012). *C. sempervirens* is a medicinal tree which is generally called as “servi” in Turkish because the leaves are used to cure hemorrhoids and diabetes, and the fruit of *C. sempervirens* is used to

*CONTACT: Ebru Erol ebruerol@bezmialem.edu.tr Bezmialem Vakif University, Faculty of Pharmacy, Department of Analytical Chemistry, İstanbul, Türkiye

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treat inflammation, toothache, and laryngitis, as well as a contraceptive and astringent. The essential oil obtained by hydrodistillation from the leaves and cones is applied topically to cure headaches, colds, coughs, and bronchitis. Its dried seeds have also been used to cure bruises, ulcers, and wounds. Due to the medical and pharmacological advantages of *C. sempervirens*, particularly its essential oil derived from leaves, it is usually applied as a cosmetic component in the industry (Batiha *et al*., 2022; Selim *et al*., 2014).

As far as we know, many studies that focus on the chemical ingredients of essential oils from *C. sempervirens* grown in the northern Mediterranean basin have been published (Batiha *et al*., 2022; Sacchetti *et al*., 2005; Tumen *et al*., 2010). Some of the research was carried out on the total phenolic potential of the polar extract of the cones of *C. sempervirens* by using spectrophotometric analysis (Selim *et al*., 2014; Semerci *et al*., 2020). However, there have also been no studies on the phenolic profiling of the seeds and cones of *C. sempervirens*, separately. The current study used $DPPH^{\bullet}$, $ABTS^{\bullet+}$ scavenging assays, CUPRAC, β-carotene/linoleic acid, and metal chelating assays to evaluate the *in vitro* antioxidant activity of the nonpolar (hexane) and polar (methanol) extracts of these different parts of *C. sempervirens*. Moreover, the phytochemical profiling to obtain detailed information about the chemical composition of the plant extracts, including the types of compounds present and their relative abundance, was carried out by using LC-HR/MS on the methanol extracts obtained from the seeds and cones of *C. sempervirens*. This information can be used to guide further investigations into the potential biological activities of these compounds, as well as to develop new products or therapies based on the natural products of the plant.

2. MATERIAL and METHODS

2.1. Plant Material

The cones of *Cupressus sempervirens* L. were collected from Florya Atatürk Forest in İstanbul-Türkiye in November 2022. The taxonomic identification of the plant was confirmed by Dr. Cagla Kizilarslan Hancer in the Department of Pharmaceutical Botanic at Bezmialem Vakif University.

2.2. Extraction Procedures

The seeds were hand-picked and isolated directly from the cones of *Cupressus sempervirens* L. The separated and air-dried plant organs were ground to a fine powder using a laboratory-type grinder. The Soxhlet extractor was used to extract 80 g of the powdered seeds using 800 mL of hexane (CSHS). The solution was filtered and concentrated to give an extract by using a rotary evaporator under reduced pressure at 38–40 °C. The remaining portions of the seeds were exposed to the same extraction technique to obtain a methanol extract after the hexane extraction (CSMS). Also, 300 g of the powdered remaining part of the cones was extracted by employing maceration techniques with 1500 mL of hexane (CSHM) and methanol (CSMM), respectively. Then, a freeze dryer was used to completely dry the extract after the removal of the metanol by using the rotary evaporator. Following the removal of the solvents under vacuum and lyophilizer, all crude extracts were stored at 4 °C in the dark until further analysis.

2.3. Antioxidant Activities

2.3.1. *Free-Radical scavenging activity (DPPH assay)*

The DPPH test was used to assess the free radical-scavenging capacity of the extracts (Blois M.S., 1958). The hexane and methanol extracts were resolved in DMSO and methanol, respectively, to prepare solutions in eight different concentrations: 6.25, 12.5, 25, 50, 100, 200, 400, and 800 µg/mL. Following the addition of the DPPH solution, the absorbance was determined at 517 nm after 30 minutes of room temperature incubation in the dark. BHA and α-

TOC were used as the standard chemicals, while methanol and DMSO were employed as the control solvents. The reaction mixture's lower absorbance showed a stronger capacity for scavenging free radicals. The data are presented as a 50% inhibition concentration in µg/mL (IC_{50}) (Ferhat *et al*., 2017).

2.3.2. *ABTS-Cation radical scavenging activity ($ABTS^{+\cdot}$ assay)*

According to the literature (Re R., 1999) the ABTS-cation radical scavenging activity of the extracts was assessed. The 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and $K_2S_2O_3$ were used to create a 7 mM $ABTS^{\bullet+}$ radical solution. Then, the same concentrations of the extracts used in the DPPH assay were tested to affect ABTS-cation radical scavenging at 734 nm after 10 min of incubation in the dark. The data are presented as a 50% inhibition concentration in µg/mL (IC_{50}) (Chemsa *et al*., 2016).

2.3.3. *Cupric reducing antioxidant capacity (CUPRAC assay)*

With a few minor adjustments, the procedure described by Apak was utilized to assess the antioxidant activity of the extracts in reducing cupric (Apak *et al*., 2004). The 50 µL of 10 mM Cu (II), 50 µL of 7.5 mM neocuproine, and 60 µL of NH_4Ac buffer (1 M, pH 7.0) solutions were added to each well in a 96-well plate. After that the same concentration, range of the extracts was used to measure the absorbance at 450 nm after 1-hour incubation at room temperature. For the purpose of comparing the activity, BHA and *α*-TOC were employed as standard antioxidants.

2.3.4. *β-Carotene/Linoleic acid assay*

The antioxidant activity of the obtained extracts was evaluated using a slightly modified version of the *β*-carotene-linoleic acid assay (Miller, 1971). For this purpose, 25 µL of linoleic acid and 200 µL of a Tween-40 emulsifier combination were added to 0.5 mg of *β*-carotene in 1 mL of chloroform. After vacuum-assisted chloroform evaporation, 100 mL of distilled water saturated with oxygen was added. Using a 96-well plate reader, the zero-time absorbance at 470 nm was determined as soon as the emulsion was applied to each tube. The emulsion system was incubated at 50 °C for two hours. Also, BHA and *α*-TOC were used as standard antioxidants.

2.3.5. *Ferrous ions chelating activity*

The chelating activity of the extracts on Fe^{2+} was determined using Ferene (Decker & Welch, 1990) with a few minor adjustments. 40 µL of 0.20 mM $FeCl_2$ was added to the extract solution, which was resolved in DMSO and methanol, respectively, to prepare solutions in eight different concentrations: 6.25, 12.5, 25, 50, 100, 200, 400, and 800 µg/mL. 80 µL of 0.5 mM ferene was added to start the reaction. After a 10-minutes incubation at room temperature, the mixture was measured at 562 nm. Also, EDTA was used as standard.

2.4. Quantification of Phenolic Compounds by LC-HR/MS

A reverse phase C18 column (150 x 3 mm x 5 µm particle size, Troyasil) and a Thermo Orbitrap Q-Exactive mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) were used for the LC-HR/MS investigations (İstanbul, Türkiye). LC-HR/MS analysis of the 100 phenolic standards was conducted with a Thermo Orbitrap Q-Exactive. The mobile phases A and B contained 1% formic acid in both the water and the methanol, respectively. The gradient programs were 50% A and 50% B for 0–1 min, 100% B for 1–3 min, 100% B for 3–6 min, 50% B for 6–7 min and 100% B for 7–15 min. The column temperature was set at 35 °C, and the mobile phase flow rate was 0.35 mL/min. The temperature and relative humidity were set at 22.0±5.0 °C and 50±15%, respectively. We selected the electrospray ionization (ESI) source for the applicable approach since it offers one of the best ionizations for small polar molecules. In the instrument's high-resolution mode, the ions with *m/z* values between 100 and 900 were scanned. By comparing retention times and HR-MS data with those of reference compounds

substances were identified. In order to lessen the repeatability issue brought on by external factors, such as ionization repeatability, in mass spectrometry measurements, dihydrocapsaicin was utilized as an internal standard for LC-HR/MS measurements. Quantitative analysis involves determining the amount of a phenolics in the extracts (µg/g extracts) using a calibration curve. All the phenolic compounds were bought and used as standards.

2.5. Statistical Analysis

All the assays were carried out in triplicate. The data were recorded as mean ± standard error meaning (SEM). Significant differences between means were determined using the student's *t*-test, while *p* values <0.05 were regarded as significant.

3. RESULTS

3.1. Strong Antioxidant Activity Profile

The hexane and methanol extracts of the seeds and cones of *C. sempervirens* were determined by five complementary methods, namely the *β*-carotene-linoleic acid assay for lipid peroxidation activity, DPPH•, ABTS•+ assays for radical-scavenging activity, and CUPRAC method and metal chelating methods as in Table 1. The antioxidant activity of the extracts studied was compared to standards such as BHA, *α*-TOC, and EDTA. According to the current findings, the activity of methanol extract obtained from seeds appears to be higher than that of cones in all antioxidant assays.

Table 1. Antioxidant activity of the extracts of *C. sempervirens* L.[a,b]

		DPPH assay	ABTS assay	CUPRAC assay	*β*-carotene/linoleic acid assay	Metal Chelating assay
		IC_{50}	IC_{50}	$A_{0,5}$	IC_{50}	IC_{50}
Seeds	CSHS	>200	411.55±4.13	149.97±1.45	>200	>200
	CSMS	24.08±1.06	6.08±0.19	18.60±0.63	30.90±1.11	>200
Cones	CSHM	>200	353.50±2.38	130.07±2.75	>200	>200
	CSMM	95.50±1.30	38.87±0.03	103.53±4.33	92.32±1.39	>200
Standards	BHA	28.59±0.06	7.23±0.01	24.49±0.19	1.34±0.04	nt
	α-TOC	36.35±0.24	27.70±0.28	134.53±0.19	2.10±0.08	nt
	EDTA	nt	nt	nt	nt	26.85±1.50

[a]IC_{50} values expressed are means ± SEM of three parallel measurements (p<0.05).
[b]IC_{50} and $A_{0.5}$ values are given as µg/mL.
[c]Reference compounds, BHA: Butylated hydroxy anisole; *α*-TOC: *α*-tocopherol; EDTA: Ethylenediaminetetraacetic acid.; nt: not tested.

In DPPH•, ABTS•+ and CUPRAC assays, both methanol extracts exhibited excellent activity, where the CSMS (IC_{50}: 24.08 ± 1.06, IC_{50}: 6.08 ± 0.19 and $A_{0.5}$: 18.60 ± 0.63 µg/mL, respectively) showed a higher activity than the standard BHA and *α*-TOC. In addition, methanol extract of the cones (CSMM) also exhibited good activity with an IC_{50}: 38.87±0.03 and $A_{0.5}$:103.53±4.33 in ABTS•+ scavenging and CUPRAC assays. The hexane extracts of the seeds and cones were less active in all assays when compared to the methanol extracts. In comparison to the other assays, all extracts demonstrated metal chelating with IC_{50}> 200 µg/mL by EDTA ferrous ions, which was used as a standard.

3.2. Qualitative and Quantitative Analysis of Phenolic Compounds by LC–HR/MS

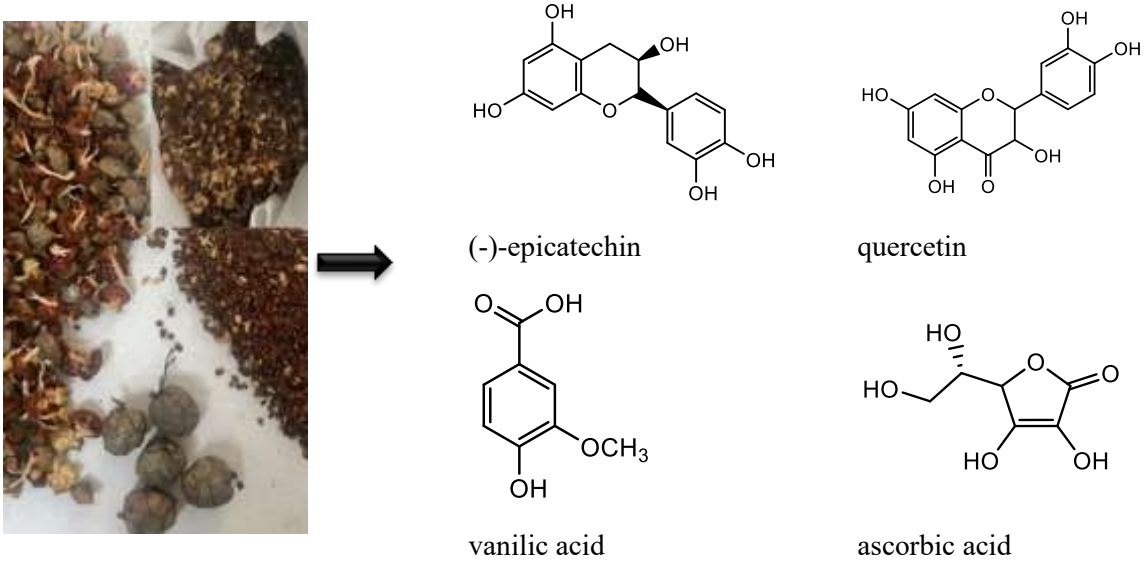
The LC-HR/MS, that stands for liquid chromatography-high resolution mass spectrometry, was used to analyze the phenolic compounds of the methanol extracts of the seeds and cones of *C. sempervirens* applying both positive and negative ionization modes. In this study, twenty-eight phenolic compounds were identified in the seeds while twenty-one phenolic compounds were identified in the cones of *C. sempervirens* (Table 2). The phenolic compounds (+)-catechin (6.735 µg/g extract), chlorogenic acid (0.018 µg/g extract), nepetin-7-glucoside (0.220 µg/g extract), scutellarein (1.743 µg/g extract), genistein (1.308 µg/g extract), kaempferol (.0383 µg/g extract), homogentisic acid (1.710 µg/g extract), and pyrogallol (0.343 µg/g extract) were identified in the seeds while they were not determined in the cones of *C. sempervirens*. And also, salicylic acid (0.115 µg/g extract) was determined only in the cones. The amounts of fumaric acid (93.518 µg/g extract), vanilic acid (37.008 µg/g extract), (-)-epicatechin (9.108 µg/g extract), quercetin (5.345 µg/g extract), hispidulin 7-glucoside (3.515 µg/g extract), hyperoside (2.783 µg/g extract), and quercitrin (2.530 µg/g extract) in the seeds are higher than those in the cones of *C. sempervirens* (Figure 1). It is well known that the identified phenolic compounds have potent antioxidant properties. For this reason, the LC-HR/MS results and the antioxidant activities were found to be correlated, indicating that the identified phenolic compounds in the sample were responsible for its antioxidant activity.

Table 2. Qualitative and quantitative analysis of the phenolic compounds in different organs of the *C. sempervirens* L. by LC-HR/MS.[a]

Phenolic Compounds	Molecular Formula	CSMS (seeds)	CSMM (cones)	U%
Ascorbic acid	$C_6H_8O_6$	8.948	27.623	3.94
(+)-Catechin	$C_{15}H_{14}O_6$	6.735	-	3.31
Chlorogenic acid	$C_{16}H_{18}O_9$	0.018	-	3.58
Fumaric acid	$C_4H_4O_4$	93.518	61.363	2.88
(-)-Epicatechin	$C_{15}H_{14}O_6$	9.108	1.458	3.17
(-)-Epicatechin gallate	$C_{22}H_{18}O_{10}$	0.065	0.073	3.05
(+)-*trans* taxifolin	$C_{15}H_{12}O_7$	0.438	0.070	3.35
Vanilic acid	$C_8H_8O_4$	37.008	32.515	3.49
Hyperoside	$C_{21}H_{20}O_{12}$	2.783	0.148	3.46
Aromadendrin	$C_{15}H_{12}O_6$	0.353	0.783	2.86
Apigenin 7-glucoside	$C_{21}H_{20}O_{10}$	1.250	0.053	3.59
Ellagic acid	$C_{14}H_6O_8$	0.188	0.185	4.20
Quercitrin	$C_{21}H_{20}O_{11}$	2.530	0.500	3.78
Myricetin	$C_{15}H_{10}O_8$	0.125	0.125	4.18
Nepetin-7-glucoside	$C_{22}H_{22}O_{12}$	0.220	-	3.07
Scutellarein	$C_{15}H_{10}O_6$	1.743	-	2.84
Quercetin	$C_{15}H_{10}O_7$	5.345	0.440	2.95
Salicylic acid	$C_7H_6O_3$	-	0.115	1.89
Naringenin	$C_{15}H_{12}O_5$	1.110	2.580	4.20
Luteolin	$C_{15}H_{10}O_6$	0.460	0.030	3.42
Genistein	$C_{15}H_{10}O_5$	1.308	-	3.28
Kaempferol	$C_{15}H_{10}O_6$	0.383	-	3.56
Apigenin	$C_{15}H_{10}O_5$	0.615	0.045	2.87
Chrysin	$C_{15}H_{10}O_4$	0.035	0.015	3.24
Homogentisic acid	$C_8H_8O_4$	1.710	-	4.35
Pyrogallol	$C_6H_6O_3$	0.343	-	4.50
Hispidulin 7-glucoside	$C_{22}H_{22}O_{11}$	3.515	0.633	4.57
Dihydrocaffeic acid	$C_9H_{10}O_4$	0.535	0.630	0.86
Chrysoeriol	$C_{16}H_{12}O_6$	0.560	0.063	2.08

[a] Values in µg/g extract.

Figure 1. The major phenolic compounds of the seeds and cones of *C. sempervirens*.



4. DISCUSSION and CONCLUSION

The phytochemical profiling of the seeds and cones of *C. sempervirens* growing in the Atatürk Forest involves the identification and quantification of phenolic compounds. There is much research on biological activity and chemical components of leaves of the cypress and its essential oils, in the literature (Batiha *et al*., 2022; Selim *et al*., 2014). However, there is lack of information and research on the cones and seeds of the *C. sempervirens,* especially on the phenolic compounds of the polar extracts. Phenolic compounds have been shown to have a wide range of health benefits, such as reducing the risk of chronic diseases such as cancer, cardiovascular disease, and neurodegenerative disorders. By analyzing the phenolic compounds present in a sample, researchers can gain insights into the potential antioxidant properties of the sample. One of the previous research on the ethanol extract obtained by maceration of seeds of *C. sempervirens* revealed that the only seven phenolic compounds together with the protocatechuic acid, (+)-catechin, and (−)-epicatechin which are major constituents by using RP-HPLC (Zengin *et al*., 2017). In the current study, the twenty-eight phenolic compounds which are the most potent antioxidant molecules were determined by using LC-HR/MS in the methanol extract of seeds (CSMS) of *C. sempervirens* which is obtained by using Soxhlet extractor. While vanilic acid and ascorbic acid were identified as major compounds in both seed and cones, (+)-catechin (6.735 µg/g extract) was analyzed in seed and naringenin (2.580 µg/g extract) was analyzed in higher amounts in cones. This can be seen as a positive result, as it helps to build upon the existing body of knowledge and reinforces the findings of the previous study.

The seeds and cones of the *C. sempervirens* were conducted *in vitro* experiments using five complementary methods to assess the antioxidant activity such as $DPPH^{\bullet}$, $ABTS^{\bullet+}$ scavenging assays, CUPRAC, *β*-carotene/linoleic acid, and metal chelating assays. The use of multiple complementary methods to assess antioxidant activity can provide a more comprehensive understanding of the antioxidant potential of seeds and cones. It can also help identify the types and quantities of antioxidants present in these plant parts and determine their potential health benefits. Especially, the seeds of the *C. sempervirens* exhibited strong antiradical potential in $DPPH^{\bullet}$ (IC_{50}: 24.08 µg/mL) and $ABTS^{\bullet+}$ (IC_{50}: 6.08 µg/mL) while showing the total antioxidant capacity with $A_{0.5}$: 18.60 µg/mL in CUPRAC assay. Moreover, an IC50 of 30.90 µg/mL obtained using the *β*-carotene/linoleic acid assay by analyzing the change in color of the

solution over time by oxidation is a measure of how methanol extract of the seeds protects β-carotene and linoleic acid. Also, Semerci investigated the DPPH free radical scavenging activity of the cones of *C. sempervirens* (Semerci *et al*., 2020).

Overall, most of the research showed the benefits of the essential oil of *C. sempervirens* seeds. Herein, investigation of the phytochemical composition and biological activity of the seeds and cones of *C. sempervirens* is an important step in understanding the potential health benefits of this plant. The study suggests that the methanol extract obtained from the seeds of *C. sempervirens* using Soxhlet extraction may contain bioactive secondary metabolites. These compounds could have potential applications in traditional medicine, as well as in the development of new drugs and nutraceuticals.

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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.

Orcid

Ebru Erol https://orcid.org/0000-0001-6342-4298

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Research Article

Assessment of antioxidant and enzyme inhibition properties of *Myrtus communis* L. leaves

Sengul Uysal[1,2*], **Kouadio Ibrahime Sinan**[3] **Gokhan Zengin**[3]

[1]Halil Bayraktar Health Services Vocational College, Erciyes University, 38280 Kayseri, Türkiye
[2]Drug Application and Research Center, Erciyes University, 38280 Kayseri, Türkiye
[3]Biochemistry and Physiology Research Laboratory, Department of Biology, Science Faculty, Selcuk University, Konya 42130, Türkiye

Abstract: This study investigated the antioxidant and enzyme inhibitory properties of *Myrtus communis* leaves. Three different solvents including methanol, 50%-methanol, and water were used to extract of *M. communis*. In addition, total bioactive compounds were evaluated by using total phenolic and total flavonoid content assays. In antioxidant assays, water extract displayed the highest antioxidant potential. The MeOH extract demonstrated the highest inhibitory effect against AChE (4.38 mg GALAE/g), BChE (1.58 mg GALAE/g), α-amylase (0.56 mmol ACE/g), and tyrosinase (132.20 mg KAE/g). The *M. communis* leaves extract could be used as a promising raw material source in food and medicine industries.

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1. INTRODUCTION

Plants are rich in phytochemicals including phenolic compounds and many pharmacological activities have been reported including antioxidant, anticancer, anti-inflammatory, and anti-hyperglycemia (Chu & Chen 2006; Kumar & Pandey, 2013; Engwa, 2018). In recent decades, plant-based foods have attracted a great deal of research attention due to their health-promoting effects (Hur *et al*., 2014; Tangyu *et al*., 2019). Additionally, the use of herbal products has increased demand as a primary source of health due to their minor side effects, efficacy, and safety (Kamboj, 2000).

Myrtus communis L., also called myrtle, is a valuable medicinal plant of the Myrtaceae family. Myrtle is used in a variety of applications in several industries including food, cosmetic, and pharmaceutical (Wannes & Marzouk, 2016). The leaves of the plant are used for hypertension, hyperglycaemia, cold, rheumatic pain, and haemorrhoids (Mine *et al*., 2019). *M. comminus* leaves have been used in traditional Turkish medicine to treat conditions such as cold, obesity, and diabetes (Tuzlacı & Bulut, 2007; Tuzlacı & Sadikoglu, 2007; Sargın, 2021). Several studies have been shown that *M. communis* leaves possessed a number of therapeutic

*CONTACT: Sengul UYSAL ✉ senguluysal@erciyes.edu.tr Halil Bayraktar Health Services Vocational College, Erciyes University, 38280 Kayseri, Türkiye

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activities including anti-bacterial, antioxidant, anti-diabetes, and anti-cancer, (Tretiakova *et al*., 2008; Amensour *et al*., 2010; Hennia *et al*., 2018; Raoof *et al*., 2019). These activities can be explained by the presence of diverse phytochemicals including phenolic acids (gallic, ellagic, caffeic acids) and flavonol derivatives (myricetin, quercetin, kaempferol derivatives) (D'Urso *et al*., 2017).

In this study, we aimed to evaluate the potential antioxidant and enzyme inhibitory properties of *M. communis*. Total bioactive compounds were also tested.

2. MATERIAL and METHODS

2.1. Plant Materials

M. communis were gathered in September 2018 at Silifke, Mersin, Türkiye. The plant sample was identified by Dr. Evren Yıldıztugay from Selçuk University. The samples were dried in the shade and the dried materials were grounded in a laboratory mill prior to extraction.

The dried leaves (5 g) were extracted by maceration with 100 mL different solvents (50% methanol, methanol, and water) for 24 h at room temperature. The extracts were evaporated with a rotary evaporator. All samples were stored at 4 °C for subsequent analysis (Uysal *et al*. 2021).

2.2. Total phenolic and Total Flavonoid Contents

The total phenolic content was tested by Folin- Ciocalteu method. The total flavonoid content was tested by $AlCl_3$ method. (Uysal *et al*., 2017). The details of methods are indicated in Supplementary materials.

2.2. Antioxidant Assays

Antioxidant properties of the extracts were performed by ABTS, DPPH, CUPRAC, FRAP, metal chelating, and phosphomolybdenum (Uysal *et al*., 2017). The details of methods are indicated in Supplementary materials.

2.3. Enzyme Inhibitory Assays

The inhibition of tyrosinase, α amylase, α-glucosidase and cholinesterase was screened. (Uysal *et al*., 2017). The details of methods are indicated in Supplementary materials.

2.4. Statistical Analysis

The details of methods are indicated in Supplementary materials.

3. RESULTS and DISCUSSION

3.1. Total Phenolic and Total Flavonoid Contents

The extracts of *M. communis* were evaluated for total bioactive contents. The total phenolic content was higher in 50%-MeOH (145.22±4.52 mg GAE/g) and water (144.45±1.38 mg GAE/g) extracts than MeOH extract (130.24±1.52 mg GAE/g) (Figure 1). However, the highest total flavonoid content was detected in the MeOH extract (57.06±0.94 mg RE/g) (Figure 2). Abdullahi *et al*. (2020) evaluated the antioxidant, antibacterial and total phenolic content of *M. communis* leaves and total phenolics ranged between 42.12 and 189 mg GAE/g in different concentrations. Our results show a higher total phenolic content than that reported by Ozcan *et al*. (2009), who found 9.9761 mg GAE/g in the extract of *M. communis* leaves. Tumen *et al*. (2012) reported the bioactive compounds of different extracts obtained from of *M. communis* leaves and berry. The total phenolic content values of four extracts of *M. communis* leaves ranged from 38.45 to 190.85 mg GAE/g. The obtained total phenolic content value in methanol extract of *M. communis* leaves is higher than our result. According to another study, the effect of drying methods on total phenolic content of *M. communis* fruits was reported by Alkaltman

et al. (2020). The highest total phenolics value (135.07 mg GAE/100g) was determined in microwave oven-dried samples. The drying effect on the phenolic content of ethanol extract obtained from *M. communis* leaves was described by Snoussi *et al*. (2021). Our results show higher total phenolic content values than those reported for ethanol extract (25.7-55.2 mg GAE/g) in this study. Snoussi *et al*. (2021) also found total flavonoid content of *M. communis* leaves in different drying methodology methods that ranged from 11.3-28.2 mgQE/g extract. Yaghoobi *et al*. (2022) reported methanol and water extracts of myrtle leaves showed higher total flavonoid content compared other solvents (ethanol and ethyl acetate). Wannes *et al*. (2010) showed that the leaf and stem of *M. communis* var *italica* contained higher amount of flavonoid compounds in comparison to flower extract.

The variability of total bioactive compounds of *M. communis* extracts could be due to the method of extraction, climatic conditions, geographical location, and polarity of solvent used (Lee Petersen, 2003; Miliauskas *et al*., 2004).

Figure 1. Total phenolic content of *M. communis* leaves extracts. Different letters in column indicate significant differences in the studied extracts (p<0.05).

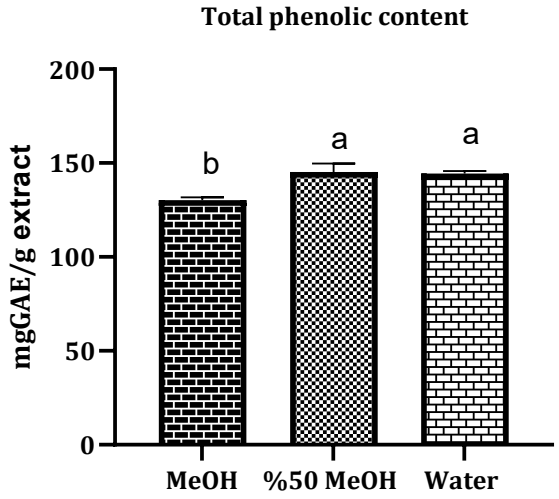
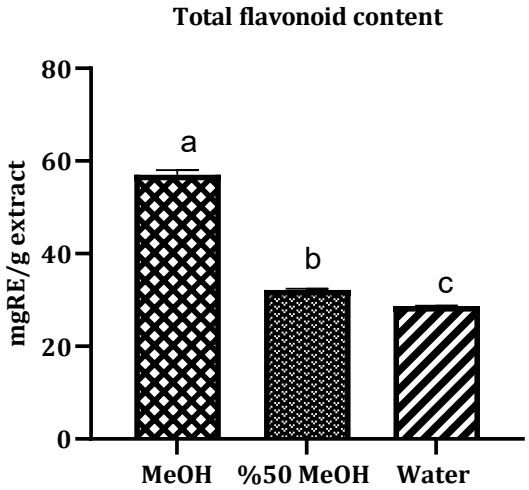


Figure 2. Total flavonoid content of *M. communis* leaves extracts. Different letters in column indicate significant differences in the studied extracts (p<0.05).



3.2. Antioxidant Capacity

Antioxidant capacity of *M. communis* leaves was assessed using complementary assays (Table 1). According to radical scavenging assays (DPPH and ABTS), the scavenger activity of the extracts decreased in the order: water> 50%-MeOH> MeOH. The reducing power activity, determined by established methods of CUPRAC and FRAP, ranged from 3.50-4.58 to 1.97-2.75 mmol TE/g, respectively. The antioxidant property of water extract was higher than that of other extracts. Antioxidant capacity of different parts from *M. communis* has been published in previous studies (Chryssavgi *et al*., 2008; Ozcan *et al*., 2009; Tuberoso *et al*., 2010; Tumen *et al*., 2012; Abdulhadi *et al*., 2020). For example, Serce *et al*. (2010) investigated DPPH activity of eight myrtles fruits. Results revealed that methanol extracts of fruits exhibited good activity, with values between IC_{50} of 2.34 and 8.24 µg/ml. Snoussi *et al*. (2021) evaluated the effect different drying methodologies on antioxidant activity of *M. communis* leaves. This study reported that the strong radical scavenging activity (DPPH IC_{50} µg/ml, ABTS IC_{50} µg/ml) was obtained from microwave dried leaves. Similarly, Alkaltham *et al*. (2021) also investigated the influence of different drying methods on antioxidant properties of *M. communis* fruits. The authors observed that dried berries extracts (DPPH: 83.01-83.55%) were more effective than the fresh berries (25.43%). In another study, the antioxidant properties of myrtle leaves cultivar were investigated by Medda *et al*. (2021). Comparison with our results is difficult because of different ways of expression of the activity.

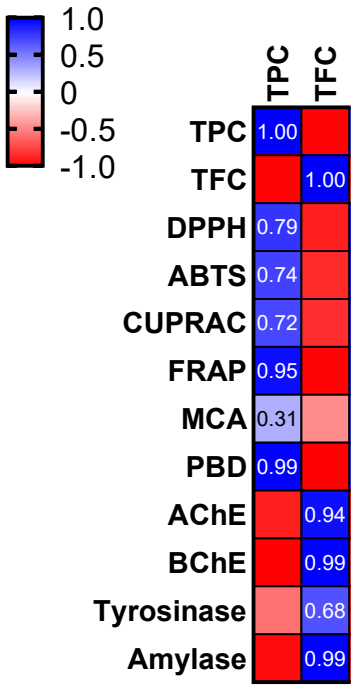
The results obtained in correlation analysis showed total phenolic content positive correlation with the antioxidant activity (Figure 3). These results are consistent with a study conducted by Medda *et al*. (2021), who found a correlation between the total phenols content and DPPH and β-Carotene.

Table 1. Antioxidant properties of *M. communis* leaves.

Assays	*Myrtus communis* -MeOH	*Myrtus communis* -50% MeOH	*Myrtus communis* -Water
DPPH (mmolTE/g)**	2.86±0.14^{c*}	3.07±0.11^{b}	3.36±0.05^{a}
ABTS (mmolTE/g)**	2.29±0.03^{c}	2.68±0.04^{b}	3.38±0.02^{a}
CUPRAC (mmolTE/g)**	3.50±0.05^{c}	3.85±0.10^{b}	4.58±0.06^{a}
FRAP (mmolTE/g)**	1.97±0.06^{c}	2.53±0.09^{b}	2.75±0.04^{a}
Phosphomolybdenum (mmolTE/g)**	4.08±0.25^{b}	4.49±0.18^{a}	4.52±0.02^{a}
Metal chelating (mgEDTAE/g)***	34.05±1.70^{c}	31.63±1.50^{b}	45.60±1.08^{a}

*Values expressed are means ±SD **TE: trolox equivalents, ***EDTAE: isodium edetate equivalents. Different letters in same row indicate significant differences in the studied extracts (p<0.05).

Figure 3. Pearson correlation values between biological activity assays and bioactive compounds (p<0.05). TPC: Total phenolic content; TFC: Total flavonoid content; ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; DPPH: 1,1-diphenyl-2-picrylhydrazyl; CUPRAC: Cupric reducing antioxidant capacity; FRAP: Ferric reducing antioxidant power; MCA: Metal chelating ability; PBD: Phosphomolybdenum; AChE: acetylcholinesterase; BChE: butyrylcholinesterase;



3.3. Enzyme Inhibitory Properties

AChE and BChE are the major enzymes responsible for the hydrolysis of acetylcholine (Fan&Chiu, 2014). α-amylase and α-glucosidase play an essential role in the hydrolysis of carbohydrate to glucose (Wang *et al*., 2013). Tyrosinase is the key enzyme in melanin biosynthetic metabolism (Pillaiyar *et al*., 2017). Inhibition these enzymes is one of the current strategies for Alzheimer's, diabetes and skin disorders management. In recent years, natural products draw more attention because of the low side effect on the treatment of these diseases (Tuzimski *et al*., 2022, Uba *et al*., 2022). Thus, the effects of *M. communis* extracts were tested on cholinesterase, α-amylase inhibition, α-glucosidase, and tyrosinase inhibitory effect. As demonstrated in Table 2, *M. communis* MeOH extract showed the highest AChE inhibition with 4.38 mg GALAE/g. In BChE assay, only MeOH extract (1.58 mg GALAE/g) displayed the activity against BChE. The α-amylase inhibition results showed that MeOH extract had significant higher activity than %50- MeOH and water extract. None of the extracts showed inhibition effect against α-glucosidase enzyme. The order of tyrosinase enzyme inhibition effect of extracts was as follows: MeOH (132.20 mg KAE/g) > 50% MeOH (124.94 mg KAE/g) > water (71.84 mgKAE/g). Tumen *et al*. (2021) studied the inhibition effect of *M. communis* leaves and fruits on different enzymes (AChE, BChE, and tyrosinase) and reported that leaves extracts showed no activity against BChE. Ibrahim *et al*. (2021) investigated the α-amylase inhibitory effect of Egyptian *M. communis* essential oil and reported inhibitory activity. Similarly, α-amylase inhibition activity of *M. communis* essential oil (IC_{50} 29.94 µg/ml) was determined by Sen *et al*. (2020). In another study, *M. communis* leaves essential oil showed AChE inhibitory activity (IC_{50} µg/ml).

The results of the correlation analysis indicated a highly positive correlation between TPC and FRAP and PBD assays (0.95, 0.99, respectively). A weak positive correlation was noted

for the total phenolic content and metal chelating ability. For enzyme inhibition assays, total flavonoid content was strong and positively associated with AChE, BChE, tyrosinase, and amylase assays (Figure 3).

Table 2. Enzyme inhibitory activity of *M. communis* leaves

Assays	*Myrtus communis* -MeOH	*Myrtus communis* -50% MeOH	*Myrtus communis* -Water
AChE (mgGALAE/g)**	4.38±0.18[a*]	3.44±0.28[b]	2.73±0.07[c]
BChE (mgGALAE/g)**	1.58±0.15	nd	nd
Amylase (mmolACE/g)***	0.56±0.02[a]	0.21±0.02[b]	0.10±0.01[c]
Glucosidase (mmolACE/g)***	nd	nd	nd
Tyrosinase (mgKAE/g)****	132.20±0.77[a]	124.94±0.67[b]	71.84±1.27[c]

*Values expressed are means±SD **GALAE:galanthamine equivalets; ***ACE: acarbose equivalents; ****KAE:kojic acid equivalents; nd: not determined. Different letters in same row indicate significant differences in the studied extracts (p<0.05).

4. CONCLUSION

This study focuses on the total bioactive compounds, antioxidant and enzyme inhibition properties of *M. communis* leaves extracts. 50 %-MeOH (145.22 mgGAE/g) and water extracts (144.45 mgGAE/g) showed similar amounts of total phenolic content. The highest amounts of total flavonoid were found in the MeOH extract. The highest antioxidant property was determined for the water extract. (DPPH: 3.36 mmolTE/g, ABTS: 3.38 mmolTE/g, CUPRAC: 4.58 mmolTE/g, FRAP: 2.75 mmolTE/g, phosphomolybdenum: 4.52 mmolTE/g, metal chelating: 45.60 mgEDTA/g). All the extracts tested showed different level of inhibition against the AChE, α-amylase, and tyrosinase enzymes. The highest activity against AChE, α-amylase, and tyrosinase was obtained with MeOH extract, followed by 50 % MeOH, and water. *In vitro* antidiabetic activity (α-amylase and α-glucosidase inhibition assays) of *M. communis* leaves was evaluated for the first time. Overall, *M. communis* leaves extracts could be regarded a possible natural source in different industries including food and pharmaceutical.

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

Sengul Uysal, Gokhan Zengin: Research concept and desing; Collection and/or assembly of data; **Sengul Uysal, Kouadio Ibrahime Sinan** and **Gokhan Zengin:** Data analysis and interpretation; Writing the article; Critical revision of the article, Final approval of the article.

Orcid

Sengul Uysal https://orcid.org/0000-0003-4562-1719
Kouadio Ibrahime Sinan https://orcid.org/0000-0002-6168-5707
Gokhan Zengin https://orcid.org/0000-0001-6548-7823

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Research Article

Antibacterial compound of *Bacillus Amyloliquefaciens* and *Bacillus Siamensis*: screening, characterization, and evaluation

Rajendrabhai Vasait[1,*], **Shital Bhamare**[1], **Sayali Jamdhade**[1], **Yogita Savkar**[1]

[1]Department of Microbiology, KAANMS ASC College, Satana Maharashtra, India 423301.

Abstract: This study was aimed at isolating potential antimicrobial compound (AMC) producing bacteria. AMC produced by a *Bacillus* species was evaluated further for its antimicrobial potential. Antimicrobial compound-producing bacteria were isolated from the soil of crop fields from the local region of Satana, Nashik (India), and tested against clinical isolates. Both isolates exhibited remarkable antibacterial potential against Gram-positive and Gram-negative clinical isolates. The AMCs of both SYS 1 and SYS 2 exhibited excellent antibacterial activity against *Salmonella paratyphi B* and *Staphylococcus aureus*. Both AMC-producing isolates were characterized and identified. *Bacillus* species SYS 1 and SYS 2 were identified as *Bacillus amyloliquefaciens* SYS 1 and *Bacillus siamensis* SYS 2, respectively. The highest antimicrobial activity of AMC produced by *Bacillus amyloliquefaciens* SYS 1 was exhibited against *Salmonella paratyphi B* (28 mm), followed by *Staphylococcus aureus* (26 mm). *Bacillus siamensis* SYS 2 AMC extracted by the solvent ethyl acetate exerted the highest antimicrobial activity against *Salmonella paratyphi B* (18 mm), followed by *Staphylococcus aureus* (16 mm). A partial characterization of the AMC was conducted and evaluated to contain amino acids and proteins. A higher total protein content of 17.9 µg/mL was estimated in the partially purified AMC of *Bacillus amyloliquefaciens* SYS 1. A detailed evaluation of the structural characteristics of AMC could prove its importance in commercial applications.

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1. INTRODUCTION

The problem of resistance against the present antibiotics in bacteria is increasing day by day (Elmaidomy, 2022). It is an alarming scenario that drug resistance is developing among the pathogenic microbes, and it is important to find effective alternative metabolites and products to overcome it (Peláez, 2003; Huan, 2020). Several studies have reported antimicrobial compounds such as antimicrobial peptides (AMPs) and bacteriocins as potential solutions to the problem of antibiotic resistance (Raheem & Straus, 2019; Moravej *et al.*, 2018; Rotem & Mor, 2009). AMPs (antimicrobial peptides) are considered potential alternatives against such resistant microbial pathogens (Rotem & Mor, 2009). Nearly 2000 varieties of AMPs from living

*CONTACT: Rajendra Vasait rd73vas@rediffmail.com Department of Microbiology, KAANMS ASC College, Satana Maharashtra, India 423301.

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organisms have been evaluated (Jenssen *et al.*, 2006; Sang & Blecha, 2008; Donadio *et al.*, 2010; Brogden & Brogden, 2011; Sumi *et al*., 2015), because AMPs are naturally occurring in humans, they serve as effective defences (Huan, 2020). Bacteriocins are ribosomally synthesized antimicrobials, short polypeptides synthesized by a number of different bacteria (Riley & Wertz, 2020; Cherif *et al.,* 2003).

The genus *Bacillus* was evaluated for its ability to produce a range of AMPs. It has considerable weightage for developing new antimicrobial compounds (Bizani *et al.,* 2005; Xie *et al.,* 2009). Many studies have found that AMPs from various *Bacillus* species are effective antimicrobial compounds (Abriouel *et al.*, 2011). *Bacillus* species produce a large number of antimicrobial compounds such as bacteriocins, polyketides, and surfactins (Wang *et al.*, 2014; Cladera-Olivera *et al.,* 2004). Application of surfactins has evolved in food, pharmaceuticals, and cosmetics as a surfactant and emulsifier (Moldes *et al.*, 2021). Bacteriocin-like substances produced by *Bacillus* species were found to be antimicrobials (Wang *et al.*, 2015). These consist of subtilin from *B. subtilis;* coagulin from *Bacillus coagulans*; bacthuricin F4, thuricin 17, entomocin 9, and tochicin from *Bacillus thuringiensis*; cerecin 7 from *Bacillus cereus*; bacillocin 490 from *B. licheniformis* (Cherif *et al.,* 2003; Aunpad & Na-Bangchang 2007; Hammami *et al.*, 2013; Stoica *et al*., 2019 & Sumi *et al*., 2015). *B. subtilis* has been reported to produce gageostatin and difficidin macrolides, that inhibit the growth of many Gram-negative bacteria (Kaspar *et al.*, 2019; Geraldi *et al.*, 2022). Lipopeptides produced by *Bacillus* are considered biosurfactants due to their biodegradability and minimal toxicity to humans, animals, and plants (Makkar & Cameotra, 2002; Jeyakumar & Zhang, 2022). *Bacillus* species is an important bacterium commonly found in soil that produces antimicrobial compounds, etc. In the present study, two *Bacillus* strains were isolated from the soil of different crop fields, and the characterization of an antimicrobial compound produced by them was conducted. The AMC produced by the isolates was evaluated against clinical isolates for antimicrobial potential.

2. MATERIALS and METHODS

2.1. Screening of Antimicrobial Compound Producing Organism

A total of 20 soil samples were collected from the rhizosphere of different crops (pomegranate, grape, sugarcane, maize, onion, etc.) in Satana, Nashik. All samples were brought to the laboratory and preserved at low temperature. During isolation, a few plates contained zones of inhibition around a few bacterial colonies in crowded growth. These organisms that produce antimicrobial compounds (AMC) were primarily screened out. From ten isolates, two potential AMC-producing organisms (exhibiting greater zones of inhibition) were further screened out for study.

2.2. Testing of Isolates Against Pathogens by Agar Diffusion Assay

The agar diffusion method has been used for the evaluation of the antibacterial potency of isolates (Piddock, 1990). In this test, an antimicrobial compound is impregnated on paper discs used for assaying (Parish & Davidson, 1993). The disc diffusion assay was used to assess each isolate's antibacterial potential against clinical pathogens. Both AMC producing isolates, SYS 1 and SYS 2, were tested against pathogens such as *E. coli, Staphylococcus aureus, Salmonella paratyphi B,* and *Pseudomonas aeruginosa.* Both AMC producing organisms, SYS 1 and SYS 2, were inoculated in nutrient broth and incubated at 37 °C for 24 hours. After incubation, the broth was centrifuged (5000 rpm for 15 min). The supernatant was collected in sterile tubes and used as crude AMC preparation for the diffusion assay. Mueller Hinton agar containing beef extract: 2.0 g/L, acid hydrolysate of casein: 17.5 g/L, starch: 1.5 g/L, agar-agar: 17.0 g/L, and pH: 7.3±0.1 was used as a basal and seed medium. Sterile basal agar plates and seed agar butts were prepared. After the pouring and solidification of seed agar, sterile filter paper discs were impregnated with the respective crude AMC preparations and placed on the surface of the

medium in each plate. Then all plates were incubated at 37 °C for 24 h. After incubation, the diameters of the zones of inhibition were recorded.

2.3. Identification of Isolates

Both AMC producing organisms were characterized by morphological and biochemical characteristics. Biochemical characterization was conducted according to Bergey's Manual of Determinative Bacteriology (Bergey & Holt, 2000).

2.4. Molecular Characterization of AMC Producing Organisms

DNA was isolated from both bacterial samples using Biopure™ kits for bacterial genomic DNA isolation. The 16S rRNA gene was amplified by PCR from the above-mentioned isolated DNA by using primers such as forward AGAGTTTGATCCTGGCTCAG and reverse TACGGTTACCTTGTTACGACTT. PCR conditions were maintained such that the first cycle was conducted at 94°C for 5 min for initial denaturing, and for 35 cycles conducted, denaturation of DNA was carried out at 94°C for 60 seconds, and 53 °C was maintained for 45 sec for annealing, as well as 68°C for 90 seconds for one extension. The final extension was conducted for one cycle at 68°C for 10 min. Amplified PCR product was subjected to electrophoresis using Agarose gel at 1% along with a 1 kb marker in TAE buffer and visualized by staining with ethidium bromide. The PCR product was purified by washing with sodium acetate and 70% ethanol and eluted from the gel. Forward and reverse sequencing reactions of PCR amplicons were carried out on an ABI 3730XL sequencer to obtain the sequence. The sequences of 16S rRNA were obtained. The assembled DNA sequence was used to carry out BLAST searches with the nr database of the NCBI. The sequences were aligned with NCBI blast sequences, and a phylogenetic tree was constructed. For the identification of both isolates, phylogenetic analysis was conducted.

2.5. Production of Antimicrobial Compound (AMC)

The production of AMC was carried out in a 1000 mL flask containing 500 mL liquid medium. The minimal medium used consists of g/L of glucose (15.00), peptone (5.00), KH_2PO_4 (1.00), $MgSO_4$ (0.1), FeSO4 (0.1), and NaCl (4.00). The medium's pH was adjusted to 7±0.02. After sterilization, the production medium was inoculated with AMC-producing organisms, *Bacillus amyloliquefaciens* SYS 1 and *Bacillus siamensis* SYS 2, in their respective flasks. The flasks were incubated at room temperature for 48 h. The broth was centrifuged, and the supernatant was collected for further study.

2.6. Salt Precipitation of AMC

It was characterised as antimicrobial peptides (AMPs) or bacteriocin like substances, admired potential antimicrobial compounds (Rotem and Mor, 2009; Pálffy *et al.*, 2009; Cotter, 2013). The *Bacillus* species have ability to synthesise a range of antimicrobials (Xie *et al.*, 2009; Bizani *et al.*, 2005). After an extensive review of reports, it was noticed that the nature of the antimicrobial compounds produced by various *Bacillus* species belongs to proteinic metabolites that may be antimicrobial peptides (AMPs). The extraction of desirable antimicrobial substances was conducted by salt and organic solvent precipitation. Salt precipitation of the AMC was carried out using the method described by Jayaraman (2007). The culture broth was centrifuged (5000 rpm for 20 min) to remove the cells. The cell free broth was subjected to salt precipitation. Solid ammonium sulphate was added slowly to the culture supernatant to reach 50 %, 60 %, and 70 % saturation and stirred for 24 h at 4 °C. The precipitate was harvested by centrifugation at 5000 rpm for 15 minutes. The precipitate was then dissolved in a 0.07 M sodium acetate buffer and dialyzed overnight at 4 °C against the same buffer. The purified residue was used for further determination of antibacterial activity and preliminary characterization (Gholam *et al.*, 2014).

2.7. Solvent Extraction

For the extraction of an antimicrobial compound, the culture medium was centrifuged at 5000 rpm for 20 minutes. The solvent precipitation of AMC was performed according to Jayaraman (2007). The cell free supernatant was mixed with organic solvents such as ethyl acetate and butanol at a ratio of 1:1 v/v. The mixture was then vigorously shaken and allowed to separate in a separating funnel, resulting in two distinct layers: the organic upper layer and the aqueous lower layer. The organic layer was separated and collected in a clean, sterile flask. Then the organic phase was subjected to evaporation in a rotary vacuum evaporator. The extracts in dry form were collected in clean, sterile containers and preserved at a low temperature in a refrigerator. These purified extracts were used for testing antimicrobial activity by the agar diffusion method.

2.8. Testing of Isolates for Antimicrobial Activity Against Pathogenic Test Organisms

The clinical isolates *E. coli, Salmonella paratyphi B, Staphylococcus aureus,* and *Pseudomonas aeruginosa* were used as test organisms in a preliminary diffusion assay to check antimicrobial potential.

2.9. Preliminary Characterization of AMC

2.9.1. *Thin layer chromatography*

The antimicrobial compounds of the two isolates were analysed by silica gel TLC. The thin layer chromatography was performed using TLC plates (Merck Silica Gel 60 F_{254}). Using a capillary tube, a row of spots of the partially purified antimicrobial compound obtained after salt and solvent extraction and known amino acids was applied along with a line 1.5 cm above the bottom of TLC plates, and the spots were allowed to dry. The TLC plate was placed vertically in a trough (the TLC solvent chamber) containing the solvents n-butanol: methanol: water (3:1:1). Solvents are allowed to travel to 80 percent of the chromatogram, and then the plates were dried in a hot air oven at 120°C for 10 min. Chromatograms were sprayed with ninhydrin solution, dried similarly as mentioned above, and observed for the presence of spots.

2.10. Qualitative Analysis of the Functional Groups of the Antimicrobial Compounds

The detection of amino acids and proteins was performed according to Jayraman (2007). For the detection of amino acids and proteins, the Ninhydrin and Folin-Lowry's tests were performed. According to Jayraman (2007), further quantitative estimation of total proteins was conducted using Folin-Lowry's method. It is a widely used and highly sensitive method for the estimation of proteins. For the total protein content estimation, bovine serum albumin was used as a standard protein. The range of concentrations of bovine serum albumin was prepared from 10 to 100 µg/mL. The reaction was conducted by mixing known concentrations of bovine serum albumin with 5 ml of freshly prepared 2% sodium carbonate in 0.1 N sodium hydroxide and 0.5% copper sulphate ($CuSO_4.5H_2O$) in 1% potassium sodium tartrate in a ratio of 50:1. Then 0.5 ml of diluted, commercially available Folin-Ciocalteau reagent was added to the mixture and incubated for 30 min. in the dark at room temperature. After reaction, the absorbance was measured at 660 nm against a blank (without bovine serum albumin). The standard curve was constructed by plotting the concentration of bovine serum albumin vs. absorbance. The amount of proteins in samples was determined using a standard curve.

3. RESULTS

3.1. Screening and Isolation of AMC Producing Organisms

Using the crowded plate technique, AMC producing organisms were screened out on the basis of the zones of inhibition exhibited from collected soil samples. During the screening, ten potential AMC producing organisms were screened out. All the isolates were bacterial and exhibited higher zones of inhibition. A preliminary disc diffusion assay was conducted to test the AMC producing isolates against clinical pathogens. Among the ten isolates, two organisms showed higher zones of antibacterial activity against all test organisms. Hence isolates designated as SYS 1 and SYS 2 which were screened out and used for further investigations.

3.2. Characterization of AMC Producing Isolates

The morphological characters detected are depicted in Table 1. The isolate SYS 1 produced mucoid, white colonies, while SYS 2 produced off-white, semi-mucoid colonies. Both isolates SYS 1 and SYS 2 were detected to have the ability to produce enzymes such as amylase, oxidase, and catalase while being unable to produce nitrate reductase. The isolates SYS 1 and SYS 2 were also tested, and they grew in the presence of a higher percentage of salt concentration. Both SYS 1 and SYS 2 isolates were tested for their ability to ferment various sugars and use different sugars as carbon sources. The results of biochemical characterization are depicted in Table 2. Morphological and biochemical characteristics of both isolated organisms suggested that isolated organisms belong to genus *Bacillus.*

Table 1. Morphological characters of the isolates SYS 1 and SYS 2.

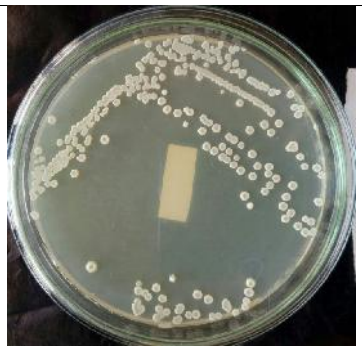
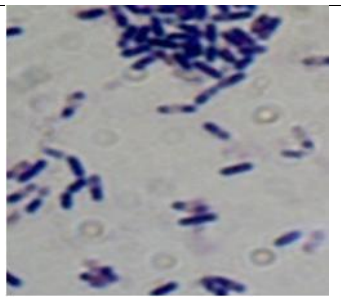
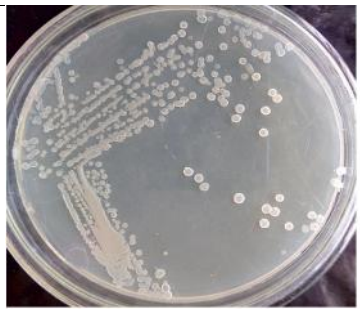
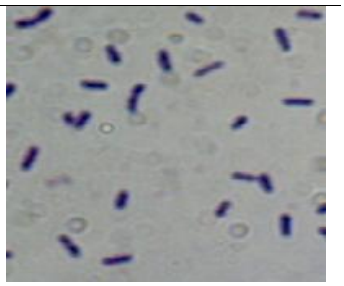
Isolate	Colony Characters		Microscopic Observation (1000x magnified image)	
SYS1	Size-5mm, colour- White circular colonies with entire margined, convex with sticky consistency		Rod shaped, Gram-positive, Motile	
SYS2	Size-4mm, colour- White circular colonies with entire margined, convex with sticky consistency		Rod shaped, Gram-positive, Motile	

Table 2. Biochemical characters of the isolates SYS 1 and SYS 2.

Biochemical Character	Isolate SYS1		Isolate SYS2	
	Sugar Fermentation			
	Acid	Gas	Acid	Gas
Arabinose	-	-	-	-
Mannitol	-	-	-	-
Glucose	+	-	+	-
Sucrose	-	-	-	-
Lactose	-	-	-	-
	Sugar Utilization			
Arabinose	+		+	
Mannitol	+		+	
Glucose	+		+	
Sucrose	+		+	
Lactose	-		-	
	Enzyme production			
Amylase	+		+	
Oxidase	+		+	
Catalase	+		+	
Nitrate reductase	-		-	
Growth in 6.5 % NaCl	+		+	

(+ Positive test; - Negative test)

3.3. Testing of Isolates for Antimicrobial Activity Against Pathogenic Test Organisms

The diameters of the zone of inhibition were measured and recorded, which are summarized in Table 3. Cell-free crude extracts of broth from both isolates (SYS 1 and SYS 2) were prepared. Both isolated organisms were found inhibitory against all pathogenic test organisms. The crude extract SYS 1 inhibited the growth of *E. coli* and *Salmonella paratyphi B* moderately. During the preliminary testing, it was also attempted to determine the inhibitory potential of cell-free crude extracts of broth from both isolates by double dilution (1:1) with sterile distilled water; however, zones of inhibition were unmeasurable against some test organisms.

Table 3. Antimicrobial activity exhibited by AMC of the isolates SYS 1 and SYS 2.

Sr. No.	Test Organism	Diameter of Zone of Inhibition (in mm)	
		SYS 1	SYS 2
1	*E. coli*	11	10
2	*Salmonella paratyphi B*	13	11
3	*Staphylococcus aureus*	6	8
4	*Pseudomonas aeruginosa*	8	9

3.4. Identification of Isolates

Amplified PCR product was subjected to electrophoresis using Agarose gel 1% along with a 1 kb marker in TAE buffer and visualized by staining with ethidium bromide (Figure 1). The assembled DNA sequence was used to carry out BLAST with the NCBI nucleotide database, and a phylogenetic tree was constructed. From the blast analysis and phylogenetic studies, isolated organisms SYS 1 and SYS 2 were identified as members of the genus *Bacillus* (Figure 2a and Figure 2b). The sequence obtained for SYS 1 was 99% identical to the partial gene sequence of 16S rRNA of *Bacillus amyloliquefaciens* strain C14, and the sequence obtained for SYS 2 was 99% identical to the partial gene sequence of 16S rRNA of *Bacillus siamensis* strain *KCTC13613*. The organisms SYS 1 and SYS 2 were identified as *Bacillus amyloliquefaciens* and SYS 2 as *Bacillus siamensis* SYS 1, respectively, with 99% similarities.

Figure 1. Gel picture of the amplicons.

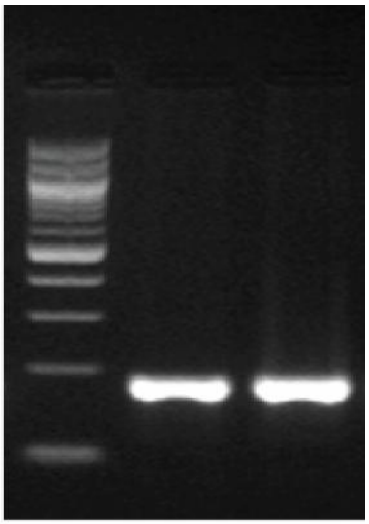


Figure 2a. Phylogenetic tree of SYS 1.

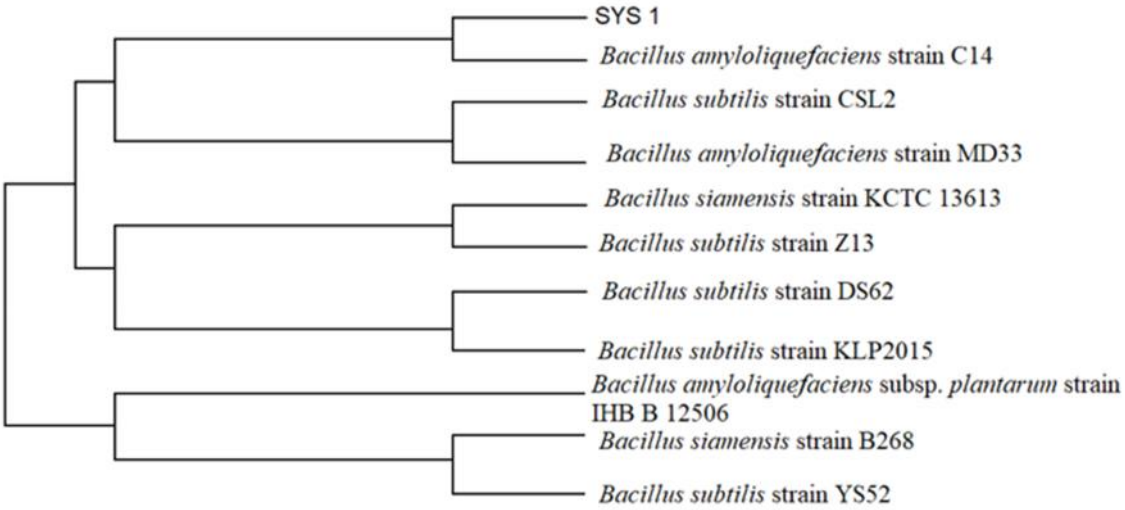
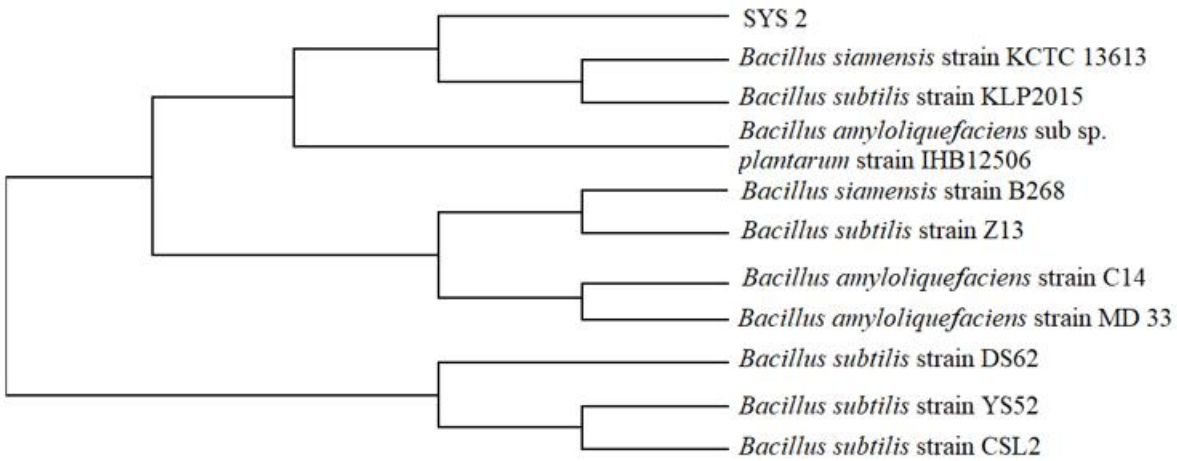


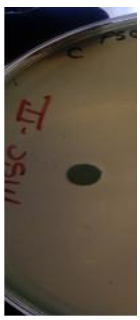
Figure 2b. Phylogenetic tree of SYS 2.

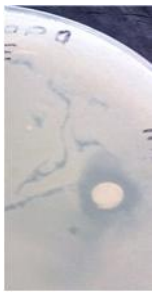


3.5. Evaluation of Antimicrobial Activity of Purified Antimicrobial Substances by *Bacillus amyloliquefaciens* SYS 1 and *Bacillus siamensis* SYS 2

The antimicrobial activity of the purified substance obtained after salt and solvent extraction was evaluated using an agar diffusion assay. Both isolates exhibited higher diameters of zones of inhibition against test organisms as compared to the crude antimicrobial compound used in the earlier assay. The diameters of the zones of inhibition obtained against test organisms are summarised in Table 4. Test organisms, *Salmonella paratyphi B* and *Staphylococcus aureus* were found highly sensitive to the antimicrobial substance produced by both isolates. The isolate, namely *Bacillus amyloliquefaciens* SYS 1 had the highest antimicrobial activity of AMC extracted by solvent ethyl acetate against *Salmonella paratyphi B*, followed by *Staphylococcus aureus*. The isolate, namely *Bacillus siamensis* SYS 2 had the highest antimicrobial activity of AMC extracted by solvent ethyl acetate against *Staphylococcus aureus*, followed by *Salmonella paratyphi B*. The butanol extract of *Bacillus amyloliquefaciens* SYS 1 exhibited maximum antibacterial activity against *Salmonella paratyphi B*, followed by *Pseudomonas aeruginosa*. The butanol extract of *Bacillus siamensis* SYS 2 species exhibited maximum antibacterial activity against *Salmonella paratyphi B*, followed by *E. coli*. The isolate, namely *Bacillus siamensis* SYS 2 (salt purified sample), had maximum antimicrobial activity against *Salmonella paratyphi B*, followed by *Staphylococcus aureus*. AMC produced by the isolate *Bacillus amyloliquefaciens* SYS 1 was detected with high antibacterial potential against *Salmonella paratyphi B* and *Staphylococcus aureus* as compared to the isolate *Bacillus siamensis* SYS 2. AMC produced by isolate *Bacillus siamensis* SYS 2 was found to produce potential antimicrobial activity against *Salmonella paratyphi B* and *Staphylococcus aureus.*

Table 4. Antimicrobial activity exhibited by AMC of the isolates SYS 1 and SYS 2.

Name of Isolate	Purified Sample of AMC	Test organisms / Diameter of Zone of Inhibition (in mm)							
		E. coli		*Staphylococcus aureus*		*Salmonella paratyphi B*		*Pseudomonas aeruginosa*	
Bacillus amyloliquefaciens	Salt Precipitation	8		8		10		6	
	Solvent Precipitation (Ethyl Acetate)	6		26		28		6	

	Solvent Precipitation (Butanol)	7		9		22		12	
Bacillus siamensis	Salt Precipitation	10		16		17		5	
	Solvent Precipitation (Ethyl Acetate)	8		16		18		6	
	Solvent Precipitation (Butanol)	12		8		14		10	

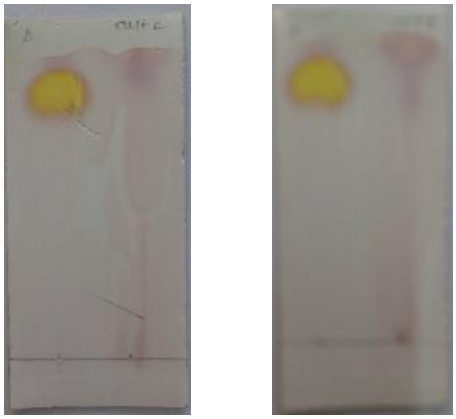
3.6. Preliminary Characterization of AMC Produced by *Bacillus amyloliquefaciens* SYS 1 and *Bacillus siamensis* SYS 2

Bacteriocins are antimicrobial peptides that inhibit or kill sensitive microorganisms (Vijayalakshmi *et al.*, 2011). *Bacillus* species are a rich source of antimicrobial peptides (Von, 1995). Hence, for the preliminary characterization of antimicrobial compounds produced by isolated organisms *Bacillus amyloliquefaciens* SYS 1 and *Bacillus siamensis* SYS 2, qualitative tests for the detection of amino acids and proteins were conducted.

3.6.1. *Thin layer chromatography* (TLC)

The characterization of antimicrobial compounds produced by the isolated organisms *Bacillus amyloliquefaciens* SYS 1 and *Bacillus siamensis* SYS 2 was conducted for the detection of amino acids. The partially purified antimicrobial compound obtained after salt and solvent extraction was analysed using pre-coated silica gel plates. The extracts were spotted on the chromatograms and evaluated as violet-coloured spots with similal Rf values for known amino acids (Figure 3). This indicated that the AMC may contain amino acids.

Figure 3. Chromatograms showing presence of amino acids.



SYS 1 SYS 2

3.6.2. *Qualitative analysis of proteins*

Folin-Lowry's and ninhydrin tests were performed for qualitative analysis of the presence of amino acids and proteins. In Folin-Lowry's and ninhydrin tests, the presence of proteins is indicated by the formation of blue and deep purple to brown colours, respectively. The results obtained are depicted in Table 5 and Figure 4a and Figure 4b.

Table 5. Tests results for detection of proteins.

Test	*Bacillus amyloliquefaciens* SYS 1 (Salt)	*Bacillus siamensis* SYS 2 (Salt)	*Bacillus amyloliquefaciens* SYS 1 (Ethyl acetate)	*Bacillus siamensis* SYS 2 (Ethyl acetate)	*Bacillus amyloliquefaciens* SYS 1 (Butanol)	*Bacillus siamensis* SYS 2 (Butanol)
Lowry Test	+	+	+	+	+	+
Ninhydrin Test	+	+	+	+	+	+

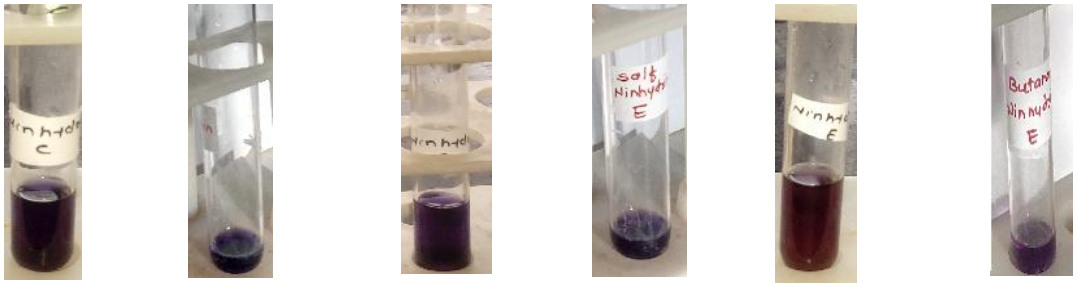
(+ Positive test; - Negative test)

Figure 4a. Detection of proteins in AMC of isolates detected by Folin-Lowry's Test.

Salt Extract | Solvent Extract (Ethyl acetate) | Solvent Extract (Butanol) | Salt Extract | Solvent Extract (Ethyl acetate) | Solvent Extract (Butanol)

Bacillus amyloliquefaciens SYS 1 *Bacillus siamensis* SYS 2

Figure 4b. Detection of proteins in AMC of isolates detected by Ninhydrin Test.



Salt Extract | Solvent Extract (Ethyl acetate) | Solvent Extract (Butanol) | Salt Extract | Solvent Extract (Ethyl acetate) | Solvent Extract (Butanol)

Bacillus amyloliquefaciens SYS 1 — *Bacillus siamensis* SYS 2

3.6.3. Estimation of Total Proteins

Using the Folin Lowrey's method the concentration of total proteins in AMC was determined. The standard curve was constructed by plotting concentration of bovine serum albumin v/s absorbance (Figure 5). The amount of proteins in samples was determined using standard curve. The concentration of total proteins determined in AMC produced by isolates is depicted in Table 6.

Figure 5. Standard curve for estimation of total proteins

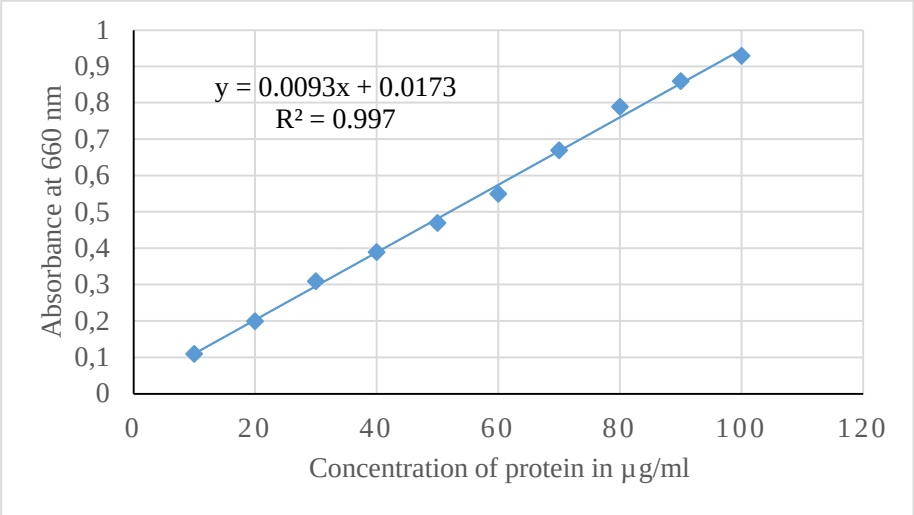


Table 6. Concentration of proteins determined in AMC produced by isolated organisms.

Sr. No.	Name of Isolate/ Sample	Concentration of Proteins (µg/ml)
1	*Bacillus amyloliquefaciens* SYS 1 Salt Extract (Ammonium sulphate)	7.7
2	*Bacillus siamensis* SYS 2 Salt Extract (Ammonium sulphate)	13.7
3	*Bacillus amyloliquefaciens* SYS 1 Solvent extraction (Ethyl acetate)	8.4
4	*Bacillus siamensis* SYS 2 Solvent extraction (Ethyl acetate)	17.9
5	*Bacillus amyloliquefaciens* SYS 1 Solvent extraction (Butanol)	14.7
6	*Bacillus siamensis* SYS 2 Solvent extraction (Butanol)	17.9

4. DISCUSSION

This study was attempted for the isolation and characterization of antimicrobial compound (AMC) producing bacteria from rhizosphere soil samples of different crops cultivated in the local region. Many members of *Bacillus* species have been reported to be capable of producing potential antimicrobial compounds (Caulier *et al.*, 2019). *Bacillus amyloliquefaciens* RO strains that inhibit different microhabitats to generate a range of antibiotics indicate the prospect of using certain strains as prospective biocontrol agents (Jeyakumar & Zhang, 2022*). Bacillus subtilis* produces subtilin and subtilosin (Stein, 2005; Pattnaik *et al.*, 2005), and *Bacillus cereus* produces cerein (Oscariz *et al.*, 1999), all of which have been shown to be effective against Gram-positive bacteria (Stoica *et al.*, 2019). Antimicrobial compound producing *Bacillus* strains was reported to exhibit inhibitory activity against *E. coli*, *P. aeruginosa*, *B. cereus*, and *S. aureus* (Geraldi *et al.*, 2022). *Bacillus megaterium L2* was reported to produce an antimicrobial compound, and an aqueous extract exerted potential antimicrobial activity against three plant pathogens (Xie *et al.*, 2021). *Bacillus* species-derived antimicrobial compounds have perspectives in sectors such as pharma, food, and agriculture (Wang *et al.*, 2014; Lin *et al.*, 2018). In the present investigation, two potential bacteria, *Bacillus amyloliquefaciens* SYS 1 and *Bacillus siamensis* SYS 2, were isolated and exhibited excellent antibacterial activity against clinical isolates, *Salmonella paratyphi B* and *Staphylococcus aureus. E. coli* and *Pseudomonas aeruginosa*, both clinical pathogens, were found to be sensitive to the AMC produced by both isolates. Initially, crude preparations of AMC from both isolated organisms showed moderate antibacterial activity. After partial purification using salt and organic solvent extraction of the AMC produced by both isolates, it exhibited admirable antibacterial activity. Purified bacteriocin from *B. subtilis GAS101* was reported to have strong antibacterial activity against *E. coli* and *S. epidermidis* (Sharma *et al.*, 2018). Preliminary characterization of AMC was conducted for the detection of amino acids and proteins, and partially purified AMC of both isolates contained amino acids and proteins. A higher 17.9 µg/mL of total protein content was estimated in the AMC of *Bacillus siamensis* SYS 2, which was extracted with the solvents-ethyl acetate and butanol.

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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

Rajendrabhai Vasait: designed the study and all authors carried out the sample collection and experiments, wrote the final version of the manuscript. **All authors:** Conducted the data analysis, read and approved the final version of the manuscript.

Orcid

Rajendrabhai Vasait https://orcid.org/0000-0001-7542-7461
Shital Bhamare https://orcid.org/0009-0005-0521-6331
Sayali Jamdhade https://orcid.org/0009-0005-9098-2056
Yogita Savkar https://orcid.org/0009-0005-5490-5635

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Review Article

Innovative methods for extraction of essential oils from medicinal plants

Yogesh Murti[1], **Divya Jain**[2], **Bhupesh Chander Semwal**[1], **Sonia Singh**[1], **Pracheta Janmeda**[2], **Pranav Bhaskar**[3,*]

[1]Institute of Pharmaceutical Research, GLA University, Mathura, Uttar Pradesh, India
[2]Department of Biosciences and Biotechnology, Banasthali University, Rajasthan, India
[3]Department of Biochemistry, Panjab University, Chandigarh, India

Abstract: Essential oils are concentrated liquids of intricate combinations of volatile substances, extracted from various plant parts. Several bioactive substances with antibacterial and antioxidant activities are abundant in essential oils. Some essential oils have also been employed in medicine. Due to the risk associated with employing synthetic preservatives, the use of essential oils as natural additives for extending the shelf life of food products has also drawn considerable attention. They are used in the pharmaceutical, cosmetic, and food industries for their functional properties. There are various methods for extraction, but both the quality and the percentage yield of essential oil never remain the same. So, innovative and non-conventional techniques of essential oils extraction from medicinal plants were evoled to get quantitative and qualitative yield. In the present article, we searched and reviewed innovative techniques used for the extraction of essential oils from medicinal and aromatic plants through electronic searches of PubMed, Medline, Wiley, Scopus, and Google Scholar. For the extraction of essential oils, several innovative/non-conventional techniques have been reported in literature. Extraction of essential oil by using innovative techniques retards the risk of losing the essential components of plants, maintains the quality, reduces chemical risk, extraction time, acts eco-friendly, and increases the percentage yield of the essential oils. This paper presents the success story of innovative extraction methods of essential oils in accordance with sustainable development and environmental protection.

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1. INTRODUCTION

Essential oils (volatile oils) are aromatic liquids that have been extracted from various plant parts, including leaves, flowers, seeds, and bark, when introduced to a solvent that dissolves volatile oil. They are good resources of numerous biologically active constituents which possess many pharmacological activities and have a significant role in the growth and development of aromatic herbs. Volatile oils confer fragrance to reproductive organs and fruits that attract pollination animals. To save plants, they resist phytophagous organisms, including viruses and phytoplasma vectors (Iriti & Faoro, 2009). They also contain different carbon- and hydrogen-

*CONTACT: Pranav Bhaskar pranavbhaskar@live.com Department of Biochemistry, Panjab University, Chandigarh, India.

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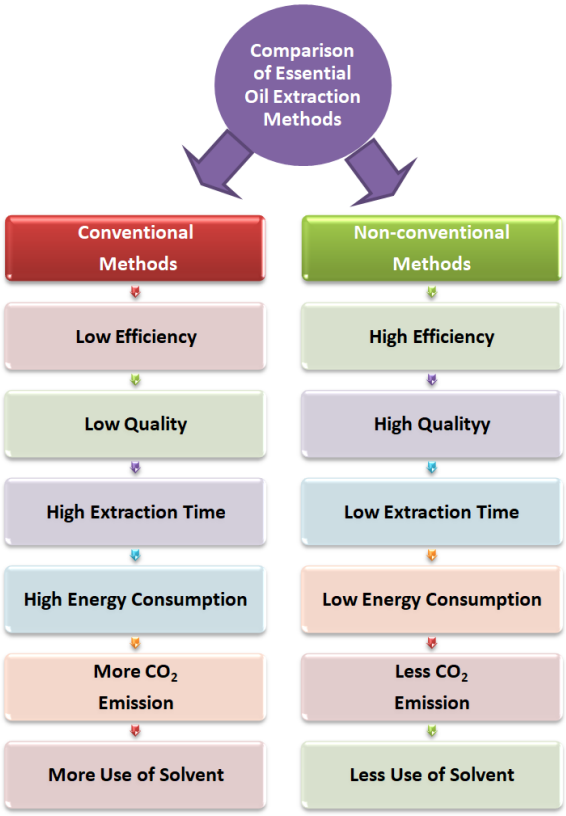
based compounds called terpene hydrocarbons. A basic hydrocarbon found in essential oils is isoprene. Mono-, sesqui-, and diterpenes are the result of combination of two, three and four isoprenes joined together respectively. The constituents of essential oils may be broadly classified as volatile and non-volatile fractions. Volatile oils are categorized into mono-/ sesquiterpene components and oxygenated derivatives along with esters, aliphatic aldehydes, and alcohols. The oxygenated derivatives of hydrocarbons are derived from isoprenoid pathways. These chemical moieties help determine the physicochemical properties, including solubility, nature, appearance, stereochemistry, and biological activities such as antimicrobial, antiviral, antioxidant, hepatoprotective, spasmolytic, analgesic, antidiabetic, and protect against cardiovascular diseases including atherosclerosis, thrombosis, and carminative (Edris, 2007; Reichling *et al.*, 2009). Many preclinical studies of essential oils have been published on various cell and animal models.

Figure 1. Comparison of conventional and non-conventional extraction methods of essential oils.



The conventional methods employed to extract volatile oils are being used worldwide owing to the fact that the essential oils are widely consumed. Selection of extraction method plays a critical role in the percentage yield of essential oils; it may affect the physiochemical properties as well. Some extraction methods are best suited to particular plant types and parts. Modern technologies have constantly been developed to overcome the limitation of traditional methods and enhance extraction efficacy, such as steam distillation, hydrodistillation, solvent extraction, maceration, carbon dioxide extraction, cold-press extraction, enfleurage, etc. However, innovations are required in conventional techniques to get the quantitative and qualitative yield of essential oils from medicinal plants. The comparison of conventional and non-conventional extraction methods of essential oils is shown in Figure 1.

2. INNOVATIVE TECHNIQUES

Innovative/ non-conventional techniques are "green" in concept. Various innovative extraction methods have been proposed (Figure 2) to overcome the limitations of traditional methods. These are less time-consuming, environmentally friendly, improve yields and quality of essential oils with less utilization of solvents and energy (Figure 3), and help avoid toxic chemicals. The conventional technology involves the transfer of heat energy via conduction and convection mechanisms which ultimately may cause variations in the temperature gradient within the product. Therefore, several novel heating techniques that contain more efficiency have been developed as summarized in Table 1 (Neetoo & Chen, 2014). Table 1 also entails the source, plant parts used, secondary metabolites of volatile oils, and their pharmaceutical importance.

Figure 2. Non-conventional and conventional extraction methods of essential oils.

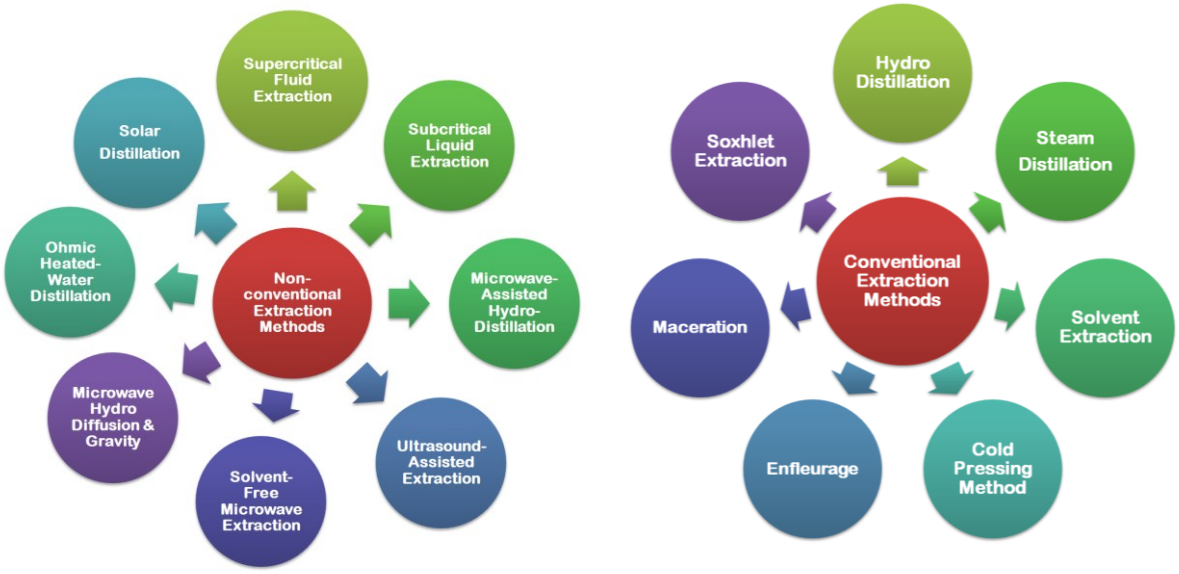
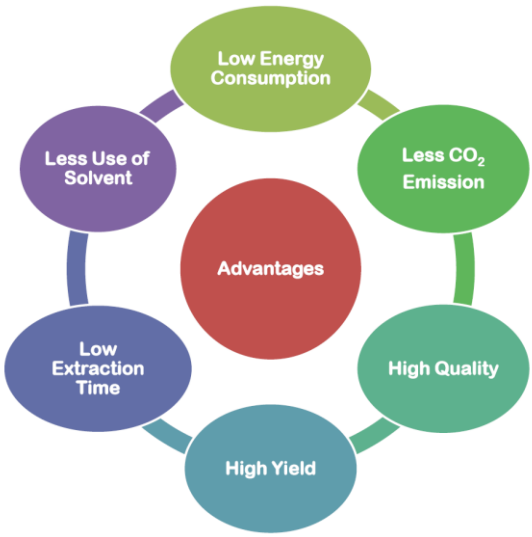


Figure 3. Advantages of innovative extraction techniques of essential oils.



2.1. Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is a sophisticated method of isolating one component from a combination utilizing a supercritical fluid as an extracting solvent composed of gases or liquids. Their dissolving power is controlled by temperature and/or pressure. However, it is employed to isolate those chemical classes of volatile oils, which cannot be advisable to extract through the steam distillation method. The schematic representation of SFE system is shown in Figure 4. The most commonly used solvent is carbon dioxide (CO_2) for several practical reasons like low critical pressure (73.8 bar) and temperature (31°C), nonflammable, noncorrosive, safe, cheap, and availability in high purity. Using the press release, it may be easily extracted from plant material (Rozzi *et al.*, 2002). One drawback is that CO_2 is nonpolar, so it cannot extract polar analytes (Pourmortazavi & Hajimirsadeghi, 2007) alone but can be used as a supercritical fluid with co-solvents such as dimethyl ether, ethane, ethylene ethanol, freons, methanol, nitrous oxide, propane, ethylene, etc. The SFE method of essential oils from various plants has been listed in Table 1. Additional benefits of this method include providing a high-quality range with advanced biological and functional properties compared to other products obtained (Cappuzzo *et al.*, 2013) by using the hydrodistillation method. This method is environmentally friendly because the nontoxic solvents used in the extraction process leave no harmful residue. The extracting solvent can be quickly recovered from the extract because of its high volatile nature. The components with high boiling points get easily extracted at low temperatures considered suitable for thermolabile components. The principal limitation of this method is the complexity of the system, which increases the cost of the equipment. Another disadvantage is that elevated pressure requires a more considerable upfront capital expenditure.

Figure 4. Schematic representation of the supercritical fluid extraction (SFE) system.

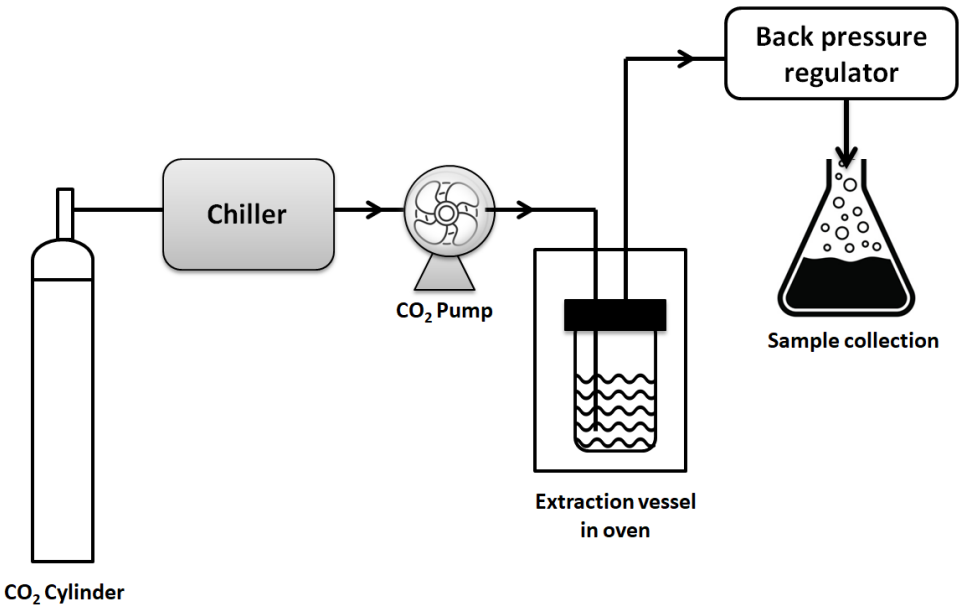


Table 1. Supercritical fluid extraction (SFE) of essential oils from various sources.

Sources (Part Used)	Common Name	Chemical Constituents	Pharmaceutical Application	References
Santolina chamaecyparissus L.; Asteraceae (Flower heads)	Cotton Lavender	1,8-Cineole, *β*-eudesmol, terpinen-4-ol, terpinolene, borneol and isobornyl acetate	Anticandidal, antibacterial and antifungal activity	(Grosso *et al.*, 2009A; Suresh *et al.*, 1997; Salah-Fatnassi *et al.*, 2017)
Mentha pulegium L.; Lamiaceae (Flowers and leaves)	Pennyroyal	*α* & *β*-Pinene, pulegone, menthol, piperitenone, isomenthol, menthone, isomenthone, neomenthol and neoisomenthol	Anti-cholinesterase, anti-Alzheimer and antioxidant activity	(Reis-Vasco *et al.*, 1999; Bektašević *et al.*, 2021)
Foeniculum vulgare Mill. subsp. *piperitum*; Umbelliferae (Flowers and unripe and ripe fruits)	Fennel	*α* & *β*-Pinene, myrcene, *p*-cymene, limonene, linalool, fenchone, estragol, camphor, *α*-phellandrene, (*E*)-anethole	Antifungal activity	(Coelho *et al.*, 2003; Ozcan *et al.*, 2006)
Coriandrum sativum L.; Apiaceae (Leaves and seeds)	Coriander	Linalool, *γ*-terpinene, *α*-pinene camphor, geranyl acetate, geraniol, and limonene	Antibacterial, antifungal, and antioxidant activity	(Grosso *et al.*, 2008; Mandal and Mandal, 2015)
Satureja montana L.; Lamiaceae (Leaves and flowers)	Winter Savory	Carvacrol, thymol, *p*-cymene, thymoquinone, *γ*-terpinene and *β*-bisabolene	Anti-cholinesterase, anti-Alzheimer, antioxidant, cytotoxic, antibacterial, and antidiarrhoeal activity	(Grosso *et al.*, 2009B; Silva *et al.*, 2009; Grosso *et al.*, 2009C; Miladi *et al.*, 2013; Skočibušić and Nada, 2008)
Satureja fruticosa Béguinot; Lamiaceae (Leaves and flowers)	Savory	Piperitenone, piperitenone oxide, pulegone and isomenthone	-	(Coelho *et al.*, 2007)
Thymus vulgaris L.; Lamiaceae (Aerial flowering parts)	Thyme	*γ*-Terpinene, linalool, thymol, *p*-cymene, and carvacrol	Antioxidant and antiviral, antiproliferative activity	(de Melo *et al.*, 2000; Grosso *et al.*, 2010; Catella *et al.*, 2021 Niksic *et al.*, 2021)
Origanum majorana L.; Lamiaceae (Leaves)	Marjoram	Terpinen-4-ol, *α* & *β*-pinene, *γ*-terpinene, camphene, *α*-terpineol, linalool, *α*-terpinene, *p*-cymol, *cis*-sabinene hydrate, spathulenol, *β*-caryophyllene,	Antimicrobial, antioxidant, anti-coagulant and antidepressant activity	(Reverchon, 1992; Va'gi *et al.*, 2005; Bağci1 *et al.*, 2017; Busatta *et al.*, 2008; Abbasi-Maleki *et al.*, 2019)

Zataria multiflora Boiss; Lamiaceae (Arial branches)	Shirazi thyme	Thymol, carvacrol, linalool, *λ*-terpinene and *p*-cymene	Antimicrobial, antioxidant and scolicidal activity	(Ebrahimzadeh *et al.*, 2003; Shafiee and Javidnia, 1997; Mahmoudvand *et al.*, 2017)
Eucalyptus loxophleba ssp. *Lissophloia;* Myrtaceae (Leaves)	*Eucalyptus*	1,8-Cineole, *α*-pinene, aromadendrene, trans-pinocarveol, and methyl amyl acetate	Antioxidant and antimicrobial activity	(Zhao and Zhang, 2014; Assareh *et al.*, 2007; Rahimi-Nasrabadi *et al.*, 2012; Aldoghaim *et al.*, 2018)
Lippia alba; Verbenaceae (Leaves & flowers)	Anise verbena	Carvone, limonene, elemol, γ-muurolene, guiaol, bulnesol and citral	Antimicrobial, antioxidant, and cytotoxic activity	(Reyes-Solano *et al.*, 2017; Stashenko *et al.*, 2004; Braga *et al.*, 2005; Lima Juiz *et al.*, 2015; Santos *et al.*, 2016; Saroj *et al.*, 2019)
Pimpinella anisum; Apiaceae (Fruits)	Aniseed	*γ* -Himachalene, trans-anethole, methylchavicol, 2-methylbutyrate, trans-pseudoisoeugenyl	Antimicrobial, antiviral, antioxidant, analgesic, muscle relaxant, antifungal, and anticonvulsant activity	(Rodrigues *et al.*, 2003; Gende *et al.*, 2009; Shojaii and Fard, 2012)
Artemisia sieberi Besser Asteraceae (Aerial parts)	Artemisa	Camphene, 1,8-cineole, *γ*-terpinene, camphor, chrysanthenone, *cis & trans*-thujone, and *cis*-chrysanthenone	Antioxidant, antimicrobial, and antifungal activity	(Ghasemi *et al.*, 2007; Ghasemi *et al.*, 2020; Mahboubi and Farzin, 2009; Aghajani *et al.*, 2014; Asgharpour and Honarvar, 2016)
Anacardium occidentale L.; Anacardiaceae (Leaves)	Cashew	Cardanol, *α*-copane, *β*-caryophyllene, cardol, *γ*-cadinene, germacrene B & D, dimethylanacardate	Antioxidant and antimicrobial activity	(Patel *et al.*, 2006; Janet *et al.*, 2015; Baptista *et al.*, 2018; Dzamic *et al.*, 2009)
Eugenia caryophyllata Thunb; Myrtaceae (Buds, leaves, and stems)	Clove	Eugenol, eugenol acetate, *trans*-caryophyllene	Antioxidant, antimicrobial and anti-inflammatory activity	(Sohlait, 2015; Wenqiang *et al.*, 2007; Ivanovic *et al.*, 2011; Mahboubi and Mahboubi, 2015; Sohilait *et al.*, 2018; Öztürk and Özbek, 2005).

2.2. Subcritical Liquid Extraction

SLE takes place when liquid reaches a pressure greater than Pc (critical pressure) but less than Tc (critical temperature). This condition is said to be in the subcritical stage. The schematic representation of the SLE system is shown in Figure 5. The solvents used to extract essential oils in this process are H_2O and CO_2. The fluid's subcritical condition has several advantages: decreased density, lower viscosity, and improved gas-liquid diffusivity. Because this extraction procedure is performed at a moderate working temperature, it is regarded as the best alternative strategy for thermolabile components. Compared to other traditional ways, this process takes 15 minutes to complete (Khmelinskii & Woodcock, 2020). The subcritical liquid extraction method of essential oils from various plants is listed in Table 2. The advantages of this method include their being simple, less time-consuming, cost-efficient, and environmentally friendly. It is a powerful alternative to the essential oils extraction technique as it enables a fast essential oil isolation process (Shirsat *et al.*, 2004). The major disadvantage of this extraction technique is that it requires high pressure to maintain the solvent in the subcritical state, which increases the operating costs.

Figure 5. Schematic representation of the subcritical liquid extraction (SLE) system.

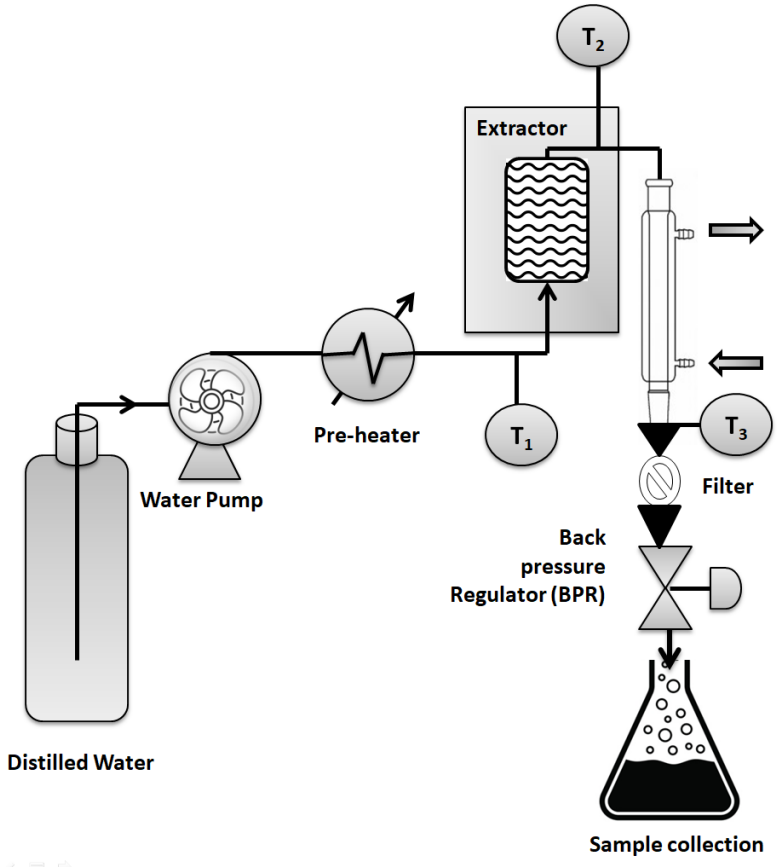


Table 2. The subcritical liquid extraction (SLE) of essential oils from various sources.

Sources (Part Used)	Common Name	Chemical Constituents	Pharmaceutical Application	References
Lavandula angustifolia Mill.; Lamiaceae (Flowers)	Lavender	*β*-Pinene, *β*-phellandrene, borneol, camphor, linalool, terpineol, linalyl acetate and 1,8-cineole	Antimicrobial, anti-inflammatory, and antioxidant activity	(Reverchon *et al.*, 1995; Zhi-linga *et al.*, 2011; Akgu *et al.*, 2000; Lakušić *et al.*, 2014; Danh *et al.*, 2012; Wells *et al.*, 2018)
Lavandula hybrida; Lamiaceae (Flowers)	Lavandin	1,8-Cineole, camphor, linalool, and linalyl acetate	Antinociceptive and gastroprotective activity	(Kamali *et al.*, 2015; Barocelli *et al.*, 2004)
Rosmarinus officinalis L.; Lamiaceae (Leaves)	Rosemary	1,8-Cineole, verbenone, α-pinene, borneol, camphor, *β*-caryophyllene	Antioxidant, antimicrobial, anti-Alzheimer, and hepatoprotective activity	(Khalili *et al.*, 2017; Nieto *et al.*, 2018; Habtemariam, 2016; Rašković *et al.*, 2014)
Zingiber officinale; Zingiberaceae (Rhizomes)	Ginger	*α*-Zingiberene, geraniol, neral, *α*-curcumene, (*Z*)-*α*-bisabolene, *β*-phellandrene and geranial	Anti-inflammatory antibacterial, antifungal, analgesic, anti-ulcer, immunomodulatory and relaxant activity	(Junior *et al.*, 2020; Mahboubi, 2019; Akinyemi and Adeniyi, 2018; Funk *et al.*, 2016)
Citrus sphaerocarpa Tanaka; Rutaceae (Peel)	Kabosu	*β*-Farnesen, auraptene, limonene and myrcene, decanal, nerol and neryl acetate	Antioxidant activity	(Suetsugu *et al.*, 2013; Minh *et al.*, 2002; Kamal *et al.*, 2013)
Chamaecyparis obtusa (Siebold & Zucc.) Endl.; Cupressaceae (Leaves)	Hinoki cypress	Bornyl acetate, elemol, *α*-pinene, 1-muurolol, (+)-limonene, and *α*-terpinyl acetate	Antibacterial, antifungal, anti-inflammatory, antioxidant, and also promote hair growth	(Jina *et al.*, 2010; Yang *et al.*, 2007; Suh *et al.*, 2016; Lee *et al.*, 2010)
Coriandrum sativum L. (Seeds)	Coriander	*γ*-Terpinene, terpin-4-ol, linalool, *α* & *β*-pinene, and borneol	Antioxidant and antimicrobial activity	Eikani *et al.*, 2007; Ghazanfari *et al.*, 2020)
Origanum majorana L.; Lamiaceae	Marjoram	Terpinen-4-ol, *γ*-terpinene, *α* & *β*-pinene, *p*-cymol, *α*-terpineol, *α*-	Antimicrobial, antioxidant,	(Busatta *et al.*, 2008; Abbasi-Maleki *et al.*, 2019; Jiménez-

(Leaves)		terpinolene, *α*-terpinene, camphene, *β*-caryophyllene, spathulenol and *cis*-sabinene hydrate	anti-coagulant and antidepressant activity	Carmona *et al.*, 1999; Va'gi *et al.*, 2005; Bağci1 *et al.*, 2017)
Zataria multiflora Boiss*;* Lamiaceae (Arial branches)	Shirazi thyme	Thymol, carvacrol, linalool, *λ*-terpinene and *p*-cymene	Antimicrobial, antioxidant and scolicidal activity	(Shafiee and Javidnia, 1997; Mahmoudvand *et al.*, 2017; Khajenoori *et al.*, 2009)
Laurus nobilis L.; Lauraceae (Leaves & fruits)	Bay	1.8-Cineole, sabinene, *β*-elemene, bornyl acetate, *α*-terpinyl acetate, *α* & *β*-phellandrene and trans-*β*-osimen	Antimicrobial, antifungal, and antioxidant activity	(Fernández-Pérez and Jiménez-Carmona, 2000; Sangun *et al.*, 2007; Fidan *et al.*, 2019; Caputo *et al.*, 2017; Mssillou *et al.*, 2020)
Aquilaria malaccensis Lamk.*;* Thymelaeaceae (Leaves)	Agar Wood	Agarospirol, guaiacol, cyclotene, mequinol, *γ*-himachalene, *α*-guaiene, *β*-agarofuran, *α*-bulnesene, kusunol, creosol, jinkoheremol, and oxoagarospirol	Antibacterial, antifungal, anti-inflammatory, analgesic, anti-diabetes, antioxidant and anticancer activity	(Samadi *et al.*, 2019; Samadi *et al.*, 2020; Tajuddin and Yusoff, 2010; Gunasekera *et al.*, 1981; Wang *et al.*, 2018)

2.3. Microwave-Assisted Hydrodistillation

This microwave-assisted hydrodistillation (MWHD) technique is used to heat the solvent in place of regular electric heating. This approach works by changing the polarity of water and then heating it with microwaves. Electromagnetic energy is converted into heat energy utilizing microwave energy directly generated through molecular interaction between aromatic plants (materials) and the electromagnetic field (Eskilsson & Björklund, 2000; Routray & Orsat, 2012; Thostenson & Chou, 1999). These waves may cause some structural alterations within the plant cells. The schematic representation of the MWHD system is shown in Figure 6. Heat and mass transfer occur in the same direction, *i.e.*, from the inner cells to the outside. The factors affecting the efficiency of this technique include time, temperature, physicochemical properties of the extracted compounds, dielectric properties of the sample mixture, and solvent type. The MWHD extraction method of essential oils from various plants has been reported in the literature (Table 3). This method is used in industry, and it provides an excellent versatile tool that covers a wide range of plant materials under suitable conditions. This method shows speedy extraction performance with low solvent consumption. It is an ecofriendly method due to the less CO_2 emission from the atmosphere (Lucchesi *et al.*, 2004; Moradi *et al.*, 2018; Ferhat *et al.*, 2006). This method offers protection from thermolabile compounds, and the efficiency is firmly based on the dielectric constant of plant material and water, respectively (Brachet *et al.*, 2002). There is only one disadvantage: the microwave technique can lead to changes in the stereochemistry of compounds and can convert them from one isomer to another (Fadel *et al.*, 2011; Norfatirah *et al.*, 2013; Jeyaratnam *et al.*, 2016).

Figure 6. Schematic representation of the microwave-assisted hydrodistillation (MWHD) system.

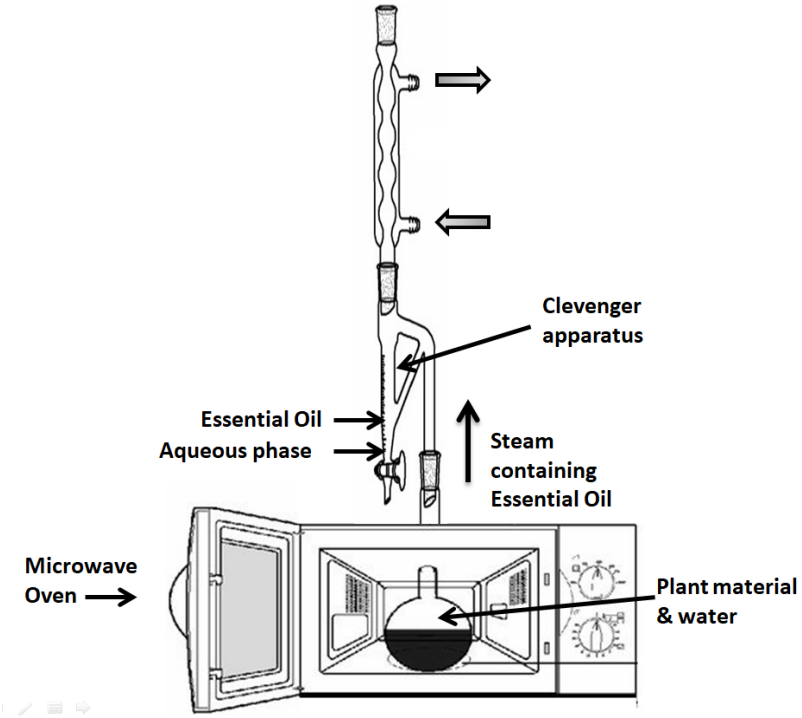


Table 3. The microwave-assisted hydrodistillation (MWHD) based extraction of essential oils from various sources.

Sources (Part Used)	Common Name	Chemical Constituents	Pharmaceutical Application	References
Rosmarinus officinalis L.; Lamiaceae (Leaves)	Rosemary	*α*-Pinene, *β*-myrcene, fenchol camphene, 1,8-cineole, linalool, camphor and borneol	Antioxidant, antimicrobial, anti-Alzheimer, and hepatoprotective activity	(Nieto *et al.*, 2018; Habtemariam, 2016; Rašković *et al.*, 2014; Moradi *et al.*, 2018; Elyemni *et al.*, 2019)
Coriandrum sativum L.; Apiaceae (Seeds)	Coriander	Linalool, *γ*-terpinene, terpin-4-ol, *α* & *β*-pinene and borneol	Antioxidant and antimicrobial activity	(Ghazanfari *et al.*, 2020)
Cinnamomum camphora L.; Lauraceae (Leaves)	Camphor tree	Sabinene, *β*-pinene, *β*-myrcene, *α* & *δ* -terpineol, 3-heptanone, 1,8-cineole, D-camphor, linalool, and *β*-thujene	Antimicrobial, anti-inflammatory, and antioxidant activity	Shang *et al.*, 2020; Lei *et al.*, 2020; Zhang *et al.*, 2019)
Pimpinella anisum; Apiaceae (Fruits)	Aniseed	*γ*-Himachalene, methylchavicol, trans-anethole, *cis* & *trans*-pseudoisoeugenyl and 2-methylbutyrate	Antimicrobial, antiviral, muscle relaxant, analgesic, antifungal, antioxidant and anticonvulsant activity	(Shojaii and Fard, 2012; Boumahdi *et al.*, 2021)
Anethum graveolens. L.; Apiaceae (Seeds)	Dill	Carvone, myristicin, *cis*-isodihydrocarvone, *cis* & *trans*-carveol, limonene, and dillapiole	Antioxidant and antimicrobial activity	(Kosar *et al.*, 2005; Ljiljana *et al.*, 2016)

2.4. Ultrasound-Assisted Extraction

The UAE method permits highly selective and escalation of volatile oils to get separated from plant material. The principle behind this method is that it develops cavitation of some tiny bubbles within the solvent system due to the passage flow of ultrasound waves which usually allow a more significant percentage of a solvent system within the plant material that enhances the surface area (Garcı´a-Pe´rez, 2006). Plant raw materials are immersed in water or another solvent (such as methanol or ethanol) and subjected to ultrasound (Figure 7) (Assami &Pingret, 2012). This technique involves extracting essential oil components from leaves, seeds, and flowers (Sereshti *et al.*, 2012). Factors that significantly affect the percentage yield and quality of the essential oils are ultrasonic frequency, duration of ultrasound treatment, immersion time, extraction temperature, the duty cycle of ultrasound, features, and size of plant materials (Sun *et al.*, 2019). The ultrasound-assisted extraction method of essential oils from various plants has been listed in Table 4. This technique is advantageous for heat-sensitive combinations due to mean temperature and saving energy. It is a suitable method of extraction to get high valuable volatile oils. The efficiency of extraction can be enhanced using this technique which may cause disruption of plant cell walls and improved mass transfer through the formation of cavitation babble effects (Entezari, 2004). The disadvantages of this method include poor purity, low efficiency, and lengthy process (Lu *et al.*, 2012; Mura *et al.*, 2015).

Figure 7. Schematic representation of the ultrasound-assisted extraction (UAE) system.

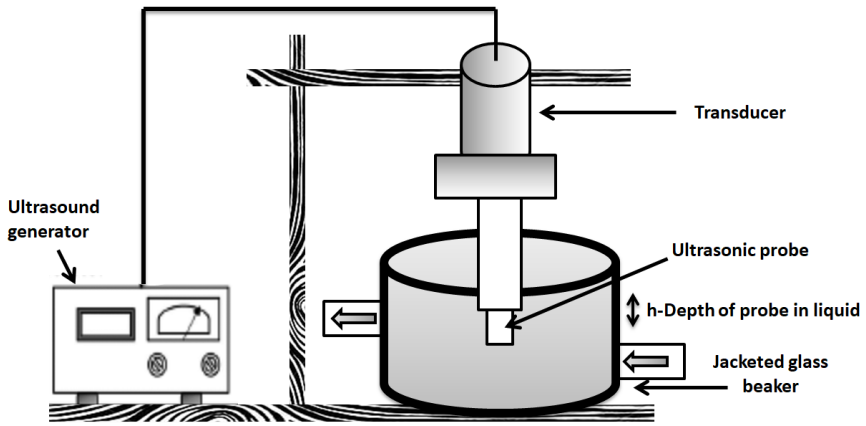


Table 4. Ultrasound-assisted extraction (UAE) of essential oils from various plant sources**.**

Sources (Part Used)	Common Name	Chemical Constituents	Pharmaceutical Application	References
Lavandula angustifolia and *L. latifolia;* Lamiaceae (Flowering stems)	Lavandin	Linalool, terpinen-4-ol, linalyl acetate, camphor, borneol, *β*-caryophyllene, lavandulyl acetate, and 1,8-cineole	Antimicrobial, antioxidant, carminative, sedative, neuroprotective, anti-depressive activity, and effective for burns and insect bites	(Périno-Issartier *et al.*, 2013; Lesage-Meessen *et al.*, 2015; Cavanagh and Wilkinson, 2002; Hancianu *et al.*, 2013)
Apium graveolens L.; Apiaceae (Seeds)	*Celery*	Limonene, *α* & *β*-selinene, sedanenolid and 3-butylphtalide	Antimicrobial and antioxidant activity	(Zorga *et al.*, 2020)
Trichoderma africanum L.; Boraginaceae (Leaves)	African barbell	Caryophyllene oxide, *γ*-eudesmol, elemol, *α*-muurolene, carvone, *β*-caryophyllene and *α*-pinene	Antimicrobial and antioxidant activity	(Jaradat *et al.*, 2016; Ahmed *et al.*, 2015)
Thymus vulgaris L.; Lamiaceae (leaves)	Thyme	Carvacrol, thymol, *p*-cymene and *γ*-terpinene	Antioxidant, antiproliferative and antiviral activity	(Grosso *et al.*, 2010; Catella *et al.*, 2021; Niksic *et al.*, 2021; Kowalski and Wawrzkowski; 2009)
Carum carvi L.; Apiaceae (Seeds)	Caraway	Carvone, limonene, *β*-myrcene, trans-carveole, γ-terpinene, trans-dihydrocarvone, *α*-pinene, sabinene, trans-*β*-ocimene, and linalool	Antibacterial, fungicidal, antioxidant, anti-acetylcholinesterase, antidiabetic and diuretic activity	(Assami *et al.*, 2012; Raal *et al.*, 2012; Begum *et al.*, 2008; Hajlaoui *et al.*, 2021)
Lavandula intermedia; Lamiaceae (Flowers)	Lavender	Linalool, cineole, camphor, 1,8- limonene, linalyl acetate and (*Z* & *E*)-*β*-ocimene	Antibacterial, antioxidant, and anti-inflammatory activity	(Ahmed *et al.*, 2015; Garzoli *et al.*, 2020; Tardugno *et al.*, 2019; Lane and Mahmoud, 2008; Wells *et al.*, 2018)
Elettaria cardamomum L. Maton; Zingiberaceae (Seeds)	Cardamom	*α*-Terpinyl acetate, *α* & *β*-pinene, *β*-myrcene and 1,8-cineole	Antimicrobial, antidiarrhoeal, and antispasmodic activity	(Morsy, 2015; Noumi *et al.*, 2018; Alam *et al.*, 2021

Origanum majorana L.; Lamiaceae (Leaves)	Sweet marjoram	Carvacrol, *o*-cymene, *α* & *β*-pinene, *β*-myrcene, *γ*-terpinene, limonene and linalool	Antimicrobial, antioxidant, anti-coagulant and antidepressant activity	(Bağcil *et al.*, 2017; Busatta *et al.*, 2008; Abbasi-Maleki *et al.*, 2019; Ebrahimzadeh *et al.*, 2003; Kowlski *et al.*, 2015)
Chamomilla recutita L.; Asteraceae (Flowers)	Chamomile	*α*-Bisabolol oxide A, *α*-bisabolol, *β*-bisabolene *α*-bisabolol oxide B, *cis*-enynebicycloether, bisabolol oxide A, chamazulene, spathulenol and (*E*)-*β*-farnesene	Anti-inflammatory, anticancer and gastroprotective activity	(Pino *et al.*, 2002; Orav *et al.*, 2010; Srivastava *et al.*, 2010)
Apium graveolens L.; Apiaceae (Seeds)	Celery	Limonene, *β*-selinene, sedanenolid, *α*-selinene, 3-butylphtalide	Antimicrobial and antioxidant activity	(Begum *et al.*, 2008; Dinç Zor *et al.*, 2017)
Allium sativum L.; Amaryllidaceae (Bulbs)	Garlic	Diallyl trisulfide, allyl methyl trisulfide, allyl methyl disulfide, allyl (*E*)-1-propenyl disulfide and diallyl sulfide	Antioxidant, anti-hypertensive, anticoagulant and anti-Alzheimer activity	(Boubechiche *et al.*, 2017; Satyal *et al.*, 2017; Hashemi *et al.*, 2019)
Mentha spicata; Lamiaceae (Leaves)	Spearmint	Limonene, carvone, menthol, carveol, isocaryophyllene, germacrene D and *β*-farnesene	Antibacterial, antidermatophytic, anticholinesterase and anti-Alzheimer activity	(Porto and Decorti, 2009; Ali-Shtayeh *et al.*, 2019)
Citrus limetta; Rutaceae (Peel)	Sweet lime	D-Limonene, bergamol, α & β-pinene, linalool, 1,8 cineole and α-terpineol	Antimicrobial, antioxidant, anti-inflammatory and anxiolytic activity	(Arafat *et al.*, 2020; Mahmud *et al.*, 2009)

2.5. Solvent-Free Microwave Extraction

Dry distillation and microwave heating energy in combination are used in this process (Chemat *et al.*, 2003; Chemat *et al.*, 2004). In this method, the moisture present in the plant material is used as a solvent (Lucchesi *et al.*, 2007). Before undergoing the extraction process, the herbal materials are moistened with water for approximately 2 hrs. The moistened materials are microwaved, and a condenser is used to collect the essential oils. The thermal stress and pressure generated within the plant tissues being treated in the case of microwave heating may cause their disruption more rapidly when compared with the traditional methods. The panel embedded within the instrument controlled the temperature, pressure, and irradiation power (Figure 8). The essential oil is dried in the desiccator and stored in a dark place. This method is used to quickly isolate volatile oils from herbs, spices, and seeds, as listed in Table 5. The main advantage of this method is the single-stage isolation and concentration of essential oil (Lucchesi *et al.*, 2004; Bayramoglu *et al.*, 2008). Other benefits of this green technique include efficiency, selectivity, and shorter time (Boubia *et al.*, 2009; Lopez-Avila *et al.*, 1994).

Figure 8. Schematic representation of the solvent-free microwave extraction (SFME) system.

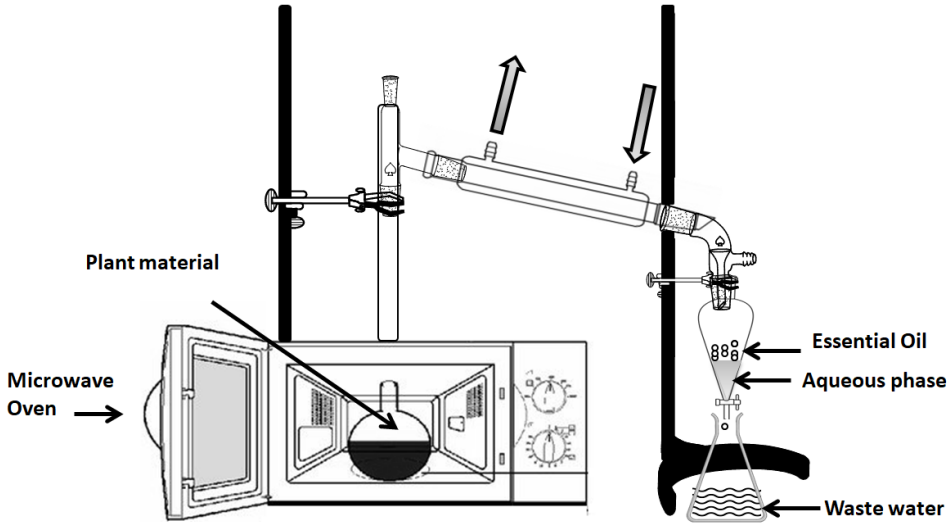


Table 5. Solvent-free microwave extraction (SFME) of essential oils from various sources.

Sources (Part Used)	Common Name	Chemical Constituents	Pharmaceutical Application	References
Ocimum basilicum L.; Lamiaceae (Leaves)	Egyptian sweet basil	Camphor, limonene, linalool, *β*-selinene, methyl chavicol and 1,8-cineole	Antioxidant and antimicrobial activity	(Chenni *et al.*, 2016)
Amomum tsaoko; Zingiberaceae (Seeds)	Chinese black cardamom	Geranial, eucalyptol, neral, Geraniol, (2*E*)-decenal and 4-indanecarbaldehyde	Antioxidant and antimicrobial activity	(Cui *et al.*, 2017; Sim *et al.*, 2019)
Foeniculum vulgare Mill.; Apiaceae (Seeds)	Fennel	*α* & *β*-Pinene, *α*-fenchene, 3-methylnonane, carvone, eucalyptol, camphor, *α*-terpinolene, *γ*-terpinene, and *cis*-anethole	Antioxidant and antimicrobial activity	(Benmoussa *et al.*, 2019; Khammassi *et al.*, 2018)
Cuminum cyminum L.; Apiaceae (Seeds)	Cumin	*α*-Thujene, *γ*-terpinene, geraniol, *α*-pinene, sabinene, (*E*)-ocimene, linalool, trans-carveole, *α*-terpinyl acetate, neryl acetate and *α*-campholenal	Antimicrobial, anti-vomiting, anti-asthma and anti-spasm activity	Wang *et al.*, 2006; Wannera *et al.*, 2010; Esmaeili, 2015)
Zanthoxylum bungeanum; Rutaceae (Fruit)	Sichuan pepper	Terpinen-4-ol, Linalool, limonene, 1,8-cineole, *γ*-terpinene, *α*-terpineol, and terpinyl acetate	Antibacterial, antifungal and anticancer activity	Wang *et al.*, 2006, Gong *et al.*, 2009; Lan *et al.*, 2014A; Lan *et al.*, 2014B; Zhu, 2011)
Melissa officinalis L.; Lamiaceae (Leaves)	Lemon balm	Geranial, neral, caryophyllene, *γ*-caryophyllene oxide, *μ*- pinene, citronella and sabinene	Anti-inflammatory, anticancer and antioxidant activity	Uysal *et al.*, 2010; Fernández *et al.*, 2020; Bounihi *et al.*, 2013; de Sousa *et al.*, 2004)
Laurus nobilis L.; Lauraceae (Leaves & fruits)	Bay	1.8-Cineole, bornyl acetate, *β*-elemene, sabinene, *α*-terpinyl acetate, *α*-pinene, trans-*β*-osimen, *α* & *β*-phellandrene	Antimicrobial, antifungal and antioxidant activity	(Sangun *et al.*, 2007; Fidan *et al.*, 2019; Caputo *et al.*, 2017; Mssillou *et al.*, 2020; Uysal *et al.*, 2010)
Melaleuca leucadendra L.; Myrtaceae (Leaves)	Cajuput	(*E*)-Nerolidol, viridiflorol, *β*-caryophyllene, (*E*)-*β*-farnesene, *α*-humulene and 1,8-cineole	Antimicrobial, antikinetoplastid, antiproliferative and cytotoxic activity	(Ismanto *et al.*, 2018; Rajendra *et al.*, 2015; Pino *et al.*, 2002; Monzote *et al.*, 2020)

Elletaria cardamomum L.; Zingiberaceae (Seeds)	Cardamom	1,8-Cineole, linalool, *α*-terpineol, *α* -terpinyl acetate, linalyl acetate	Antibacterial, antiseptic, carminative and antispasmodic activity	(Lucchesi *et al.*, 2007; Al-Zuhair *et al.*, 1996)
Origanum vulgare L.; Lamiaceae (Leaves and flowers)	Oregano	Thymol, *γ*-terpinene, Carvacrol, *β*-caryophyllene, terpinen-4-ol, *β*-myrcene, and *trans*-sabinene hydrate	Antimicrobial, antioxidant, anti-inflammatory, antidiabetic and anticancer activity	(Lucchesi *et al.*, 2008; Leyva-López *et al.*, 2017)
Dryopteris fragrans; Dryopteridaceae (Leaves and stems)	Fragrant woodfern	Esculetin, isoscopoletin, methylphlor-butyrophenone, aspidinol and albicanol	Antioxidant, anticancer and anti-inflammation activity	(Li *et al.*, 2012; Zhao *et al.*, 2014; Zhang *et al.*, 2018)
Rosmarinus officinalis L.; Lamiaceae (Leaves)	Rosemary	1,8-Cineole, *α*-pinene, verbenone, borneol camphor and *β*-caryophyllene	Antioxidant, antimicrobial, anti-Alzheimer and hepatoprotective activity	(Nieto *et al.*, 2018; Habtemariam, 2016; Rašković *et al.*, 2014; Okoh *et al.*, 2010)
Haplophyllum robustum Bge; Rutaceae (Aerial parts)	-	Sabinene, *β*-phellandrene, 1,8-cineole, camphor, terpinene-4-ol, *β*-pinene, 3,5-dimethoxy toluene	Antioxidant activity	(Moradalizadeh *et al.*, 2013; Gholivand *et al.*, 2012)

2.6. Microwave Hydro-diffusion and Gravity

Microwave hydro-diffusion and gravity (MHG) is one of the novel green methods of extracting volatile oils. This technology utilizes microwaves and earth gravity to harvest and extract volatile oils that hydro diffuse from the inner cell areas to the exterior of the plant material. The schematic representation of the MHG extraction system is shown in Figure 9. It is typically carried out at atmospheric pressure with no solvent added. It was designed for small-scale experimentation and processing (Vian *et al.*, 2008). This technique is used for expeditious isolation of volatile oils, as listed in Table 6. The advantage of this method is that it is economical, requires less energy, is highly efficient and does not require any solvent/water (Lucchesi, 2005). The extraction time is in minutes compared to hydrodistillation, which takes hours (Vian *et al.*, 2008).

Figure 9. Schematic representation of the microwave hydro-diffusion and gravity (MHG) extraction system.

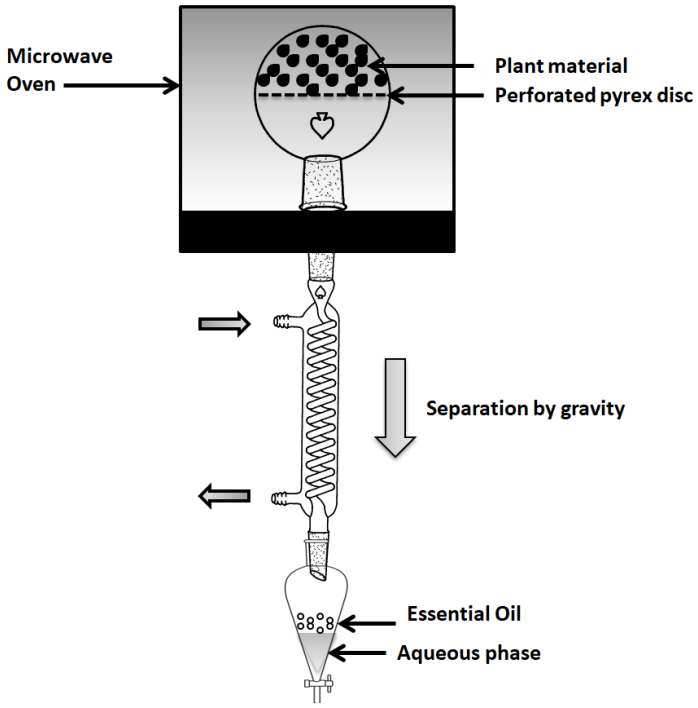


Table 6. Microwave hydro-diffusion and gravity (MHG) method of extraction of essential oils from various sources.

Sources (Part Used)	Common Name	Chemical Constituents	Pharmaceutical Application	References
Mentha spicata L.; Lamiaceae (Leaves)	Spearmint	Limonene, carvone, menthol, carveol, isocaryophyllene, germacrene D and *β*-farnesene	Antibacterial, antidermatophytic, anticholinesterase and anti-Alzheimer activity	(Ali-Shtayeh *et al.*,2019; Vian *et al.*, 2008)
Mentha pulegium L.; Lamiaceae (Flowers and leaves)	Pennyroyal	*α* & *β*-Pinene, pulegone, piperitenone, menthol, iso & neo-menthol, menthone, iso-menthone, and neoiso-menthol	Anti-cholinesterase, anti-Alzheimer and antioxidant activity	(Bektašević *et al.*, 2021; Vian *et al.*, 2008)
Zingiber officinale; Zingiberaceae (Rhizomes)	Ginger	Geraniol, geranial, *β*-phellandrene, neral, *α*-curcumene, *β*-sesquiphellandrene, (*Z*)-*α*-bisabolene and *α*-zingiberene	Anti-inflammatory antibacterial, anti-ulcer, antifungal, analgesic, immunomodulatory and relaxant activity	(Mahboubi, 2019; Akinyemi and Adeniyi, 2018; Funk *et al.*, 2016; Asofiei *et al.*, 2017)
Calophyllum inophyllum L; Calophyllaceae (Seeds)	Tamanu	*α*-Thujen, *α* & *β*-pinene, camphene, limonene, *γ*-terpinene, myrcene and *p*-cymene	Anti-inflammatory, antibacterial, wound healing, anti-HIV and antioxidant activity	(Rakotoarivelomanana *et al.*, 2018; Emmanuel *et al.*, 2019)
Cuminum cyminum L.; Apiaceae (Seeds)	Cumin	*α*-Thujene, 1,8-cineole, *α*-pinene, sabinene, limonene, terpinolene, (*E*)-ocimene, geraniol, *γ*-terpinene, linalool, *α*-campholenal, trans-carveole, linalyl acetate, *α*-terpinyl acetate and neryl acetate	Antimicrobial, anti-vomiting, anti-asthma and anti-spasm activity	(Wannera *et al.*, 2010; Esmaeili, 2015; Benmoussa *et al.*, 2018)

2.7. Ohmic Heated Water Distillation

Ohmic heated water distillation (OHWD) is a revolutionary process for isolating essential oils that use ohmic or Joules' heating (Shirsat *et al.*, 2004), and it requires less power (per mL) (Gavahian *et al.*, 2012). Controlling treatment homogeneity necessitates the most accurate modeling inputs. The schematic representation of the OHWD extraction system is shown in Figure 10. The heating rate is proportional to the square of the electric field strength and the conductivity of the medium. This technique is used for expeditious isolation of various volatile oils, as listed in Table 7. Ohmic heating is a highly energy-efficient and safe technology. It gives rapid and relatively homogenous heating. The quality of essential oils extracted by this method is good. The main disadvantage of ohmic heating is the high cost, the corrosion in the electrodes, and constant cleaning (Zareifard *et al.*, 2003; Goullieux & Pain, 2005).

Figure 10. Schematic representation of the Ohmic heated water distillation (OHWD) extraction system.

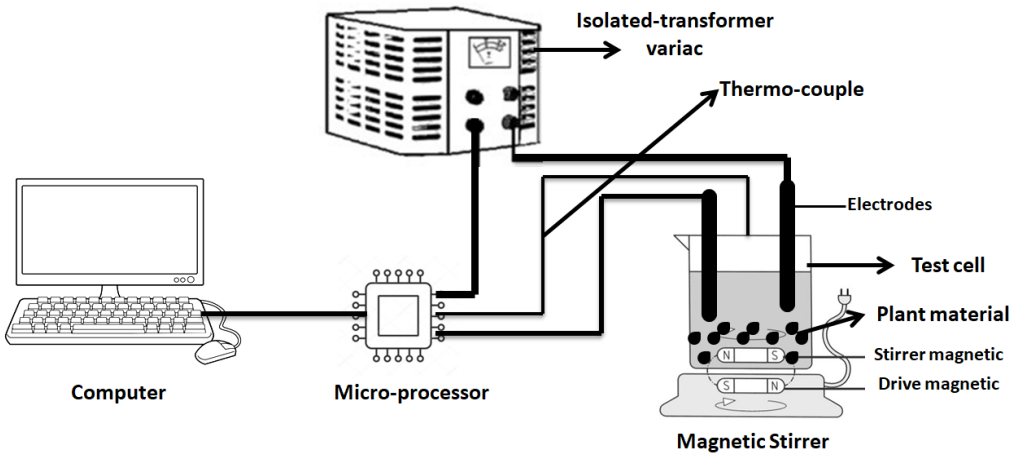


Table 7. Ohmic heated water distillation (OHWD) method of extraction of essential oils from various sources.

Sources (Part Used)	Common Name	Chemical Constituents	Pharmaceutical Application	References
*Thymus daenensi*s Celak; Lamiacae (Aerial parts)	Thyme	Thymol, carvacrol,*γ*-terpinene and *p*-cymene	Antimicrobial, antioxidant, insecticidal and immunomodulatory activity	(Tavakolpour *et al.*, 2017; Pirbalouti *et al.* 2013; Hadipanah and Khorami, 2016; Mohammad and Krenn, 2015)
Cymbopogon nardus; Poaceae (Grass)	Citronella	Citronellal, citronellol, neral, geraniol, linalool, *γ*-cadinene, trans-*β*-caryophyllene and citronellyl acetate	Antimicrobial, anthelmintic, antioxidant, anticonvulsant antitrypanosomal and wound healing activity	(Hazwan *et al.*, 2012; Sharma *et al.*, 2019)
Carum copticum L.; Umbelliferae (Seeds)	Ajowan	*α*-Thujen, *p*-cymene, thymol, *α* & *β*-pinene, 1,8-cineole, carvacrol and *γ*-terpinene	Antioxidant and antiaflatoxigenic activity	(Hashemi *et al.*, 2016; Ghadimian and Esmaeili, 2016; Kahkha *et al.*, 2014)
Melaleuca leucadendra L; Myrtaceae (Leaves, twigs and flowers)	Weeping paperbark	(*E*)-Nerolidol, (*E*)-*β*-farnesene, viridiflorol, *β*-caryophyllene, and *α*-humulene	Antimicrobial, antifungal, antikinetoplastid and antiproliferative activity	(Rajendra *et al.*, 2015; Monzote *et al.*, 2020; Iswahyono *et al.*, 2021; Zhang *et al.*, 2019)
Mentha piperita; Lamiaceae (Leaves)	Peppermint	Menthol, 1,8-cineole, menthone, *β*-pinene, (+/-)-menthyl acetate, *β*-caryophyllene and limonene	Antioxidant and antimicrobial activity	(Gavahian *et al.*, 2017; Gavahian *et al.*, 2015; Schmidt *et al.*, 2009; Mimica-Dukić *et al.*, 2003)
Lavandula angustifolia; Lamiaceae (Flowers)	Lavender	*β*-Pinene, linalool, camphor, terpineol, *β*-phellandrene, borneol, linalyl acetate and 1,8-cineole	Antimicrobial, anti-inflammatory and antioxidant activity	(Akgu *et al.*, 2000; Lakušić *et al.*, 2014; Danh *et al.*, 2012; Wells *et al.*, 2018; Gavahian and Chu, 2018)
Thymus vulgaris L.; Lamiaceae (Aerial flowering parts)	Common thyme	*p*-Cymene, linalool, thymol, *γ*-terpinene and carvacrol	Antioxidant, antiviral and antiproliferative activity	(Grosso *et al.*, 2010; Catella *et al.*, 2021; Niksic *et al.*, 2021; Gavahian *et al.*, 2012)
Zataria multiflora Boiss; *Lamiaceae* (Arial branches)	Shirazi thyme	Thymol, carvacrol, linalool, *λ*-terpinene and *p*-cymene	Antimicrobial, antioxidant and scolicidal activity	(Shafiee and Javidnia, 1997; Mahmoudvand *et al.*, 2017; Gavahian *et al.*, 2011)
Syzygium aromaticum (L.) Merrill et L.M. Perry; Myrtaceae (Buds)	Clove	Eugenol, eugenyl acetate, *δ*-cadinene *α*-caryophyllene, and *β*-elemene	Antioxidant, antifungal and antimicrobial activity	(Tunç and Koca, 2019; Selles *et al.*, 2020; Kaur *et al.*, 2019)

2.8. Solar Distillation

Solar energy serves in the agriculture field by saving money and reducing the environmental pollution. New technology has been developed to improve the efficiency of the distillation process by utilizing renewable energy sources such as sunlight. This method is used about the same amount of heat energy per unit weight of plant material (Garg & Prakash, 2006). A Scheffler fixed steam receiver, condenser, focus concentrator, oil separator, distillation still, and other components are used in solar distillation (Figure 11). The amount of energy available for the distillation process is determined by the sun intensity and the solar distillery's thermal and optical efficiency. It is a low-cost method for extracting essential oils from medicinal plants. Essential oils from different plant sources are extracted using a solar distillation system like eucalyptus leaves, peppermint leaves, clove buds, fennel seeds, basil, lavender, cumin, cardamom, orange, lemon, rosemary, citrus, Cymbopogon, etc. (Table 8) (Al-Hilphy *et al.*, 2022; Radwan *et al.*, 2020; Afzal *et al.*, 2017; Yen and Lin, 2017).

Figure 11. Schematic representaiton of the solar distillation (SD) extraction system.

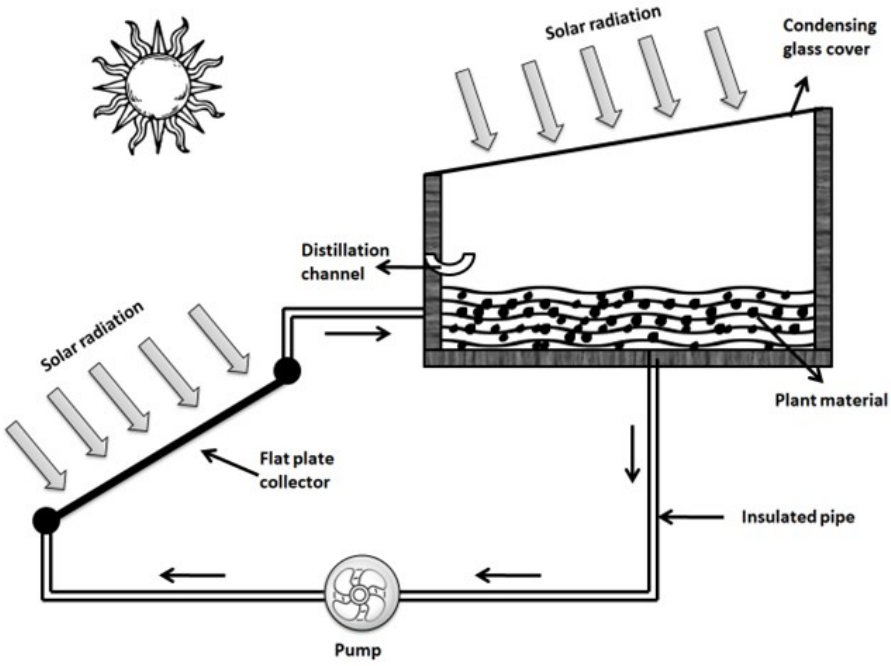


Table 8. Solar distillation (SD) method of extraction of essential oils from various sources.

Sources (Part Used)	Common Name	Chemical Constituents	Pharmaceutical Application	References
Eucalyptus camaldulensis; Myrtaceae (Leaves)	River red gum	Eucalyptol, *α*-phellanderene, *β*-pinene, arommadenderene, terpineol-4-ol	Antimicrobial activity	(Al-Hilphy et al., 2022; Afzal *et al.*, 2017; Lima *et al.*, 2013)
Mentha piperita; Lamiaceae (Leaves)	Peppermint	Menthol, 1,8-cineole, menthone, *β*-pinene, (+/-)-menthyl acetate, *β*-caryophyllene and limonene	Antioxidant and antimicrobial activity	(Afzal *et al.*, 2017; Schmidt *et al.*, 2009; Mimica-Dukić *et al.*, 2003)
Pinus (Roxburghii)		*α*-Pinene		(Afzal *et al.*, 2017)
Lavandula angustifolia; Lamiaceae (Flowers)	Lavender	*β*-Pinene, *β*-phellandrene, borneol, camphor, linalool, terpineol, linalyl acetate, terpineol-4-ol, cymene and 1,8-cineole	Antimicrobial, anti-inflammatory, and antioxidant activity	(Radwan *et al.*, 2020; Lakušić *et al.*, 2014; Danh *et al.*, 2012; Wells *et al.*, 2018)
Cymbopogon citratus (DC) Stapf.*.;* Poaceae (Leaves)	Lemon grass, oil grass	Geranial (citral a), neral (citral b), myrcene	Antioxidant, antileishmanial, antispasmodic, analgesic, anti-inflammatory, anti-pyretic, diuretic, anticonvulsant, and sedative activity	(Yen and Lin, 2017, Hanaa *et al.*, 2012, Santin *et al.*, 2009, Blanco *et al*., 2009)
Syzygium aromaticum; Myrtaceae (Buds)	Clove	Eugenol, eugenyl acetate, *δ*-cadinene *α*-caryophyllene, and *β*-elemene	Antioxidant, antifungal and antimicrobial activity	(Al-Hilphy *et al.,* 2022, Selles *et al.*, 2020; Kaur *et al.*, 2019)
Rosmarinus officinalis L.; Lamiaceae (Leaves)	Rosemary	1,8-Cineole, verbenone, α-pinene, borneol, camphor, *β*-caryophyllene	Antioxidant, antimicrobial, anti-Alzheimer, and hepatoprotective activity	(Hilali *et al.*2018, 2018; Habtemariam, 2016; Rašković *et al.*, 2014)
Ocimum basilicum; Lamiaceae (Leaves)	Egyptian sweet basil	Camphor, limonene, linalool, *β*-selinene, methyl chavicol and 1,8-cineole	Antioxidant, and antimicrobial activity	(Nannaware *et al*., 2022)
Elettaria cardamomum; Zingiberaceae (Seeds)	Cardamon/ cardamum	1,8-Cineole, *α*-terpinyl acetate, sabinene, *β*-linalool, *α* & *β*-pinene, and *β*-myrcene	Antimicrobial, aphrodisiac, astringent, digestive, stomachic, stimulant, and diuretic activity	(Al-Hilphy *et al.*, 2022, Alam *et al.*, 2021; Noumi *et al.*, 2018)

3. CONCLUSION

Various essential oils can be utilized as natural food additives and emanate from various sources. Many researchers have proven the effectiveness of innovations in traditional methods to extract volatile oils from various plant materials. The creation, improvement, and scale-up of these advances from the laboratory to pilot and industrial-scale all need modeling the experimental data. Reverchon *et al.*, 1999 and Sovová, 2005 provided some of the most successful ways. The objective of research scientists in the twenty-first century is to stimulate advances in traditional essential oil extraction processes that increase the yield and quality of volatile oils for aromatherapy and pharmaceutical uses. Essential oils' mechanisms of action must be understood to determine their efficacy as phytotherapeutic agents. On the other hand, innovative approaches, including microwave, ultrasound, ohmic heat, and solar energy-assisted extraction, can be coupled with conventional extraction methods for efficacious production of essential oils. Further research is necessary to find essential oils with novel bioactivities or functionalities.

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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

Yogesh Murti and Divya Jain: Equally contributed to conceiving the presented idea for the article, performed the literature search, analyzed the data, and wrote the original draft. **Bhupesh Chander Semwal and Sonia Singh**: Drew the figure and tables and edited the manuscript. **Pracheta Janmeda**: Provided critical feedback. **Pranav Bhaskar**: Provided critical feedback, edited the paper, and helped shape the final draft for submission.

Orcid

Pranav Bhaskar https://orcid.org/0000-0001-7449-7222
Yogesh Murti https://orcid.org/0000-0002-5278-0681
Divya Jain https://orcid.org/0000-0001-7220-5687
Bhupesh Chander Semwal https://orcid.org/0000-0003-1503-2745
Sonia Singh https://orcid.org/0000-0003-4351-6587
Pracheta Janmeda https://orcid.org/0000-0003-0500-4636
Pranav Bhaskar https://orcid.org/0000-0001-7449-7222

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Research Article

Ultrasonic assisted propolis extraction: characterization by ATR-FTIR and determination of its total antioxidant capacity and radical scavenging ability

Umit Erdogan [1,*]

[1]Isparta University of Applied Sciences, Rose and Aromatic Plants Application and Research Center, 32260, East Campus 32260 Çünür, Isparta, Türkiye

Abstract: In the current study, ultrasonic assisted ethanolic extract of propolis **was** discussed in detail, including their total phenolic content, total antioxidant capacity and radical scavenging capacity. For this purpose, we determined the total antioxidant capacity of propolis extract by CUPRAC and FRAP) assay. At the same time, the free radical scavenging capacity of propolis extracts was investigated via the DPPH˙ and CUPRAC- hydroxyl radical scavenging (HRS) methods. The chemical constituents of propolis extract were characterized by ATR-FTIR. The results revealed that propolis is rich in total phenolic components (189 mg GAE /g extract). According to the CUPRAC assay, the total antioxidant capacity of propolis extract was calculated to be 2.43 ± 0.07 mmol TE/g-propolis extract. FRAP value of propolis extract was determined as 1.11 mmol TE/g-propolis extract. DPPH• scavenging activity of propolis extract was calculated to be 0.71± 0.002 mmol TE / g - extract. On the other hand, according to the CUPRAC method, HRS capacity of propolis extract at different concentrations (5-10 µg/mL) was determined as 68.1% and 77.64 %, respectively. Research findings showed that propolis extract has a strong radical scavenging potential. The FTIR spectra of the functional groups originating from the phenolic compounds in the propolis extract were as expected.

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1. INTRODUCTION

Propolis is a natural product collected by bees from the cones and barks of trees, buds and shoots of plants. The content of propolis varies depending on the region where it is collected and the season. It has strong antimicrobial (Choi *et al*., 2006), antiviral, anti-inflammatory (Kujumgiev *et al*., 1999), antioxidant (Mohammadzadeh *et al.,* 2007), regenerative effects, and anticancer (Kimoto *et al.,* 2001), containing a mixture of oils, pollen, special resin and waxy substances in its composition (Osés *et al*., 2016). Propolis generally contains various chemical compounds such as polyphenols (flavonoids, phenolic acids and their esters), terpenoids, steroids and amino acids (Kumazawa *et al.,* 2004). Propolis is used in traditional medicine, cosmetics and food industry due to the pharmacological activity of its bioactive components (Banskota *et al*., 2001; Chaillou & Nazareno, 2009; Dezmirean *et al*., 2020).

*CONTACT: Umit Erdogan ✉ umiterdogan.sdu@gmail.com Isparta University of Applied Sciences, Rose and Aromatic Plants Application and Research Center, 32260, East Campus 32260 Çünür, Isparta, Türkiye

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Antioxidant components could remove free radicals and prolong shelf life by delaying the lipid peroxidation process, which causes food and pharmaceutical products to deteriorate (Halliwell, 1996). An inquiry of normally happening antioxidant ingredients from plant sources may prompt the advancement of novel medicines, which may diminish the danger of long-term infections brought about by free radicals (Abuja & Albertini, 2001).

The antioxidant activity of propolis deserves attention due to the phenolic components it contains. Since propolis contains a high proportion of phenolic components, it has significant antioxidant activity. Phenolic compounds represent the largest group of propolis components, depending on the amount and type (Oroian *et al*., 2020).

Ultrasound-assisted extractions (UAE) is a new and easy-to-use technique for obtaining bioactive molecules from various sources (Carreira-Casais *et al*., 2021; Jha & Sit, 2021). The intensity of the ultrasonic energy generates more vibrations in the sample components, facilitating the transport of the target molecules from the solid to the liquid solvent medium (Samaram *et al*., 2015). Due to the high yield with short extraction time and the use of a small amount of solvents, the UAE technique is superior to other techniques. In addition, it is an ideal option in the food industry as it can be made quickly, efficiently and at low temperatures (Madhu *et al*., 2019).

In the present study, in addition to identifying the presence or absence of functional groups of phenolic compounds of ultrasound-assisted propolis extracts by ATR-FTIR spectroscopy, the total antioxidant capacity and radical scavenging capacity of propolis extract were determined using various *in vitro* antioxidant methods. For this purpose, we determined the total antioxidant capacity of propolis extract according to "Cupric ion Reducing total Antioxidant Capacity" (CUPRAC) and Ferriccyanide (Fe^{3+}) Reducing Antioxidant Power (FRAP) assay. At the same time, the radical scavenging capacity of propolis extracts was investigated according to the 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^{\bullet}$) and CUPRAC- hydroxyl radical scavenging (HRS) methods.

2. MATERIAL and METHODS

2.1. Chemicals

Copper(II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$), catalase from bovine liver (2000-5000 U mg^{-1} solid), and Neocuproine (Nc-$C_{14}H_{12}N_2$), were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ethanol (96%) was purchased from ISOLAB Laborgeräte GmbH (Eschau, GERMANY). Ammonium acetate (NH_4Ac), iron(II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), sodium salicylate ($C_7H_5NaO_3$), Potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$), hydrogen peroxide (H_2O_2, 30 wt.%), Iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), and trichloroacetic acid (TCA), were purchased from Merck (Darmstadt, Germany).

2.2. Ultrasound-Assisted Extraction

Propolis samples collected in the region of Isparta, Türkiye (satellite coordinates: 38° 1' 25.5288" North and 30° 52' 22.3032" East) were then dried in the dark until processed. Propolis samples were prepared prior to extraction by then grinding with a coffee grinder (Sinbo SCM 2934-Türkiye). A total amount of 4 g of powdered propolis was soaked in 40 mL of 96% ethanol in a sealed bottle (Cavalaro *et al.,* 2019). The experimental conditions of the extraction procedure were as described previously by Samaram *et al*., (2014). The collected supernatants were filtered from the residue and dried by evaporating the solvents with a rotary evaporator (IKA RV 10 digital, IKA, Germany) at 50 ºC under vacuum.

2.3. Fourier Transform Infrared Spectroscopy (ATR-FTIR) Analysis

The infrared spectra were scanned on an JASCO FT/IR 4700 spectrophotometer (Jasco Co., Tokyo, Japan) at 4 cm^{-1} resolutions in frequency range between 4000 and 400 cm^{-1}.

2.4. *In Vitro* Antioxidant Activity Assays and Total Phenolic Content

It is recommended that the antioxidant activities of foods be compared by more than one method in terms of the mechanisms, selectivity, sensitivity, and applicability of the assays utilized to determine their antioxidant capacity (Apak *et al.,* 2004), For this purpose, we applied DPPH (Bener *et al*, 2022), CUPRAC (Apak et al., 2006), CUPRAC-HRS (Özyürek *et al*., 2008), and FRAP (Berker *et al*., 2007) methods to measure the antioxidant capacity and radical scavenging capacity of propolis extract. In each method, all tests were repeated three times for propolis extract and evaluated with a UV-Vis spectrophotometer (UV-1280, Shimadzu, Japan). A calibration curve was constructed using Trolox and results were expressed as mmol TE /g extract for each method.

Total phenolic content of propolis extract was determined via the the Folin–Ciocalteu method (Li *et al*., 2008). A calibration curve was constructed using gallic acid equivalents (GAE) and results were expressed as mg GAE /g extract.

2.5. Statistical Analysis

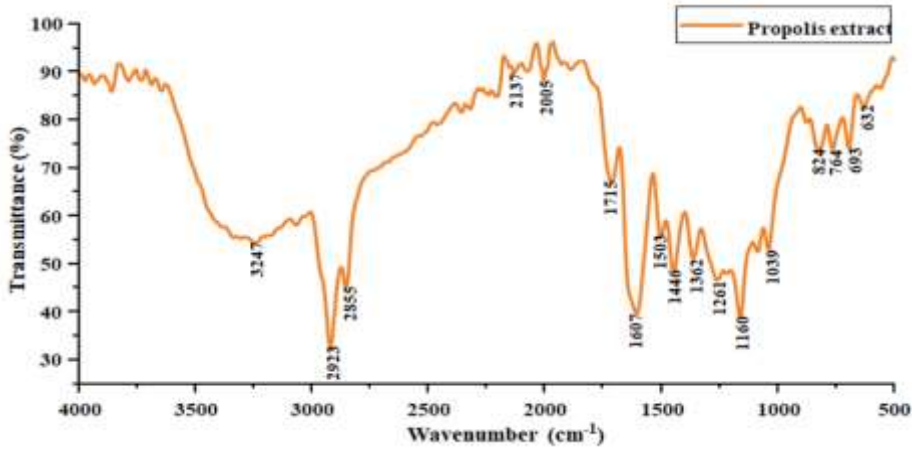
The presented data (mean ± standard deviation) resultedfrom at least three independent experiments and analyzed by SPSS (version 23 for Windows 10 pro, SPSS Inc.). The values were analyzed by one-way analysis of variance (ANOVA) and the post hoc Tukey's test, with significance set at $p < 0.05$.

3. RESULTS and DISCUSSION

3.1. Characterization of Propolis Extract by ATR–FTIR

The FT-IR spectra of the propolis extract are presented in Figure 1. It revealed that the FTIR spectrum of the propolis extract had considerable bands at 2923 and 2855 cm^{-1} corresponding to symmetrical and asymmetrical C-H stretching, respectively (Figure 1). Intense patterns located between 1362 and 1039 cm^{-1} illustrate C–O stretching and C–OH bending resulted from alcohols, ethers, esters and carboxylic acids representing functional groups in phenolic compounds which are found in propolis extracts (Soltani *et al.,* 2017). The stretching of the C=O, which originates from the stretching vibration of and C=C from the stretching of the aromatic rings, was 1715 and 1607 cm^{-1}, respectively. In the 3247 cm^{-1} region, a very large broad band was observed, corresponding to the absorption of the OH functional group representing alcohols. The IR spectra of the propolis extract were consistent with previously reported spectra in the literature (da Silva *et al*., 2018). More specifically, the spectra of functional groups originating from the phenolic compounds found in the propolis extract were as expected.

Figure 1. FTIR spectra of propolis extract.



3.2. Total Phenolic Content

As in natural food products, the type and amount of phenolic substances determine the majority of the compounds responsible for antioxidant activity in propolis. Total phenolic content was determined according to the Folin method. According to this analysis; high phenolic content indicates high antioxidant activity. In the studies presented in the literature, it was reported that there was a strong correlation between the folin method and different antioxidant methods (CUPRAC, ABTS/persulfate, FRAP), because all of these methods were electron transfer based assays (Çelik et al., 2008). According to the test used to measure the total amount of phenolic substances, the total amount of all ethanol-soluble phenolic and polyphenolic substances was determined, since the Folin reagent forms a colored complex with all phenolic compounds such as phenolic acids, flavonoids, flavanols, anthocyanins. The total phenolic content of the propolis extract was calculated to be 189.17 ± 3.004 mg/g (GAE/g-extract). However, different results have been reported in the literature. Gulcin *et al*., (2010) reported that the total phenolic content of propolis varied between 31.2 mg/g and 302 mg/g GAE.

Table 1. Antioxidant activity and total phenolic content of propolis extract.

Sample	CUPRAC value (mmol TE/ g-extract)	FRAP value (mmol TE/ g-extract)	DPPH value (mmol TE/ g-extract)	TPC (mg/g-extract)
Propolis extract	2.43 ± 0.07	1.11 ± 0.13*	0.71± 0.002	189 ± 3.004

*Mean ± standard deviation. Abbreviations: CUPRAC, cupric ions (Cu^{2+}) reducing antioxidant capacity, DPPH, 2,2-diphenyl-1-picrylhydrazyl TPC, total phenolic content, FRAP, Ferriccyanide (Fe^{3+}) Reducing Antioxidant Power

3.3. Radical Scavenging Capacity and Total Antioxidant Capacity of Propolis Extract

In our current study, we evaluated total anxidant capacity and the radical scavenging activity of propolis extract by DPPH, CUPRAC, FRAP, and modified CUPRAC – Hydroxyl radical scavenging (HRS), methods. The total antioxidant capacity and free radical scavenging activity of propolis extract according to the applied methods were presented in Table 1 and Figure 2.

Based on the ability of DPPH, a stable free radical, to lighten in the presence of antioxidants, the DPPH test is a direct, practical and reliable method for measuring radical scavenging activity (Hasan *et al.,* 2009). The DPPH is a stable free radical absorbing at 517 nm wavelength. Therefore, it can be said that when the antioxidant donates its electron to DPPH, and this causes the absorption of DPPH radical solution to decrease at 517 nm (Bozkurt *et al.,* 2020). Researchers often express the values of DPPH radical scavenging activity of herbal extracts as % scavenging or IC50. In the current study, the DPPH radical scavenging activity of propolis extract was expressed as mmol trolox equivalents per gram of extract. For this purpose, molar absorption coefficient of TR compound (ε_{TR}: 21600 L mol^{-1} cm^{-1}) was determined in the DPPH method and free radical scavenging activity of propolis extract was calculated to be 0.71 ± 0.002 mmol TE / g - extract (Table 1). In a study reported in the literature, the DPPH radical scavenging activity of propolis extracts in the ultrasonic- assisted extraction in 80% ethanol medium was calculated to be 1.03 mmol TE/g-dry sample (Ulloa *et al*., 2017). The difference in the measured DPPH values could be attributed to the region where the propolis samples were collected, the ethanol concentration used in the extraction, the extraction time and the temperature.

The CUPRAC assay is a method based on the estimation of the total amount of antioxidants as a function of the reduction of copper ions (II). Using bis(neocuproine) copper(II)chloride, a chromogenic redox reagent, the total amount of antioxidants, both hydrophilic and lipophilic, can be easily determined. The CUPRAC method refers to the electron donating power of the antioxidant. Contrary to DDPH, the higher absorbance values recorded at 450 nm depending on the intensity of yellow-orange color formation in the Cuprac method indicate higher antioxidant capacity. According to the CUPRAC assay, the total antioxidant capacity of

propolis extract was calculated to be 2.43 ± 0.07 mmol TE/g-extract (Table 1). However, it was determined that the total antioxidant capacity of propolis samples collected from different geographical regions of Turkey was measured between 0.71 and 8.24 mmol TR/g- propolis extract by the CUPRAC method. Of all these data, it can be deduced that the total antioxidant antioxidant capacity of propolis samples varies according to the geographical region where it was collected and the vegetation.

In the FRAP method, the reducing capacity of propolis extract was accomplished using Fe^{3+} to Fe^{2+} reduction assay. In this analysis, the light color of the FRAP test solution changed to dark colors depending on the concentration of the substance that showed antioxidant activity (Erdoğan, 2022). The presence of reducing agents, which act as antioxidants in the samples, causes the Fe^{3+}/ferricyanide complex to be reduced to the iron form. Thus, Fe^{2+} can be traced by measuring the formation of Prussian blue of pearl at 700 nm (Gülçin *et al*., 2006). The absorbance values of propolis extract and reference antioxidant substances at different concentrations at 700 nm were presented in Table 2. The higher absorption value measured by the FRAP method at 700 nm indicates a higher reduction capacity. The data in Table 2 revealed that BHA had the highest FRAP value at 450 µg/mL concentration, followed by BHT and propolis extract, respectively. However, the absorbance value measured at 700 nm increased depending on the concentration. In addition, the FRAP value of propolis extract was calculated to be 1.11 mmol trolox equivalent / g -propolis extract.

When the in vitro antioxidant methods used to determine the antioxidant capacity of propolis are compared, For the total antioxidant capacity of propolis extract, it was determined that the FRAP value (1.11 mmol TE/g extract) was higher than the DPPH value (0.71 mmol TE/g extract), while it was lower than the CUPRAC value (2.43 mmol TE /g extract).

Table 2. Total reducing power of different concentrations (150–450 µg/mL) of propolis extract, BHA, and BHT

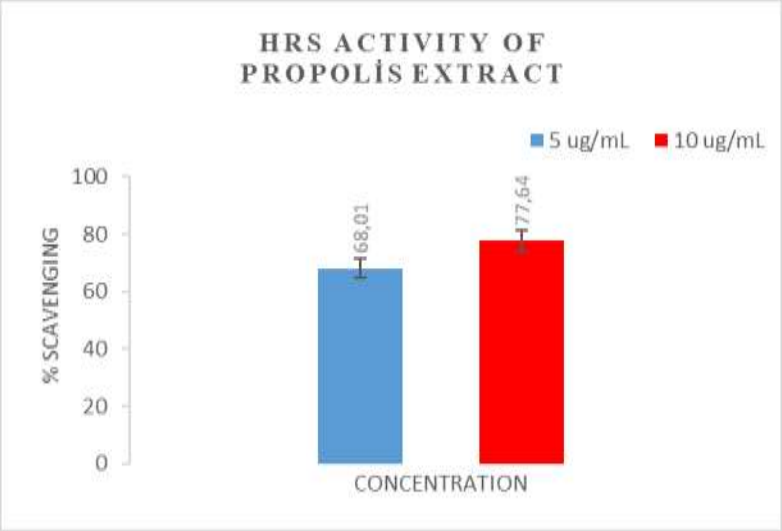
Concentration (µg/mL)	*FRAP value (at 700 nm)*		
	BHA	BHT	Propolis extract
150	0.828 ± 0.041[a]	0.523 ± 0.008[b]	0.237 ± 0.003[c]
300	1.367 ± 0.052[a]	0.840 ± 0.025[b]	0.446 ± 0.012[c]
450	2.704 ± 0.017[a]	0.968 ± 0.071[b]	0.587 ± 0.044[c]

Mean ± standard deviation. Different letters (a, b and c) in each row indicate significantly different ($p < 0.05$).

According to the modified CUPRAC assay, HRS capacity of propolis extract at different concentrations (5-10 µg/mL) was calculated to be 68.1% and 77.64 %, respectively (Figure 2). Research findings showed that propolis extract has strong radical scavenging potential. Free radicals are destructive molecules that break down living cells and cause aging and diseases. Free radicals are molecules with an unpaired electron. Most free radicals in our organism are radicals composed of molecular oxygen. Molecular oxygen tends to form highly reactive oxygen species (ROS) due to its diradical nature. Reactive oxygen species (ROS) are superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$), which are formed in small amounts during normal oxygen metabolism (Erdoğan & Erbaş, 2021). ROS is also responsible for damaging crucial biomolecules, including nucleic acids, lipids, proteins and carbohydrates, and might cause DNA damage that can lead to mutations (Ak & Gülçin, 2008). Among ROS, $OH^{\bullet}$ is the most dominant in terms of oxidative activity. $OH^{\bullet}$ is the most toxic radical known, as it can oxidize all biological macromolecules composed of lipids, proteins and nucleic acids at almost diffusion-limited rates (Özyürek *et al*., 2008). In a study previously reported in the literature, it was reported that propolis extract was more effective in delaying the oxidation of olive oil compared to synthetic antioxidants such as BHA and BHT (Erdoğan 2023). The data presented in the present study revealed that propolis extracts

exhibited a great ability to scavenge a toxic radical such as $OH^{\bullet}$, even at very low concentrations.

Figure 2. Hydroxyl radical scavenging (HRS) activity of propolis extract.



4. CONCLUSION

In the current study, propolis extracts were discussed in detail, including their total phenolic content, total antioxidant, and radical scavenging activity. The data showed that propolis is rich in total phenolic content. Using *in vitro* antioxidant methods, propolis was found to possess strong free radical scavenging capacity and antioxidant properties. The characteristic FT-IR spectra of the propolis extracts confirmed the functional groups originated from the phenolic compounds of the propolis extracts. In the light of the data obtained in this study, more detailed studies can be carried out for the purification of individual phenolic compounds of propolis and that will provide some critical insights into the use of the bioactive components of propolis for different applications such as pharmaceutical, cosmetic and food industry. However, due to the undesirable properties of conventional solvents used in propolis extraction, the next step in research efforts should focus on finding green solvents that can provide high extraction yield and effective antioxidant results, such as ethanol.

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Orcid

Ümit Erdogan https://orcid.org/0000-0002-6627-4472

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Research Article

Essential oil composition of *Bellardia trixago* (L.) All. (Orobanchaceae) from Türkiye

Gurkan Semiz[1,*], **Batikan Gunal**[2]

[1]Department of Biology, Faculty of Science, Pamukkale University, Denizli, Türkiye

Abstract: *Bellardia trixago* (L.) All. (syn. *Bartsia trixago* L.) is a hemiparasitic plant that is mainly native to the Mediterranean area. Formerly, the genus was considered to be a part of the Scrophulariaceae family, but it is currently classified as belonging to the family Orobanchaceae. *Bellardia* species are used in traditional medicine by the local people to cure backache, menstrual problems and as an antipyretic and against the human immunodeficiency virus. Various populations of *B. trixago* exhibit qualitative and quantitative variations in the chemical composition of the plant. This research described the chemical composition of *B. trixago* from Dazkırı, Afyon/Türkiye. The essential oil of *B. trixago* was obtained by hydro-distillation method and the oil content was analyzed by GC-MS. Cembrene (51.7%) was identified as the major component and the other most abundant components were phellandral (15.4%) and α-terpineol (14.5%). To the best of knowledge, no research has ever been performed on *B. trixago*'s essential oil from Türkiye.

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1. INTRODUCTION

Bellardia trixago (L.) All. (syn. *Bartsia trixago* L.) is a taxon of the Orobanchaceae family (Chase, 2003) and the only member of the monotypic *Bellardia* genus in Türkiye. *B. trixago* is a species native to the Mediterranean Basin and North Africa, but it can also be distributed in other parts of the world with similar climates (Tutin *et al,* 1972; Hedge 1978). Formerly, the genus was considered to be a part of the Scrophulariaceae family, but it is currently classified as belonging to the family of the hemiparasitic Orobanchaceae (Thieret 1967; Olmstead 2002). *B. trixago*, a facultative hemiparasitic plant, is reported to produce a variety of bioactive metabolites and infect ruderal plants that have no detrimental effects on agriculture (Press *et al,* 1993; Uribe-Convers & Tank 2016). Although *B. trixago* is a photosynthetic plant, it is considered a parasitic plant because of its ability to obtain carbohydrates from other plants

*CONTACT: Gurkan Semiz ✉ gsemiz@pau.edu.tr Pamukkale University, Faculty of Sciences, Department of Biology, Denizli, Türkiye

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(Carafa *et al,* 1980). The aroma of the plant is described as similar to the smell of honey (Esteban *et al,* 1996). Different types of natural compounds, including glycosides, flavonoids, and terpenes were determined by their chemical composition (De Pascual Teresa *et al,* 1978, 1982; Ersöz *et al,* 1988; Tomas-Barberan *et al,* 1990). There are also some studies on the use of different extracts of *B. trixago* as an insecticide (Pascual-Villalobos & Robledo 1998; Formisano *et al,* 2008). Additionally, Barrero and co-workers showed that various populations of *B. trixago* exhibit qualitative and quantitative variations in the chemical composition of the plant (Barrero *et al,* 1988; 2011). *Bellardia* species are also used in traditional medicine by the local people to cure backache, menstrual problems and as an antifebrile in Peru (Velasco-Negueruela *et al,* 1995), and against HIV/AIDS in Uganda (Lamorde *et al,* 2010). Although the bioactive content of *B. trixago* has been determined by different studies, no study has been found in the literature on the chemical composition of the essential oil of *B. trixago* in Türkiye (Bianco *et al,* 1976; Barrero *et al,* 1988; Ersöz *et al,* 1988; Tomas-Barberan *et al,* 1990). In this study, the chemical contents of the essential oil of *B. trixago* were shown for the first time in Türkiye.

2. MATERIAL and METHODS

2.1. Plant Materials and Essential Oil Collection

B. trixago was collected from its natural population (Dazkırı, Afyon-Türkiye) during its flowering season in 2018. The collected specimen was identified by Prof. Gurkan SEMIZ and voucher specimens (GSE 2013) were deposited in the Biology Department of Pamukkale University. The aerial plant samples, which were air-dried for a week in a shady and cool place, were cut into small pieces. The procedure of essential oil collection using the Clevenger apparatus was followed by Semiz *et al,* 2022.

2.2. GC-MS Analysis

The relative chemical profile of the *B. trixago* essential oil was determined on GC-MS (Hewlett-Packard GC-7820A, MSD-5975). The HP-5MS capillary column, 30 m long, was selected (ID 0.25 mm, film thickness 0.25 mm, Hewlett Packard) and the chromatographic conditions to obtain terpenes were followed by Semiz *et al,* (2018). Briefly, the temperature was set from 50°C to 250°C at 5°C min^{-1}, using helium as the carrier gas (flow rate 1.2 ml/min); SCAN technique (mass numbers from m/z 30 to 350 were recorded; signal ions in monitoring; 93, 133, 136, 161, and 204 m/z) was used; the samples of 1 µl were injected automatically and in the splitless mode. The compounds were identified by comparing their mass spectra and retention indices with those in NIST and Wiley electronic libraries.

3. RESULTS

In this study, the chemical composition of the essential oil from *B. trixago* was characterized by GC-MS. The essential oil constituents of *B. trixago* were dominated by diterpenes. Sixteen compounds representing 97.0% of the oil were detected in *B. trixago*. Essential oil yield was found as 0.27%. The relative amounts of chemical compounds in the essential oils are presented in Table 1. The main constituent of *B. trixago* was determined as cembrene (51.7%). The other major compounds were determined as phellandral (15.4%) and α-terpineol (14.5%).

Table 1. Essential oil composition (%) of *B. trixago.*

No	RRI[a]	RRI[b]	Compounds[c]	%
1	980	980	1-Octen-3-ol	1.0
2	1029	1029	D-Limonene	0.1
3	1032	1031	1,8-Cineole	0.2
4	1060	1059	γ-Terpinene	0.1
5	1099	1099	Linalool	0.9
6	1144	1143	Camphor	0.2
7	1159	1158	Isoborneol	0.6
8	1165	1164	δ-Terpineol	0.1
9	1168	1166	Borneol	0.5
10	1183	1183	*p*-Cymen-8-ol	2.9
11	1190	1189	α-Terpineol	**14.5**
12	1219	1219	Fenchyl acetate	5.4
13	1255	1255	Linalool acetate	1.0
14	1274	1274	Phellandral	**15.4**
15	1956	1947	Cembrene	**51.7**
16	2086	2087	Cembratrienol	2.4
TOTAL				**94.5**

[a] RRI: Relative retention indices measured to against *n*-alkanes on HP-5MS column, [b] Retention indices from literature (Adams 2007; Babushok *et al*, 2011; Chizzola *et al,* 2021), [c]Compounds listed in order of their elution. The values in bold indicate the highest amounts.

4. DISCUSSION and CONCLUSION

Aromatic and medicinal plant species and their essential oils were described as sources of secondary metabolites and have been frequently used for centuries in traditional medicine (Deans & Svoboda, 1990). In addition to serving as a natural source for acquiring and isolating compounds with pharmacological uses, plants can also be used to make herbal medicines for use in conventional or alternative medicine. At this point, *B. trixago* is an ethnomedicinal plant, which is used by local people in many parts of the world for different purposes. However, the information available in the literature on the essential oil of *B. trixago* is quite limited. In a study conducted by Formisano and her co-workers (2008) in Italy, the most abundant compounds from the essential oil of *B. trixago* were (*E,E*)-farnesyl acetone (42.1%), trixagol (8.0%), and 4-vinyl guaiacol (5.4%). In another study conducted to determine the volatile components of some aromatic plants by automatic thermal desorption technique, it was determined that trixagoyl acetate (56.1%), trixagol (25.4%) and trixagoene (4.22%) were the three most abundant compounds among the volatile organic compounds of *B. trixago* (Esteban *et al,* 1996). In the same study, according to the simultaneous distillation-extraction technique, the most abundant compounds of *B. trixago* were determined as trixagol (25.4%), trixagoene (4.22%) and 3,4-dihydro-γ-ionone (14.12%). The large variations between the findings and the earlier publications may be the consequence of genetic, environmental and collection-time variations. In conclusion, our findings show that environmental and climatic factors have an impact on the chemical profile of the essential oils of *B. trixago*.

It may be advantageous to use essential oils in medicinal, cosmetic, and industrial fields after considering their pharmacological qualities and their constituent parts. It is safe to employ essential oils and the components they contain in studies to find novel antibacterial treatments that may be effective against pathogenic microorganisms. We hope that these findings will stimulate more research into the chemistry of *Bellardia* species and that terpene-based chemical content profiling of the species may be helpful in taxonomic studies.

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Authorship Contribution Statement

Gurkan Semiz: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing-original draft. **Batikan Gunal**: Methodology and Validation.

Orcid

Gurkan Semiz https://orcid.org/0000-0003-0276-8542
Batikan Gunal https://orcid.org/0000-0002-6126-6510

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Research Article

Phytochemical screening and *in vitro* assessments of antioxidant and cytotoxic potentials of extracts from *Aesculus hippocastanum* L. green fruit mesocarps

Tulay Askin Celik[1,*], **Ozlem Sultan Aslanturk**[1]

[1]Aydın Adnan Menderes University, Faculty of Science, Department of Biology, Central Campus, Aydın-Türkiye

Abstract: In this study, the in vitro antioxidant and cytotoxic effects of water and methanol extracts obtained from the green fruit mesocarp of *Aesculus hippocastanum* L. (Hippocastanaceae) were investigated. Phytochemical content of the methanol extract and the water extract were determined by qualitative methods; antioxidant activity was determined by DPPH free radical scavenging test, and total antioxidant capacity was determined by phosphomolybdate test. The effects of the extracts on proliferation and cell viability of BJ normal human foreskin fibroblasts were also evaluated by the WST-8 cell viability test.

Qualitative phytochemical screening results showed that the methanol extract contains phenols, tannins, flavonoids, and saponins, but no alkaloids and anthraquinones. On the other hand, phenols, flavonoids, anthraquinone, and saponins were found in the water extract, tannins and alkaloids could not be detected.

In addition, an increase in antioxidant activity was also observed with each increasing concentration of methanol and water extract. When the antioxidant capacity and free radical scavenging activity of methanol and water extracts were compared, it was determined that the methanol extract was more effective than that the water extract. The WST-8 trial results showed that both water and methanol extracts obtained from the green fruit mesocarp of *A. hippocastanum* did not have cytotoxic effects on BJ cells, on the contrary, treatment concentrations of 10, 20 and, 30 μgmL^{-1} increased cell proliferation significantly at the 24-hour work.

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1. INTRODUCTION

Medicinal plants, according to the World Health Organization (WHO), have bioactive chemicals that can be used for therapeutic purposes or synthesize metabolites that can be used to make effective medications (Paul *et al.,* 2018). A number of reports have been published in the literature regarding the antibacterial, anti-inflammatory, and wound healing efficacy of different plants, but the vast majority has yet to be investigated (Thakur *et al.,* 2011). Phytochemicals are naturally occurring chemicals in plants, providing a protective function against bacteria, viruses, fungi, the harms of free radicals, insects, and herbivores that feed on

*CONTACT: Tulay ASKIN CELIK ✉ tcelik@adu.edu.tr Aydın Adnan Menderes University, Faculty of Science, Department of Biology, Central Campus, Aydın-Türkiye

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them and pose any environmental threat (Molyneux *et al.,* 2007; Curran, 2018). Medicinal plants generally contain phenolic compounds, including flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans, and lignin. Many of the beneficial effects of medicinal plants are attributed to their high polyphenolic content. These molecules are known to reduce cellular oxidative stress, which plays a crucial role in the pathogenesis of a range of diseases including cancer and cardiovascular diseases.

Aesculus hippocastanum L. (Hippocastanaceae), commonly known as Horse chestnut, is native to Western Asia, but today it is widely distributed and cultivated all over Europe. (Zhang *et al.,* 2010; Roy *et al.,* 2011; Geetha *et al.,* 2013). *A. hippocastanum* seeds are used for abdominal pain, stomach ache, cold, hemorrhoids, arterial stiffness, rheumatism, edema, diarrhea, antihemorrhagic, and antipyretic treatment (Baytop, 1999; Comitte on Herbal Medicinal Products, 2011; Tuzlacı, 2016) . The bark of *A. hippocastanum* has been used as a tonic, narcotic, and antipyretic, and also to induce sneezing. The plant constituents utilized medicinally come from the fruits (Roy *et al.,* 2011). In the bark extract of *A. hippocastanum*, coumarin derivatives such as triterpenoid saponins (aescin, prosapogenin), proanthocyanidin A2 and coumarins (esculin and fraxin), scopoline are present.There are flavonoids such as quercetin, kaempferol, astragalin, isoquercitrin, rutin, leucocyanidine and essential oils such as oleic acid, linoleic acid (Sirtori, 2001). Amino acids, allantoin, argyrin, carotin, choline, citric acid, epicatechin, leucodelphinidin, phytosterol, resin, scopoletin, tannin, and uric acid are among the other ingredients. Horse chestnut extract has also been shown to have antioxidant effects (Braga *et al.,* 2012; Vaskova *et al.,* 2015; Kováˇc et al, 2020; Owczarek *et al.,* 2021) and produce contraction force in fibroblasts, which is important for skin regeneration (Wilkinson & Brown, 1999). Due to these effects, horse chestnut extracts are used in skin products (Thakur *et al.,* 2011).

The plant material is applied, mainly externally such as bath infusions, creams, ointments, and suppositories, to conditions connected with vascular damage and defective blood clottings, such as venous insufficiency, hemorrhoids, cutaneous capillary fragility, as well as oedemas, small bruises, and limited skin and subcutaneous tissue inflammations (Comitte on Herbal Medicinal Products, 2011). *A. hippocastanum* seeds and their derivates might be thought of as an opencast mine of natural different chemicals that play a variety of roles in various biological activities in several ways.

While there are many reports on the antioxidant and cytotoxic activity of *A.hippocastanum* (AH) leaf, seed, and the seed coat, there are no *in vivo* or *in vitro* research samples about the effects of green fruit-mesocarp extracts. This experimental study was conducted to determine the phytochemical content and antioxidant activity of *A. hippocastanum* green fruit mesocarp methanol and water (decoction) extracts. In addition, the viability and proliferation of BJ normal human foreskin fibroblasts cells were investigated using the *A. hippocastanum* green fruit mesocarp methanol and water extracts.

2. MATERIAL and METHODS

In this study, the in vitro antioxidant and cytotoxic effects of water and methanol extracts of the green fruit mesocarp of *Aesculus hippocastanum* L. (Hippocastanaceae) were investigated. Phytochemical content of the methanol extract and the water extract were determined by qualitative methods. Antioxidant activity was determined by DPPH free radical scavenging test, and total antioxidant capacity was determined by phosphomolybdate test. The effects of the extracts on proliferation and cell viability of BJ normal human foreskin fibroblasts were also evaluated by the WST-8 cell viability test.

2.1. Chemicals and Reagents

The chemicals 1,1-diphenyl -2-picryl-hydrazyl radical (DPPH), sulphuric acid, disodium hydrogen phosphate (Na_2HPO_4), ammonium molybdate, ascorbic acid, dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and WST-8 cell viability assay kits were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Plant Material and Extracts of *A. hippocastanum* Fruit-Mesocarp (AHFME)

The fresh fruit mesocarp of *A. hippocastanum was* used in this study. The fresh fruit mesocarp of horse-chestnut was collected during the autumn season of 2017 from the Ankara Province of Türkiye. The fruit is a large, round and greenish capsule, covered with small pungent spines, opening into three valves and containing a large shiny brown seed. The fresh fruit shell is a green, spiky capsule that contains one (rarely two or three) nut-like seeds known as conkers or horse-chesnuts. The fresh fruit-mesocarps were washed 2-3 times with water and dried at room temperature.

The dried AHFME was finely powdered with a blender before being used for extraction. 50g of ground dry AHFME was added to 500mL of methanol and the extraction process was conducted. The extraction process was carried out at room temperature until the solvent became colorless (24-48 h). After the methanol extraction, the extract solution was filtered. After the methanol extraction, plant material (AHFME) was dried and subjected to water (decoction) extraction (Domínquez, 1973; Ravishankara *et al.,* 2002; Miliauskas *et al.,* 2004). Then the water extract was filtered as well. The methanol extract was evaporated using a rotary evaporator at 50^{o}C under decreased pressure. The water extract was lyophilized. The extracts were stored at -20^{o}C until they were used in experiments.

2.3. Preliminary Phytochemical Screening

The quantitative phytochemical screening tests were performed to determine various active components that are likely to be present in *A. hippocastanum* fruit-mesocarps extracts (AHFME). The test details are as follows:

2.3.1. Detection of phenols

The method of Ravishankara *et al.,* 2002 was used. The methanol and water extracts of AHFME prepared in ethanol were spotted on a filter paper in beakers. After that, the spots were applied to a drop of phosphomolybdic acid reagent and were exposed to ammonia vapors. The presence of polyphenols was indicated by the appearance of fresh radish blue color.

2.3.2. Detection of tannins

10% alcoholic ferric chloride (FeCl3) is added to 2-3mL of the extract of the methanol by doing so, the specific coloring was observed which is the dark blue or greenish-gray color indicative of the presence of tannins in the extracts (Ravishankara *et al.,* 2002).

2.3.3. Detection of alkaloids

A drop of the extracts prepared in methanol was spotted in a small piece of precoated TLC plate and the plate was sprayed with Dragendorff's reagent if the coloring of the solution is orange-reddish it indicates the presence of alkaloids in the extracts (Ravishankara *et al.,* 2002).

2.3.4. Detection of anthraquinones

With 10% ferric chloride solution and 1mL of concentrated hydrochloric acid, about 50mg of the extracts were heated. The extracts were cooled and then filtered, and the filtrates were shaken with diethyl ether. The extracts of ether were further extracted with strong ammonia. If there is aqueous layer coloration in pink or deep red indicates the presence of anthraquinones (Ravishankara *et al.,* 2002).

2.3.5. Detection of saponins

Hot water was added to the test tubes containing 10mg of extract and the tubes were vortexed for 30 seconds. Foamy appearance indicates the presence of saponins in extracts (Ravishankara *et al.,* 2002).

2.3.6. Detection of flavonoids

A piece of magnesium ribbon and 1mL of concentrated hydrochloric acid was added to 2-3 mL of the extracts dissolved in methanol. If pink-red or red coloration is observed it is indicative of flavonoids in the extracts (Ravishankara *et al.,* 2002).

2.4. Antioxidant activity of AHFME

2.4.1. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH free radical scavenging potential assay is based on the stable DPPH scavenging activity (Brand-Williams *et al.,* 1995). 1 mL of 0.1 mM DPPH methanol solution was added to 3 mL of different concentrations 10, 20, 30, 50, 75, 100, and 150 μgmL^{-1} of extracts in methanol. The mixture was shaken vigorously and kept at room temperature. Using a microplate reader (Elisa Reader, Biotek Co, USA) the absorbance of the mixture was measured at λ = 517 nm after 30 min. Ascorbic acid was used as the standard. The experiments were carried out three times. DPPH radical scavenging activity of the extracts was calculated using the following equation:

DPPH Scavenging capacity (%) = $[(A_0-A_1)/A_0] \times 100$

[Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the *A. hippocastanum* fruit-mesocarps methanol and water extracts].

The actual decrease in absorption induced by the test was compared with the positive controls. The EC_{50} (concentration providing 50% inhibition) values were calculated by using the dose inhibition curve in the linear range by plotting the extract concentration versus the corresponding scavenging effect.

2.4.2. *Phosphomolybdate assay* (*Total antioxidant capacity*)

The total antioxidant capacity (TAC) of the extracts was carried out with the phosphomolybdenum method (Umamaheswari & Chatterjee, 2008). About 0.1 mL of each plant extract sample was added to 1 mL of reagent solution (0.6 M sulphuric acid, 4 mM ammonium molybdate, and 28 mM sodium phosphate). Tubes containing the mixture were covered with aluminum foil and incubated for 90minutes in a water bath at 95°C. The mixture was then left to cool at room temperature. The absorbance of the solution was measured at 765 nm against a blank. Ascorbic acid was used as the standard. The higher absorbance values indicated the higher total antioxidant potential of the plant extracts.

All the experiments were carried out in triplicate and were repeated three times. The total antioxidant capacity (TAC) was estimated using the following formula:

Total antioxidant capacity (%) = [(Abs. of control−Abs. of the sample/(Abs. of control] × 100

Abs: absorbance of sample;

Abc: absorbance of control

2.5. Cell Culture and Proliferation Assay (WST-8 assays)

BJ cells (Human normal foreskin fibroblast cell line; ATCC CRL-2522) were obtained from Dr. Mehtap Kılıç Eren, Aydın Adnan Menderes University, Faculty of Medicine, Department of Medical Biology, Aydın/Türkiye and used for *in vitro* studies. WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2Htetrazolium, monosodium salt] is a

water-soluble tetrazolium salt used for assessing cell metabolic activity that produces corresponding formazan dye that absorbs at 460nm. WST-8 is typically used as a cell viability indicator in cell proliferation assays.

The cells were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Germany), penicillin (100IUmL^{-1}), and streptomycin (100 µgmL^{-1}) under a humidified atmosphere of 5% CO_2 at 37 °C until confluent. The cells were trypsinized and proliferation assays were carried out in 48 well plates. BJ cells were seeded into 48 well plates (5x10^3 cells per well) and incubated to form a partial monolayer for 24 h. After 24 h of incubation, the cells were treated with 10, 20, 30 µgmL^{-1} of AHFME for 24h respectively. The negative control cells were grown in a culture medium only and 10% crystalin has used as the standard. At the end of each of the treatment times, cellular viabilities were determined with WST-8 assay method (Tominaga *et al.,* 1999). The absorbance was read at 450nm for WST-8 assay by using a microplate reader (Elisa Reader, Biotek Co, USA). All of the absorbance values were compared to the control samples (without any compound) which represented 100% viability.

Cell viability (%)= [(As-Ab)/(Ac-Ab)] x100

As: Absorbance of sample

Ab: Absorbance of blank

Ac: Absorbance of control

2.6. Statistical Analysis

The analysis was executed by using the statistical package for the social sciences (SPSS version 20.0 Armonk, NY: IBM Corp) SPSS IBM 20. All measurements were carried out in triplicate and expressed as mean ± standard deviation. Statistical significance was determined using a one-way analysis of variance (ANOVA). The normality of variables was evaluated using the Kolmogorov-Smirnov Z test. The statistical differences between the control and treatment groups were carried out using the non-parametric Mann-Whitney Test (for independent samples). The correlations between different variables were determined using the Spearman Rank Correlation Test. P values less than 0.05 were considered as significantly different ($p \leq 0.05$).

3. RESULTS

3.1. Phytochemical Screening

Phytochemical screening results are presented in Table 1. Phytochemical screening results showed that AHFME methanol extracts contain phenols, tannins, flavonoids, and saponins, and do not contain alkaloids and anthraquinones. It was also determined that flavonoids, anthraquinones, and saponins were present in the water extract and tannins and alkaloids were not present.

Table 1. Phytochemical screening of methanol and water *extracts of A. hippocastanum* green fruit mesocarps

Active Compounds	Tests	Methanol Extract	Water Extract
Phenols	Phosphomolybdic acid test	++	+
Tannins	Braemer's test	+	-
Alkaloids	Dragendroff's test	-	-
Flavonoids	Shinoda's test	++	+
Anthraquinones	Bornträger test	-	++
Saponins	Frothing test	+	++

- absent of active compound; + moderate amount; ++ high amount after added reagent

3.2. Antioxidant Activity of *A. hippocastanum* Green Fruit Mesocarps Extracts

3.2.1. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The antioxidant potential of the methanol and water extracts of *A. hippocastanum* green fruit mesocarp (AHFME) was evaluated based on their ability to scavenge stable free DPPH radicals. This test is based on the change of the color of the DPPH solution from purple to yellow due to the scavenging of stable free DPPH radicals. This change from purple to yellow was measured at 517nm (Brand-Williams *et al.,* 1995). A stronger yellow color indicates the ability of the extract to scavenge free DPPH radicals more and thus a stronger antioxidant potential.

The antioxidant potential of the different extracts from the methanol and water extracts from *A. hippocastanum* fruit mesocarps was assessed using different methods and the results are reported as values of IC_{50} (Table 2). The antioxidant activity and radical scavenging activity of the extracts differed depending on the concentration.

When the free radical scavenging effect of *A. hippocastanum* green fruit mesocarp extracts of methanol and water was compared in terms of EC_{50} values, the EC_{50} value of the water extract was found to be lower than that of the methanol extract. Therefore, it was determined that the antioxidant activity of the water extract was slightly higher. However, it has been determined that the antioxidant activity of both the methanol and water extract is significantly lower than that of ascorbic acid.

3.2.2. Phosphomolybdate assay (Total antioxidant capacity)

The phosphomolybdate assay is used routinely for the evaluation of extracts' antioxidant capacities. In this assay, phosphomolybdate (MoVI) is reduced to phosphate/MoV complex in the presence of an antioxidant (Umamaheswari & Chatterjee, 2008). The total antioxidant capacities o the AHFME methanol and water extracts measured are given in Table 2. The total antioxidant capacity of the AHFME methanol extract started to increase from the concentrations of 50µgmL^{-1} and reached 76.14±0.018% at its highest concentration (150 µgmL^{-1}). The EC_{50} value of the methanol extract is found to be 42.37 µgmL^{-1}, and the EC_{50} value of the water extract is found to be 87.95 µgmL^{-1}. However, the total antioxidant capacity of AHFME water extract could only exceed 50% at concentrations of 100 and 150 µgmL^{-1}.

The total antioxidant capacity of ascorbic acid is determined as 25.00%, 79.46%, and 89.71% at 10, 25, and 50 µgmL^{-1} concentration, respectively and the EC_{50} value is 17.65µgmL^{-1}. Although AHFME water extract also shows antioxidant activity, it has been found to show significantly lower than that of both ascorbic acid and methanol extracts (Table 2).

Table 2. DPPH scavenging activity and total antioxidant capacity of methanol and water extracts of *A. hippocastanum* green fruit mesocarps

Groups	Concentrations	DPPH Scavenging activity (% ± SD)	DPPH Scavenging activity (EC_{50})	Total antioxidant capacity (% ± SD)	Total antioxidant capacity (EC_{50})
Ascorbic acid	10 µgmL^{-1}	47.14 ± 0.021*	12.82 µg/mL	25.00 ± 0.005	17.65 µg/mL
	25 µgmL^{-1}	70.80 ± 0.007*		79.46 ± 0.027	
	50 µgmL^{-1}	73.91 ± 0.001*		89.71 ± 0.021	
ME	10 µgmL^{-1}	11.42 ± 0.061	57.57 µg/mL	36.87 ± 0.017*	42.37 µg/mL
	20 µgmL^{-1}	12.85 ± 0.053		39.85 ± 0.009*	
	30 µgmL^{-1}	15.24 ± 0.047		46.27 ± 0.007*	
	50 µgmL^{-1}	49.06 ± 0.003*		57.72 ± 0.001*	
	75 µgmL^{-1}	63.35 ± 0.002*		72.42 ± 0.030*	

	100 µgmL^{-1}	71.42 ± 0.002*		74.78 ± 0.070*	
	150 µgmL^{-1}	72.67 ± 0.001*		76.14 ± 0.018*	
WE	10 µgmL^{-1}	19.05 ± 0.025	41.85 µg/mL	16.67 ± 0.039	87.95 µg/mL
	20 µgmL^{-1}	25.24 ± 0.096		18.95 ± 0.003	
	30 µgmL^{-1}	26.19 ±0.020		20.55 ± 0.002	
	50 µgmL^{-1}	58.39 ± 0.004*		31.95 ± 0.013	
	75 µgmL^{-1}	66.46 ± 0.001*		40.10 ± 0.004*	
	100 µgmL^{-1}	67.70 ± 0.006*		74.27 ± 0.099*	
	150 µgmL^{-1}	68.94 ± 0.035*		75.94 ± 0.035*	

*p<0.05 ME: Methanol extract; WE: Water extract

3.3. Cell Viability and Proliferation Assay

The effect of different concentrations of the AHFME methanol and water extracts (10, 20, and 30µgmL^{-1}) on the cell viability and proliferation of BJ fibroblasts cells are shown in Table 3. WST-8 assay results showed that BJ cells treated with three different concentrations (10, 20, and 30 µgmL^{-1}) of the AHFME methanol extract for 24 hours, significantly increased cell viability and proliferation of BJ cells compared to control and crystalin (p<0.05). Similar results were observed in BJ cells treated with concentrations of 10 and 30 µgmL^{-1} of AHFME water extract, excluding 20 µgmL^{-1} (p<0.05) for 24h, as they were also statistically significant (Table 3). When the methanol and water extracts were compared for their effects on the cell vitality and proliferation of BJ cells, the methanol extract was found to be more effective. Crystalin (10%) did not have a significant effect on the vitality/proliferation of BJ cells (Table 3).

Table 3. Effects of methanol and water *extracts of A. hippocastanum* green fruit mesocarps on proliferation and viability of BJ human fibroblast cells.

Groups	Concentrations	Viability/Proliferation WST-8 assay at 24 h (%±SD)
Control	---	100.00±0.00
Crystalin (standart agent)	% 10	97.80±0.300
ME	10 µg/mL	121.22±0.220*
	20 µg/mL	100.14±0.187
	30 µg/mL	112.23±0.130*
WE	10 µg/mL	102.86±0.040
	20 µg/mL	97.70±0.215
	30 µg/mL	105.08±0.537

ME: Methanol extract; WE: Water extract. Data are the mean ± SD of three separate determinations. Values expressed are means ± SD of three parallel measurements (*p<0.05).

4. DISCUSSION and CONCLUSION

This study's aim is to determine the phytochemical content of the methanol and water extracts of *Aesculus hippocastanum* green fruit mesocarps (AHFME), which have not been studied much before, and also to investigate the effects of these extracts on the cell viability and proliferation of BJ cells. The results of the study showed that the AHFME methanol extracts contain phenols, tannins, flavonoids, and saponins, and do not contain alkaloids and anthraquinones. It was determined that phenols, flavonoids, anthraquinones, and saponins were present in the water extract and tannins and alkaloids were not present (Table 1). Other studies on *Aesculus hippocastanum* show that the most commonly found ingredients in the *Aesculus hippocastanum* seed extract are escin and prosapogenin. *A.hippocastanum* seeds also contain

fatty acids such as flavonoids, tannings, amines, amino acids, uric acid, phytosterol, resins, citric acid epicatechin, leukocyanidine, oleic and linoleic acid, and kaempferol derivatives (Makuch & Matlawska, 2013). Leaf extracts include carbohydrates and anthocyanin (Paterska *et al.,* 2017; Idris *et al.,* 2020), while flowers contain coumarins such as esculin, esculetin, scopoletin, and fraxetin. *A. hippocastanum* immature fruit pericarps have been reported to contain saponins, flavonoids, and other phytochemicals (Ertürk, 2017).

Antioxidants inhibit the polymerization chains caused by substances that delay oxidation, free radicals, and other subsequent oxidizing reactions (Halliwell &Aruoma, 1991). When plants are exposed to extreme conditions, they activate the antioxidant mechanisms linked to the synthesis of phenolic compounds such as flavonoids, tannins, and other secondary metabolites (Oscar *et al.,* 2020). These chemicals are produced by plants to defend themselves from microorganisms and oxidative stress. However, nowadays some data indicate that these phytochemicals also often shield humans from different diseases. Some of the medicinal plants' positive effects are due to their high polyphenolic contents. Such compounds minimize oxidative cellular stress, which plays an important role in the pathogenesis of various diseases, including cancer and cardiovascular diseases (Sagdıcoglu Celep *et al.,* 2012).

Low IC_{50} values show a high activity antioxidant which means that the specific dissolvent mixture allowed enormous amounts of metabolites with antioxidant activity (Feghhi-Najafabadi *et al.,* 2019; Aslantürk *et al.,* 2017; Aşkın Çelik & Aslantürk, 2018; Uzunhan & Aşkın Çelik, 2018). The results of the DPPH scavenging assay revealed that the fruit mesocarps methanol extract from *A. hippocastanum* possesses a higher DPPH radical scavenging activity than that of the water extract. In this study, we examined the crude extract of AHFME to identify its antioxidant activity, but the effects of the individual molecules it contains were not examined.

The phosphomolybdate assay provides a method for reducing MoVI to phosphate/MoV complex by electron transport. This reduction will affect many natural products, including phenols and flavonoids (Ahmed *et al.,* 2015). In our analysis, concentrations of the methanol extracts showed a higher overall antioxidant capacity than of the water extract (Table 2). This difference in activity among extracts may be because of the phytochemical variations in the extracts. Additionally, the solvent polarities used for extractions are distinct, since methanol is an organic solvent, and water is an inorganic solvent. Such solvent properties may have contributed to a difference between the extracts in their overall antioxidant efficiencies.

Nowadays *in vitro* cytotoxicity and/or cell viability analyses, have appeared as an alternative to animal experiments and have been more favored because of their ease of use, speed, standardization, low cost, and compatibility with findings of *in vivo* studies (Aslantürk, 2018). Tetrazolium-based analyses (i.e., MTT, MTS, XTT, WST-1, and, WST-8 assays) measuring cytotoxicity via mitochondrial activity are commonly used to evaluate cell proliferation, cell viability (Taşkın *et al.,* 2020), and drug cytotoxicity, especially in cancer cells studies (Berridge *et al.,* 2005). The WST-8 test kit is an easy-to-use tool for evaluating the activation or inhibition of cell proliferation in an *in vitro* model. The test is based on NADH's extracellular reduction of WST-8, provided by transporting trans plasma membrane electron and electron mediator in the mitochondria. WST-8 reduction creates a water-soluble formazan that dissolves directly into the medium of culture, reducing the need for a further stage of the solution. WST-8 is more stable and less cytotoxic than the other salts of tetrazolium, making it particularly useful for longer incubation times, and its sensitivity detection is higher than of other tetrazolium salts (Aslantürk, 2018).

The effect of AHFME of ME and WE extracts on the proliferation and viability of BJ cells are present in Table 3. WST-8 test results showed that the AHFME methanol and water extract were not cytotoxic on BJ cells, except for 20 μgmL^{-1} concentration of the water extracts.

Many phytochemicals show a non-monotonic dose/concentration-response called a biphasic dose-response, with dose and time-dependently. These phytochemicals, which cause biologically opposite effects at different doses and/or durations, are called hormetic compounds (Mohapatra *et al.,* 2015). In cells treated with extracts, the reason for the concentration-independent increase and decrease in proliferation may be the hormetic compounds present in the extracts.

Crystalin (10%) also does not have an important effect on cell viability/proliferation. Interestingly, the ME and WE extract exhibited the highest antiproliferative effect on BJ fibroblast cells depending on the dose. The mitogenic effect is important for fibroblasts, which are important cells involved in wound healing and the production of extracellular matrix components (EMC). A study by Sagdıcoglu Celep *et al.,* (2012) found that the *A. hippocastanum* bark extract (0.01 mg/mL) increased cell proliferation in 3T3 healthy fibroblast cells (120%). The data obtained in our study are similar to the findings of that study. There are study examples showing that *A. hippocastanum* seed extracts have anticancer effects and inhibit proliferation. As the cell proliferation phase progresses, fibroblasts become the predominant cells at the wound site and play an important role in wound contraction to restore the integrity of injured tissue (Aksoy, 2020). Especially, for the wound-healing phase, fibroblast concentration, proliferation and migration are very significant (Addis *et al.,* 2020). Dermal fibroblasts are injury-responsive protective cells. Fibroblast proliferation and movement to the region where significant events occur in the wound healing process (Addis *et al.,* 2020) and stimulation of the development of fibroblast cells are a valuable models for investigating *in vitro* activities of wound healing. The data we obtained as a result of our study showed that the AHFME methanol and water extracts increased proliferation in BJ fibroblast cells. These results suggest that AHFME extracts may have wound-healing effects and it may be important to conduct new studies on this subject.

Aesculus hippocastanum L. seed extracts are known to heal venous ulcers, increase contraction force in fibroblasts, and have antioxidant and anti-aging effects. However, to our knowledge, there are not many *in vitro* studies to evaluate the phytochemical content, antioxidant activity, and effect of *A. hippoastanum* green fruit mesocarps extracts on cell viability and proliferation. The results of this study show that horse chestnut fruit mesocarps extracts have high antioxidant content and increase cell proliferation.

The use of natural antioxidants as a potential preventive for free-radical mediated diseases has become a very important issue for improving the quality of life. Different studies demonstrate the significant antioxidant activity of the ethanolic extract of *A. hippocastanum* in both the models utilized for the free radical scavenging activity. The antioxidant activity of different extracts obtained from *A. hippocastanum* can be attributed to the presence of different phenolic compounds found in extracts and the synergistic effects of other compounds. The results of this study showed that green fruit mesocarps extracts of *A.hippocastanum* have antioxidant activity and may also be useful in evaluating these parts, which are not usually used as waste products. Further studies are required to isolate, identify and elucidate the structure of the bioactive compound particularly responsible for the antioxidant activity of this plant. In addition, the extracts used in our study increased the proliferation of BJ cells *in vitro*. Further *in vitro* and *in vivo* studies are required to determine the effects of these extracts on wound healing and tissue regeneration.

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

Tulay Askin Celik has designed the study, collected the data, reviewed and edited the manuscript. **Ozlem Sultan Aslanturk** has performed laboratory analysis and statistical analysis of the study.

Orcid

Tulay Askin Celik https://orcid.org/0000-0001-6891-9089
Ozlem Sultan Aslanturk https://orcid.org/0000-0002-2503-0164

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Research Article

The antifungal effect of propolis extract against cotton wild disease (*Verticillium dahliae* Kleb.)

Melike Mutlu Yilmaz[1,*], **Yesim Kara**[1], **Oktay Erdogan**[2]

[1]Department of Biology, Faculty of Science, Pamukkale University, Kımıklı Campus, 20070, Denizli, Türkiye
[2]Department of Organic Farming Business Management, Faculty of Applied Sciences, Pamukkale University, 20680 Çivril, Denizli, Türkiye

Abstract: The aim of this study is to investigate the antifungal activity of propolis against *V. dahliae* Kleb. under both *in vitro* and *in vivo* conditions. Firstly, the inhibitory effect of the propolis on mycelial growth in Potato Dextrose Agar (PDA) media containing its ethanol extract (PE) at various concentrations (0.003, 0.06, 0.125, 0.25, 0.5, and 1 ppm/mL) was investigated under *in vitro* conditions. Then to assess the effect of PE on non-defoliating (PHCVd3 isolate) and defoliating (PHCVd47 isolate) pathotypes of *V. dahliae*, the varieties Giza 45 (resistant), Carmen (tolerant), and Acala SJ2 (susceptible) treated with PEE (1 ppm/mL) were observed in the plant growth chamber up to the 4-6 leaf stage. The whole *in vitro* experiments were carried out with three replicates, and the studies *in vivo* experiment were with five replicates depending on a completely randomized parcels design. The most effective dose of PEE with1 ppm/mL dose resulted in 75.2% suppression against the PHCVd3 isolate, while the effect of the same dose against the PHCVd47 isolate was 74.4%. The lowest disease severity index (DSI) values against PHCVd3 and PHCVd47 isolates in cotton cultivars treated with PE were 1.34 and 1.64 in the Giza 45, respectively, and the highest DSI values were 3.80 and 3.90 in the Acala SJ2 cultivar *in vivo* experiment, respectively. The findings indicate that PE treatment has a promising effect against cotton wilt disease that could be combined with known plant protection strategies.

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1. INTRODUCTION

Cotton (*Gossypium* spp.) is an industrial crop grown worldwide in tropical and subtropical warm-climate regions. Cotton fiber is used as a raw material for the textile industry, and cotton pulp and seed husks are used as animal feed. In addition, the seed's residual linter is utilized by the cellulose and chemical industries, the military industry, and the filling business (Gokdogan *et al.,* 2016). Cotton is grown on 35 million hectares in around 90 countries worldwide, and an average of 26.7 million tons of lint cotton is produced in these areas. Türkiye is the sixth largest cotton producer in the world after India, China, the United States, Brazil, and Pakistan (USDA, 2021). In Türkiye, cotton is grown in 477.000 hectares in 4 main regions (Southeastern

*CONTACT: Oktay Erdogan ✉ oktaye@pau.edu.tr Department of Organic Farming Business Management, Faculty of Applied Sciences, Pamukkale University, 20680 Çivril, Denizli, Türkiye

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Anatolia, Aegean, Çukurova, and Antalya), yielding 2.2 million tons of seed cotton yield. Some nations, including Türkiye, produce around 80% of the cotton used worldwide (TSI, 2021).

Wilt disease caused by *Verticillium dahliae* Kleb is one of the stress factors affecting yield and fiber quality traits in cotton cultivation (El-Zik, 1985). *V. dahliae* Kleb. causes wilting of more than 400 plant species (vegetables, legumes, ornamental plants, industrial plants, fruit trees and weeds, and so on), especially cotton (Berlanger & Powelson, 2000). In regions where cotton is grown, the pathogen can remain in the soil as microsclerotia for about 15 years and cause a wilt disease that can lead to significant yield losses (Chen *et al.,* 2016).

Nowadays, places where cotton is grown have both defoliating and non-defoliating pathotypes of the disease. The non-defoliating pathotype results in less leaf shedding by inducing wilting, while the defoliating pathotype causes the cotton plants to shed their leaves completely and die (Bejarano-Alcazar *et al.,* 1995). Of two pathotypes detected in our country, 93% of the defoliating pathotype is in the Aegean region, and 77% of the non-defoliating pathotype has been reported in the Çukurova and Southeastern Anatolia regions (Göre, 2007).

First, the fungus blocks the movement of water and other minerals from the root to the leaves and tissues. Then it causes wilting, desiccation, reduced photosynthesis, shedding of small bolls, and changes in yield and fiber quality characteristics, starting with the lower leaves (Agrios, 2005). The disease reported causes a yield loss of 480 million bales in the US between 1990 and 2014 (Lawrence *et al.,* 2016).

Currently, there is no effective and economical chemical control against Verticillium wilt. Alternative control methods are necessary for the control of the disease. Propolis and bee products with antimicrobial properties are considered to be one of the alternative measurements against plant pathogens. Understanding of the interaction between disease agent and the host plant is important in view of the disease control (Koral & Türktaş, 2018).

Apitherapy is one of the ways of using bee products to treat or prevent diseases from ancient to modern times. Beeswax, honey, honey milk, pollen, bee larvae, bee venom, and propolis are some bee products used in apitherapy. The propolis contains a large number of active chemicals as it exhibits a variety of biological and pharmacological activities, including antibacterial, antifungal, antiviral, antitumor, and anti-inflammatory effects (Kujumgiev *et al.,* 1999; Basim *et al.,* 2006; Pereira *et al.,* 2008). In general, raw propolis consists of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen, and 5% various other substances including organic residues (Cirasino *et al.,* 1987). The ethanolic extract of propolis (EEP) is the most widely used preparation and over 200 compounds have been identified (Burdock, 1998). Galangin, caffeic, gallic acid, and quercitin are just a few examples of the flavonoids and aromatic acids found in propolis and its preparations that are beneficial in their biological action. Lisa *et al.* (1989) reported that propolis ethanol extract inhibited the growth of 60 yeast isolates and 38 fungal isolates. Some researchers have reported in their studies that propolis has antifungal effects against various plant-pathogenic fungi (Yanar *et al.,* 2005; Özdemir *et al.,* 2010; Curifuta *et al.,* 2012; Manty *et al.,* 2014; Manty, 2015; Araujo *et al.,* 2016; Er, 2021; Çakar *et al.,* 2022). Hegazi *et al.* (2014) reported that geography, plant species, and harvest timing all impact the propolis's biological activity.

This study aims to investigate the antifungal activity of propolis collected from Muğla region against defoliating and non-defoliating pathotypes of Verticillium wilt (*V. dahliae* Kleb.) under both *in vitro* and *in vivo* conditions.

2. MATERIAL and METHODS

2.1. Plant Material, Fungal Pathogen and Propolis Sample

Cotton varieties resistant to Verticillium wilt Giza 45 (*Gossypium barbadense* L.), tolerant Carmen (*Gossypium hirsutum* L.), and susceptible Acala SJ2 (*G. hirsutum*) were used as plant material (Bolek *et al.,* 2005; Erdoğan *et al.,* 2014). The raw propolis of the study was sourced from Muğla province in 2021. Isolates of PHCVd3 (non-defoliating pathotype) and PHCVd47 (defoliating pathotype) were obtained from the Plant Protection Laboratory, Department of Plant Protection, Hatay Mustafa Kemal University.

2.2. Preparation of Propolis Ethanol Extract (PEE)

Raw propolis was purchased from Muğla, frozen at -18°C in the laboratory condition, and then chopped into tiny pieces while still frozen. It was first made from a 1:3 mixture of propolis and ethyl alcohol, which was ground up in a blender for 2 minutes before being homogenized in an ultrasonic bath for two days. Then it was prepared from 80% ethyl alcohol (80 mL ethyl alcohol + 20 mL clean water (Rios) = 100 mL). Inverting and mixing at least twice daily, the homogenized mixture was kept in a dark area for five days. At the end of this time, the extract was filtered using Whatman No. 1 filter paper, and PEE was prepared by isolating the propolis components from the wax. The alcohol in each combined filtrate was concentrated by rotary evaporation using an IKA RV10-Germany rotary evaporator and then cooled to +4°C.

2.3. Calculation of Total Phenolic Substance, Total Antioxidant Activity, and Total Flavonoid Content of PEE

According to Oruç *et al.* (2021) separation of phenolic compounds in propolis samples and high-performance liquid chromatography (HPLC) analyses were performed. A C18 column (Inertsil ODS-3.5 mm, 4.6 x 150 mm) was used to separate the propolis samples.

By reducing the ferric ion in the presence of antioxidants, the FRAP technique (Ferric Reduction/Antioxidant Power) becomes (Fe(III)-TPTZ-2,4,6-Tris(2-Pyridyl)-S-Triazine)-based on TPTZ synthesis. To do this, 100 mL sample and 3 mL of the FRAP reagent (300 mM pH 3.6 acetate buffer, 10 mM TPTZ and 20 mM $FeCl_3$ (10:1:1) were combined, and after 4 minutes this combination formed at a maximum absorbance of 593 nm (Benzie & Strain, 1999). The standard graph was created with different concentrations of $FeSO_4.7H_2O$ (31, 25, 62.5, 125, 250, 500 and 1000 M). Results are presented as antioxidant potency equivalent to $FeSO_4.7H_2O$.

The 4-keto and C-3 or C-5 hydroxyl groups (or both) of the flavonoids combine to form a stable acidic complex that forms the basis of the Fukumoto and Mazza method, also known as the aluminum chloride colorimetric method (Fukumoto & Mazza, 2000). The standard graphic was created using quercetin at different concentrations (1, 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.015625 mg/mL). The amount of flavonoid substance corresponding to quercetin was detected, as shown by the graphic constructed using absorbance values at 415 nm versus concentration.

2.4. Determination of the Antifungal Effect of PEE *In Vitro* on Pathotypes of *Verticillium dahliae*

To evaluate the mycelial growth in PDA (Difco) media containing propolis at various concentrations (0.003, 0.06, 0.125, 0.25, 0.5 and 1 ppm/mL) under *in vitro* conditions, the effect of PEE on both *V. dahliae* pathotypes was noted. A variable concentration of propolis was added to the sterilized PDA medium before being dispensed in 20 mL portions into sterilized Petri plates (100 mm). PDA medium with PEE was kept at room temperature for 24 hours. After that, propolis was used to inoculate the 7-day-old cultures of both pathotypes of *V. dahliae* grown in PDA medium with 5 mm mycelial discs cut with a mushroom drill. Petri dishes were

cultured at 24 ± 1ºC for 7-10 days. Only the pathogen was inoculated into the control Petri plates. Growth inhibition rates were separated using calipers after the pathogen had grown in the control plates and the treated petri plates, *in vitro* experiment was performed using a fully randomized parcels design with three replicates, and was replicated twice. The following formula was used to calculate the level of antifungal activity of propolis (Deans & Svoboda, 1990).

Percentage of mycelial growth inhibition (%) = [(dc-dt/dc)] x 100

where dc is the average mycelial growth diameter in the control petri plate (mm) and dt is the average mycelial growth diameter in the treatment petri plate (mm).

2.5. Determination of the Antimicrobial Effect of PEE *In Vivo* against *Verticillium dahliae*

The *in vivo* pot experiment included drilled, fungicide-free seeds of cotton varieties resistant Giza 45, tolerant Carmen, and susceptible Acala SJ2. First, the autoclave-sterilized (1 hour at 121°C) soil mixture (1/3 soil + 1/3 sand + 1/3 peat) was filled into sterile plastic pots (10 cm diameter). Then 4 cotton seeds were planted in each pot (2 mL/seed) coated with the effective dose of propolis extract (1 ppm/mL). Pots were cultivated in a plant development plant growth chamber with 12 hours light and 12 hours dark at 24 ± 1ºC. Then, when the cotton seedlings reached the cotyledon stage, thinning was performed and one seedling was left in each pot. The plant maintenance procedures were completed on schedule and the cotton seedlings were grown until they had 4-6 leaves.

To determine the susceptibility of PEE-coated cotton cultivars to *V. dahliae* (Erdoğan *et al.,* 2014) two-week-old spores cultured in broth medium (0.01 g $FeSO_4.7H_2O$, 0.5 g $MgSO_4.7H_2O$, 2 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g KCl, and 7.5 g sucrose, 1 L sterile distilled water) cultured isolates of PHCVd3 and PHCVd47 were filtered through 2 layers of cheesecloth and mycelium and pieces of agar were removed from the suspension and then the spore concentration was adjusted to 4×10^6 spores/mL using a Thoma slide in the light microscope (Leica) and used for the inoculation of cotton plants. The plants were transplanted into new plastic pots (10 mL) with spore solution when they reached the 4-6 leaf stage. Only sterile distilled water was added to the plastic pots bottoms as a control. The pot experiment was performed with five replicates in a fully randomized parcels design in the plant growth room (under a 12-hour light/12-hour dark cycle at 24 ± 1ºC). The severity of the disease on cotton plants was evaluated by the wilt index according to the percentage of affected leaves using a scale of 0 to 5 (0 = no symptoms, 1 = chlorosis in the lower leaves, 2 = moderate (30-50% of the leaves) wilt with severe chlorosis, 3 = moderate wilting and necrosis, 4 = severe (more than 50% of leaves) wilting and necrosis, 5 = dead plant) after about 3-5 weeks (Tsror *et al.,* 2001). The following formula was used to calculate the Disease Severity Index (DSI) value caused by *V. dahliae* and the data obtained from the pot experiment were transformed using Arcsin (Karman, 1971).

$$DSI= (a \times 0) + (b \times 1) + (c \times 2) + (d \times 3) + (e \times 4) + (f \times 5) / M$$

where a, b, c, d, e, and f are the plant numbers with degrees 0, 1, 2, 3, 4, and 5, respectively, and M is the overall plant number.

2.6. Statistical Analysis

The data were analyzed by performing the ANOVA (one-way analysis of variance). Statistically significant differences between mean values were determined using Least Significant Differences (LSD) Test ($p \leq 0.01$). All statistical analyses were performed using JMP software version 13 (SAS Institute Inc., Cary, NC, USA).

3. RESULTS

3.1. Antifungal Effect of PEE on Mycelial Growth of Pathotypes of *Verticillium dahliae*

The effects of PEE concentrations on mycelial growth and inhibition rates of non-defoliating (PHCVd3 isolate) and defoliating (PHCVd47 isolate) pathotypes under *in vitro* conditions are given in Table 1. The PEE doses were found to be significant according to the statistical analysis results ($p \leq 0.01$) of the *in vitro* experiment. At various concentrations, PEE reduced mycelial growth in both non-defoliating (PHCVd3 isolation) and defoliating pathotypes (PHCVd47 isolate). PEE doses showed percentage inhibition rates of the non-defoliating pathotype (PHCVd3 isolate) ranging from 18.30 to 75.20%. The 1 ppm/mL dose produced the most potential antifungal effect (75.20%), followed by the 0.5 ppm/mL dose (61.80%). The defoliating pathotype (PHCVd47 isolate) showed inhibition rates from PEE dosages between 13.00 and 74.40%. The highest antifungal effect was determined at 1 ppm/mL dose (74.40%), followed by 0.5 ppm/mL dose (61.00%). Compared to the other treated petri dishes, the mycelium diameter in the control petri dish was statistically different (Table 1).

Table 1. Antifungal effect of PEE on mycelial growth of pathotypes of *Verticillium dahliae*.

Concentration (ppm/mL)	PHCVd3 isolate		PHCVd47 isolate	
	Mycelial growth (mm)*	MGI	Mycelial growth (mm)*	MGI
0.003	16.17 B	18.30	17.25 B	13.00
0.06	15.08 C	23.90	15.17 C	23.70
0.125	11.50 D	42.00	11.67 D	41.30
0.25	9.00 E	54.60	9.17 E	53.90
0.5	7.58 F	61.80	7.75 F	61.00
1	4.92 G	75.20	5.08 G	74.40
Control	19.83 A	0.00	19.88 A	0.00
CV $_{(0.01)}$	2.43		2.32	
LSD	0.52		0.50	

Each observation is based on three replicate plates. Arcsine transformation was performed prior to statistical analysis. *Mean values followed by different letters within the column are significantly different according to LSD Test ($p \leq 0.01$). MGI: Mycelial growth inhibition rate (%)

3.2. Determination of The Antimicrobial Effect of PEE *In Vivo* against *Verticillium dahliae*

The effects of the effective dose of PEE (1 ppm/mL) on PHCVd3 and PHCVd47 isolates of *V. dahliae* Kleb. in disease-resistant Giza 45, tolerant Carmen and susceptible Acala SJ2 cultivars under pot conditions are given in Table 2. The statistical analysis of the pot experiment's data revealed that cotton variety differences were significant ($p \leq 0.01$). The range of 1.34 to 3.80 was discovered for the mean disease severity index values in cotton cultivars treated with PEE for the PHCVd3 isolate. The lowest disease severity index value was found in the resistant Giza 45 variety (1.34) and the tolerant Carmen variety (1.82), and these varieties were statistically in the same group. The mean disease severity index values for the PHCVd47 isolate ranged from 1.64 to 3.90. The resistant Giza 45 variety came in first with a disease severity index value of 1.64, followed by the tolerant Carmen variety (2.32). When both disease pathotypes were present, the Acala SJ2 cultivar had the highest Disease Severity Index value (3.80-3.90) (Table 2).

Table 2. Disease severity index values in cotton plants treated with PEE after PHCVd3 and PHVd47 inoculation.

Variety	PHCVd3 isolate DSI*	PHCVd47 isolate DSI*
Acala SJ2 (Susceptible)	3.80 A	3.90 A
Carmen (Tolerant)	1.82 B	2.32 AB
Giza 45 (Resistant)	1.34 B	1.64 B
CV $_{(0.01)}$	3.25	4.34
LSD	1.10	1.66

Each observation is based on five replicate plates. Arcsine transformation was performed prior to statistical analysis. *Mean values followed by different letters within the column are significantly different according to LSD Test ($p \leq 0.01$). DSI: Diseases severity index value.

3.3. Total Phenolic Analysis, Total Antioxidant Analysis and Total Flavonoid Substance Amount Values of Propolis

The propolis samples used in the HPLC-DAD analyses of the studies are given in Table 3. It was determined that propolis samples contained high levels of phenolic chemicals. According to the HPLC-DAD results of propolis, phenological chemicals such as galangin, pinosembrine, quercetin, chrysin and naringenin were found in significant amounts (Table 3).

Table 3. HPLC-DAD analysis results of propolis.

Identified phenolic compounds	Amounts found (µg/mL) *
Gallic acid	30.28
Epigallocatechin gallate	24.34
Caffeic acid	292.55
p-Coumaric acid	116.68
trans-Ferulic acid	86.00
trans-Isopherulic acid	225.25
3-4-Dimethoxycinnamic acid	142.16
Quercetin	468.02
trans- Cinnamic acid	44.29
Naringenin	367.28
Apigenin	287.01
Kaempferol	172.73
Krisin	419.76
Pinosembrine	958.08
Galangin	959.83
Caffeic acid phenethyl ester	2102.26
trans- Chalcone	443.85

*Analysis results include µg/g amounts of liquid propolis in 1 mL.

The total antioxidant capacity of propolis used in the study is given in Table 4. Table 4 shows that propolis has a high level of overall antioxidant ability.

Table 4. Total antioxidant capacity of propolis.

Sample	Total antioxidant capacity FRAP
liquid ethanolic propolis	222.85±1.67 Mmol $FeSO_4.7H_2O$/mL
Raw propolis	197.79±2.593 (mmol $FeSO_4.7H_2O$/g)

FRAP: Ferric reducing/antioxidant power.

The results of the total content of flavonoids in propolis are given in Table 5. Flavonoids, which are the main source of antioxidants, have analytical results suggesting that the propolis used in the study has an effective content (Table 5).

Table 5. Total amount of flavonoid substance of propolis.

Sample	Total amount of flavonoid substance mg QE/g
liquid ethanolic propolis	10.18±0.06 mgGAE/mL
Raw propolis	4.779±0.140 mgQE/g

4. DISCUSSION and CONCLUSION

In vitro studies conducted in petri dishes the various doses of PEE inhibited both pathotypes to varying degrees. The highest antifungal effect was obtained at 1 ppm/mL dose between 75.20% and 74.20% in non-defoliating (PHCVd3 isolate) and defoliating (PHCVd47 isolate) pathotypes, respectively. In a similar study Kurt and Şahinler (2003) reported that increasing concentrations of PEE reduced mycelial growth of the pathogens tested and the effect of PEE on *V. dahliae* was 84.8% and 83.3% at 1.0 and 0.5 ppm concentrations, respectively. Gallez *et al.* (2014) have shown the inhibitory effect of the propolis ethanol extract (PEE) which inhibited mycelial growth by 70-78% of *Didymella bryoniae* and *Rhizotocnia solani in vitro*. They have suggested to have its fungistatic effect. Abd-El-Kareem *et al*. (2017) have reported that the antifungal effect of PEE increased with increasing doses, and 10% EEP dose inhibited sclerotid germination by 91% of *Sclerotinia sclerotiorum*. In another study, 3% propolis ethanol extract strongly inhibited the mycelial growth of the green mold disease agent *Penicillium digitatum* in lemon, and an inhibition zone was formed (Abo-Elyousr *et al*., 2021). Türk *et al*. (2022) showed that mycelial growth of *F. oxysporum* decreased depending on PEE concentrations increased, PEE collected from Muğla at the highest concentration (50 ppm) was 77.81% against *F. oxysporum*, and PEE collected from Denizli had the lowest antifungal effect (64.52%). The reason for the varying antifungal effects of propolis extract against different or same pathogens is due to its chemical content, which is significantly depending on the plant flora where the propolis content has been supplied (Ali & Kunugi, 2020).

As known in previous studies, PEE was used against many phytopathogenic fungi *in vivo*, whereas fewer studies were conducted against *V. dahliae*. Soylu *et al*. (2008) have reported, all doses of PEE prepared in 70% ethanol prevented disease emergence in citrus fruits artificially infected with *Penicillium digitatum*, PEE at 100 mg/mL concentration reduced natural disease emergence by 100% *in vivo* conditions. In a previous study, the results showed that 3% dose of Iraqi origin PEE prevented the rot caused by *P. digitatum* on oranges for three weeks at room temperature (Matny, 2015). In another study, the effectiveness of PEE increased as the concentration increased. The studies carried out *in vivo* conditions indicated that 5% concentration of PEEs completely prevented fruit infections, 3% and 5% concentrations of PEE were also effective against quince brown rot disease (*M. fructigena*) that can be used as an edible coating (Özyiğit *et al.,* 2018). Er (2021) tested PEE under *in vivo* conditions. An alcohol-based propolis extract at a concentration of 60 mg/mL applied to seeds seed + foliar cabbage showed up to 97.9% protection against *A. brassicola*. Spraying seeds + leaves with the same concentration of water-based propolis extract gave the highest antimicrobial effect with of

91.6% inhibition. The raw propolis used in the study was rich in view of phenolic compounds, high in total antioxidant capacity and, effective in total flavonoid substance content. In the studies carried out, propolis has been reported as inhibitory compound against selected plant pathogenic fungi due to the antifungal properties of phenolics, flavonoid aromatic acids in its chemical composition (Bancova *et al.,* 2000). Kordali *et al.* (2009) stated that terpenes have an antifungal effect against plant pathogenic fungi and this effect changes depending on the type and structure of the molecule. In the studies carried out on the chemical composition of propolis, researchers reported that propolis contains chemical compounds such as myristic acid, benzoic acid, benzyl alcohol, vanillin, cinnamic acid, pinosembrin, pinobanksin, quercetin, galangin, apigenin, chrysin, caffeic acid, acacetin, campheride, and isovaniline (Burdock, 1998; Salomão *et al.,*2004; Uzel *et al.,* 2005). Keskin & Kolaylı (2018) reported that the total phenolic content of Anatolian propolis ranges between 16.13-178.34 mg GAE/g for raw propolis. In a similar study conducted, the total phenolic content of propolis obtained from different regions of Anatolia was found to be between 2748 mg GAE/100 g and 19969 mg GAE/100 g (Ozdal *et al.,* 2019). Our findings revealed that samples with high total phenolic content had also high antioxidant effects. In accordance with our findings, Aygun (2017) reported that although the chemical composition of propolis is complex, its antimicrobial effect is due to flavonoids from phenolic acids, phenolic acid esters and terpenes.

In the pot experiment, seed application of PEE suppressed the non-defoliating and defoliating pathotypes of Verticillium wilt disease agent. PEE was a promising treatment against both pathotypes of *V. dahliae* on the resistant Giza 45 cultivar and then in the tolerant Carmen cultivar, which was determined according to the disease severity index values. In this context, the combination of resistant variety + propolis can be suggested against Verticillium wilt disease as biological control, which is the best alternatives within the scope of integrated control. However, we need detailed studies related to assessing the effective dose of PEE in cotton varieties and Verticillium wilt under field conditions. The results of the propolis analysis showed that it is rich in phenolic compounds with a high total antioxidant capacity, and that the total amount of flavonoid substances is the reason for being effective against the pathogen. However, no studies have been conducted on the propolis extract's mechanism of action. For this purpose, detailed studies should be carried out to determine the mechanism of action and plant growth promoting effects of PEE, which is found to be effective. The study is pioneer to determine the effects of PEE to cotton seed against Verticillium wilt under pot conditions. Seed coating studies carried out with simple laboratory facilities are both effective and its application commercially uncomplicated. Therefore, the results obtained from the study will shed light on biological control studies for the future.

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Declaration of Conflicting Interests and Ethics

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

Authorship Contribution Statement

Melike Mutlu Yilmaz: Literature survey, Data collection, Edit data. **Yesim Kara:** Methodology, Supervision, Resources, Reading and Editing of article. **Oktay Erdogan:**

Methodology, Supervision, Data analyses, Writing, Reading and Editing the original draft of the article.

Orcid

Melike Mutlu Yilmaz https://orcid.org/0000-0002-8510-2107
Yesim Kara https://orcid.org/0000-0002-7027-3667
Oktay Erdogan https://orcid.org/0000-0003-1466-3035

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The botanical study, phytochemical composition, and biological activities of *Laurus nobilis* L. leaves: A review

Yazid Khaled Khodja[1,2], Mostapha Bachir-bey[3,*], Messaoud Belmouhoub[4], Rachid Ladjouzi[5], Farid Dahmoune[6,7], Bachra Khettal[2]

[1]Department of Biology, Faculty of Natural Sciences and Life, Ziane Achour University, Djelfa, Algeria
[2]Laboratory of Plant Biotec. and Ethnobotany, Fac. of Natural Sci. and Life, University of Bejaia, Bejaia, Algeria
[3]Laboratory of Applied Biochemistry, Department of Food Sciences, Faculty of Natural Sciences and Life, University of Bejaia, Bejaia, Algeria
[4]Department of Medicine, Faculty of Medicine, University of Constantine 3, 25000 Constantine, Algeria.
[5]Laboratory of Applied Microbiology, Dep. of Microbiology, Fac. of Natural Sci. and Life, Uni. of Bejaia, Algeria
[6]Department of Biology, Faculty of Natural Sciences and Life and Earth Sciences, University of Bouira, Algeria
7Laboratory of Biomathematics, Biochemistry, Biophysics and Scientometrics, Fac. of Natural Sciences and Life, University of Bejaia, Bejaia, Algeria

Abstract: *Laurus nobilis* is native to the southern Mediterranean region. It is a small tree from the Lauraceae family. The leaves of *L. nobilis* are the most exploited part of the plant due not only to the high produced quantity but to the large benefits and extensive use in different fields including culinary, cosmetic, therapeutic, and pharmacologic. The various beneficial health properties attributed to bay leaves are related to the presence of various bioactive compounds. Chemically, they contain numerous essential elements, some vitamins, and many secondary metabolites such as essential oils (cineole, linalool, and eugenol), phenolic compounds, particularly phenolic acids (ferulic, protocatechuic, and caffeic acids, etc.) and flavonoids (such as catechin, kaempferol, apigenin, quercetin, and their derivatives), and alkaloids (noraporphins and aporphins). Laurel leaves are not only used to flavor dishes, but present several beneficial properties that justified their traditional use against numerous illnesses, particularly for rheumatism, indigestion, and diarrhea. Bay leaves are an essential component of several industrial applications including agrifoods, cosmetics, and pharmaceuticals. Due to the presence of cited chemical constituents in bay leaves, various biological and pharmacological properties have been reported such as antioxidant, antibacterial, fungicidal, antiviral, insecticidal, wound healing, antimutagen, anticonvulsant, analgesic, anti-inflammatory, and immunostimulatory activities. This review provides an overview of *L. nobilis* leaves, beginning with botanical aspects, including its preparation and composition, followed by a discussion about the most abundant bioactive compounds, and finally the traditional uses and therapeutic effects.

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*CONTACT: Mostapha Bachir-bey ✉ mostapha.bachirbey@univ-bejaia.dz Laboratory of Applied Biochemistry, Department of Food Sciences, Faculty of Natural Sciences and Life, University of Bejaia, Bejaia, Algeria

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1. INTRODUCTION

The use of plants for therapeutic purposes is a growing practice. Although the use of medicinal plants is continuously increasing, there the use of plants for therapeutic purposes is a growing practice. Despite the large number of investigations on medicinal plants, there are still many aspects to explore in this area (Ahmad *et al.*, 2022; Barroso *et al.*, 2018; Elefe, 2021). Besides their content of important nutrients, plants also synthesize bioactive compounds that have preventive properties against various diseases. These plants are now industrially transformed into various products for pharmaceutical, food, perfume, and cosmetic uses (Ambrose *et al.*, 2016; Yasin *et al.*, 2020).

Condiments are used to add flavor to foods and for preservation purposes due to their antioxidant and antibacterial properties. Condiments such as bay laurel are widely used in various products (Morais *et al.*, 2009). The noble laurel, much appreciated for the condiment benefit of its leaves, is one of the aromatic plant species, which is experiencing a resurgence of interest for its use in traditional medicine and the pharmaceutical, agrifood, and cosmetic industries. The increased demand for products resulting from the use of bay leaves has significantly increased its global production (Chaaben *et al.*, 2015).

Laurel (*L. nobilis*, family Lauraceae) is an evergreen tree that has been used for 1000 years and is an essential ingredient in cooking and many traditional uses (Hanif *et al.*, 2020). The leaves are used in fresh or dried form to flavor culinary preparations and scented and aromatic essential oil in perfumery. Laurel has been traditionally used for years in traditional medicine, due to its various pharmacological activities, including antimicrobial, antioxidant, anticancer, insecticide, and antifungal (Bendjersi *et al.*, 2016; Nabila *et al.*, 2022; Zibi *et al.*, 2022). This tree is native to the southern Mediterranean region. It is cultivated commercially for its aromatic leaves in Algeria, Türkiye, Morocco, Portugal, Spain, Italy, France, and Mexico. It is widely cultivated in Europe and the United States as ornamental (Guenane *et al.*, 2016).

L. nobilis is a widely studied medicinal plant that is the subject of numerous studies concerning its phytochemical compounds (Caputo *et al.*, 2017; Chahal *et al.*, 2017; Khaled Khodja *et al.*, 2021) and its therapeutic virtues such as the treatment of several neurological pathologies, dermatological and urological as well as gastrointestinal diseases such as epigastric bloating, impaired digestion, gas and belching (Khaled Khodja *et al.*, 2020). To gather recent knowledge concerning *L. nobilis*, this review is devoted to the phytochemical composition and traditional uses of bay leaves.

2. BOTANICAL STUDY

2.1. Origin

L. nobilis is native to the Mediterranean region (Buto *et al.*, 1990). The ancient Greeks and Romans used it as a condiment and medicine. In Greek mythology, it was considered sacred, which is why in ancient Greece, receiving a wreath made of bay leaves was considered an honor. Olympic winners, poets, victors, and heroes received the crown to wear on their heads. This habit was also accepted by the Romans (Ballabio & Goetz, 2010).

The name *Laurus* was derived from the Latin word "*laureola*", which means laurel wreath. The word "baccalaureate", whose Latin root comes from bacca lauri meaning bay of laurel, refers to the laurel wreath offered to heroes in antiquity. The Romans spread the species to parts of Europe; the first settlers introduced it to the New World. Today, the species is cultivated in the Mediterranean region, Russia, Central America, and, the southern United States (Elzebroek & Wind, 2008).

2.2. Botanical Classification

The laurel, *L. nobilis* L., belongs to the Lauraceae family. It is also known as the laurel sauce or the laurel of Apollo. The Laurales constitute a large order which brings together 9 families and about 3000 species. The main families of this order are Calycanthaceae, Lauraceae, and Monimiaceae. The Lauraceae family comprises more than 55 genera and 2500-3500 species (Trofimov *et al.*, 2022). The genus *Laurus* includes three major species: *Laurus azorica*, also called *Laurus canariensis*, growing in the forests of the Azores islands; *L. nobilis*, in the Mediterranean region and *Laurus novocanariensis*, present on the island of Madeira, the Canaries and Morocco (Ballabio & Goetz, 2010). The botanical classification of *L. nobilis* L. is reported in Table 1.

Table 1. Botanical classification of *L. nobilis* L. (Quézel & Santa, 1962).

Kingdom	Plantae
Under the reign	Vascular plants
Branch	Spermaphytes
Sub-Branch	Angiosperms
Class	Dicotyledonous
Subclass	Magnolideae
Order	Laurales
Family	Lauraceae
Genus	*Laurus*
Species	*L. nobilis* L.

2.3. Botanical Description

Laurel is an evergreen shrub or tree up to 12 m tall in the wild and cultivation is usually pruned to 2-3 m tall. The species naturally has several trunks. The bark of the stem and branches is dark brown to almost black (Elzebroek & Wind, 2008). The foliage of *L. nobilis* is evergreen with a dark green color above and lighter below. The leaf shape is elongated, even lanceolate, with pointed tips and a short petiole. The blade has a slightly thickened, wavy edge that curves inward. The leaves are approximately 3 to 5 cm wide by 10 cm long. Hairy at first, they then take on a shiny and hairless appearance (Geerts *et al.*, 2002) (Figure 1).

Laurel is a dioecious plant, that is, the male and female flowers are on separate feet. Flowering takes place from March to May. The inflorescence is made up of small umbels of four or five axillary flowers. It is creamy-white to greenish-white in color, unlike other Lauraceae which are trimers, the flower of the genus *Laurus* is a dimer, which can be seen more easily on a floral diagram. As a bud, the flowers are enclosed in an involucre of bracts. Since the petals and sepals are not distinct, we will speak of tepals. The tepals are arranged in two whorls, with a slightly smaller size for those located internally (Figure 1).

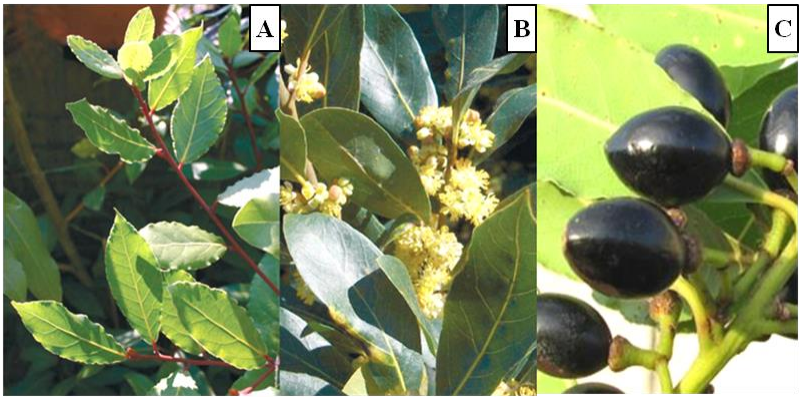
The fruit is a fleshy aromatic drupe, 10-15 mm long, ovoid, bright green at first and purplish black when ripe in autumn. It is made up, from the outside to the inside of the pericarp, mesocarp, and endocarp and contains a single seed, formed by two cotyledons rich in fat. The first two parts are dark, a few millimeters thick and makeup about 36% of the weight of the fresh fruit, the remainder, 64%, being made up of the endocarp and the seed. The berries remain on the plant all winter, sometimes until spring, which may coexist with the new flowering (Ballabio & Goetz, 2010) (Figure 1).

2.4. Geographical Distribution

Laurel is cultivated in different ecological and climatic conditions. Well-drained, moist soil with a pH ranging from 4.5 to 8.2 and humid atmospheric conditions near the sea are better

conditions for rapid growth (Kemp, 1983; Patrakar *et al.*, 2012). The cultivation of laurel grows in the following countries: Türkiye, Algeria, Morocco, Portugal, France, Spain, Greece, India, Pakistan, other Southeast Asian countries, some Pacific Islands, Australia, America Central, Mexico, southern United States, and Canary Islands (Parthasarathy *et al.*, 2008).

Figure 1. Morphological appearance of the different organs of *L. nobilis*: branch (A), flowers (B), fruits (C) (Chaaben *et al.*, 2015; Elzebroek & Wind, 2008).



2.5. Leaf Treatment

2.5.1. *Harvesting of leaves*

Laurel leaves can be harvested all year round thanks to the fact that the plant is evergreen. In the Mediterranean region, the optimum time for harvesting is in the autumn season. For example, in Türkiye, Greece, and the former Yugoslavia, bay leaves are harvested from August to October, for Morocco and Portugal the recommended harvest period is July to August. Harvesting should be done in optimal conditions avoiding dew, humidity, and heavy rains, as these can accelerate deterioration and discoloration and thus result in a poor quality product. The collection of bay leaves is usually done by hand or using small agricultural tools such as rakes (Ambrose *et al.*, 2016).

2.5.2. *Drying of leaves*

Drying is the oldest method used for preserving food. By definition, drying is the operation aimed at evaporating free water from a foodstuff to minimize microbial growth and chemical and enzymatic reactions (Cantín *et al.*, 2011). Drying is a process that can bypass the seasonal overproduction of crops and spread their availability throughout the year. In addition, the fresh plant cannot be cost-effectively supplied to all places in the world, due to the high water content, it suffers deterioration caused by the growth of microorganisms and biochemical changes. The elimination of water by dehydration reduces this growth and keeps the organoleptic characteristics of the plant (Ouafi *et al.*, 2015).

Small farmers and large producers of bay leaves wash the leaves after harvest and then proceed to drying. Leaf moisture levels are reduced to less than 10% using drying to improve the stability of their quality in storage (Ambrose *et al.*, 2016; Díaz-Maroto *et al.*, 2002). Drying of bay leaves is carried out by several methods such as sun drying, in shade, artificial process using dryers, or in hot air. Sun drying is an easy and inexpensive method, but direct sunlight can cause leaf discoloration and the use of high temperatures in other drying methods can cause a loss of volatiles. Drying in the shade or artificial drying is particularly recommended to achieve better quality (Ambrose *et al.*, 2016; Cakmak *et al.*, 2013; Sellami *et al.*, 2011).

Hot air drying is a method applicable when air drying cannot be practiced due to atmospheric conditions. This method also allows a considerable reduction in drying time. In addition to these traditional methods, new drying methods have recently been introduced, such as oven and

microwave drying; these can both comply with microbiological safety and food quality regulations and reduce the energy costs of drying (Cakmak *et al.*, 2013; Díaz-Maroto *et al.*, 2002; Kuzgunkaya & Hepbasli, 2007; Sellami *et al.*, 2011).

2.6. Composition and Nutritional Value

A bay leaf has a sharp, bitter taste. The difference in fragrance and aroma is due to the presence of essential oils in the leaves (Sumono & Sd, 2008). Fresh leaves have a water content of around 50% (Cakmak *et al.*, 2013), on the other hand, dry leaves contain 5-10% water, 65% carbohydrate, 8-11% protein, 5 9% fat, and 4% ash. The oil contains over 140 different components (Elzebroek & Wind, 2008).

Four sugars (fructose, sucrose, glucose, and trehalose), three polysaccharides (alginate, fucoidan, and laminaran), and three organic acids (oxalic, malic, and ascorbic acids) were detected in bay leaves. As they also contain several fatty acids; palmitic acid being the main one, followed by linoleic acid (Alejo-Armijo *et al.*, 2017).

Four tocopherols (α, β, γ, and δ) are detected in bay leaves and seeds. The α and γ-tocopherols are the most abundant in the leaves, while β-tocopherol predominates in the seeds. In both bay organs (leaves and seeds), δ-tocopherol is the least abundant component (Chahal *et al.*, 2017). The nutritional composition of the leaves of *L. nobilis* is presented in Table 2.

Table 2. Composition and nutritional value of *L. nobilis* leaves (Ambrose *et al.*, 2016).

Constituent	Value per 100g of dry matter
Water (g)	5.44
Energy (kcal)	313
Protein (g)	7.61
Carbohydrates (g)	74.96
Ash (g)	3.62
Fat (g)	8.36
Total saturated fatty acids (g)	2.28
Total monosaturated fatty acids (g)	1.64
Total polyunsaturated fatty acids (g)	2.29
Calcium (mg)	834
Iron (mg)	43
Magnesium (mg)	120
Phosphate (mg)	113
Potassium (mg)	529
Sodium (mg)	23
Zinc (mg)	3.70
Folate (µg)	180
Niacin (mg)	2.005
Riboflavin (mg)	0.421
Thiamine (mg)	0.009
Vitamin A, IU (IU)	6185
Vitamin A (µg)	309
Vitamin B (mg)	1.740
Vitamin C (mg)	46.5
Vitamin E (mg)	139

3. Bioactive Compounds of *L. nobilis* Leaves

An antioxidant is any substance present at a low concentration compared to that of the oxidizable substrate, significantly delays or prevents the oxidation of this substrate. Antioxidants are substances that can have an endogenous origin such as enzymes (superoxide dismutase, catalase, and glutathione peroxidase) and transition metal chelating proteins (transferrin, ferritin, and ceruloplasmin) and an exogenous origin (molecules antioxidants including phenolic compounds, carotenoids, vitamin C, and certain trace elements such as copper, zinc, selenium which are essential for the activity of antioxidant enzymes). These different antioxidants can neutralize or reduce the oxidation caused by reactive oxygen species (Pincemail *et al.*, 2002; Ghulam Yasin *et al.*, 2020).

Reactive oxygen species can have an endogenous origin following their generation by metabolic reactions in the cytosol, membranes, and mitochondria or exogenous such as pollution, ozone, tobacco, radiation, pesticides, and drugs (Pincemail *et al.*, 1998). These reactive species cause a direct action on biological molecules (lipids, proteins, DNA, and carbohydrates), but also secondary damage due to the cytotoxic and mutagenic properties of the metabolites released in particular during the oxidation of lipids (Deby-Dupont *et al.*, 2002).

3.1. Essential Oils

Over 150 components have been identified in bay leaf essential oil by GC-MS, with 1,8-cineole generally being the major component. The other main compounds are α-pinene, β-pinene, sabinene, limonene, and linalool (Figure 2) (Chahal *et al.*, 2017). Other parts of the plant, other than leaves, have also been explored regarding their volatile composition. Thus, the essential oils of bay fruits (Abu-Dahab *et al.*, 2014), seeds (Zolfaghari *et al.*, 2013), flowers (Moghtader & Salari, 2012), stems, and bark have also been studied repeatedly, although to a lesser extent than leaves. 1,8-cineol (26 to 51%), α-terpinyl acetate (5% to14%), and a-pinene (4 to 6%) were the main components of the essential oil of the stem and fruits (Chalchat *et al.*, 2011).

Compounds like eugenol (11 to 12%), methyl-eugenol (9% to 12%), and elemicin (1% to 12%) are important for the spicy aroma of noble bay leaves and they are used as important indicators in determining the quality of these leaves (Hanif *et al.*, 2020). This essential oil can also be used as a conservator of many foodstuffs such as table oils (Ordoudi *et al.*, 2022).

3.2. Phenolic Compounds

Polyphenols or phenolic compounds group together a vast set of more than 8000 molecules, divided into ten chemical classes, which all have one point in common: an aromatic ring with 6 carbon atoms that carry a variable number of hydroxyl functions (Harborne & Baxter, 1999). Many compounds such as flavones and flavonol found in bay leaves exhibit antioxidant activity (Hanif *et al.*, 2020).

Phenolic compounds in plants are also very important, as their hydroxyl groups confer scavenging capacity (Naczk & Shahidi, 2004). Plant materials rich in phenolic compounds are increasingly used in the food industry, as they delay the oxidative degradation of lipids and improve the quality and nutritional value of food as well as for antimicrobial activity (Nithya *et al.*, 2016; Zerrouki & Riazi, 2021). *L. nobilis* present all phenolic classes including flavonoids, phenolic acids, tannins (proanthocyanidins), and lignans (Dobroslavić *et al.*, 2022). Phenolic compounds of *L. nobilis* leaves are subjected to quantitative and qualitative variations depending on the region of harvesting, method of extraction, and solvent used for phenolics recovery (Table 3).

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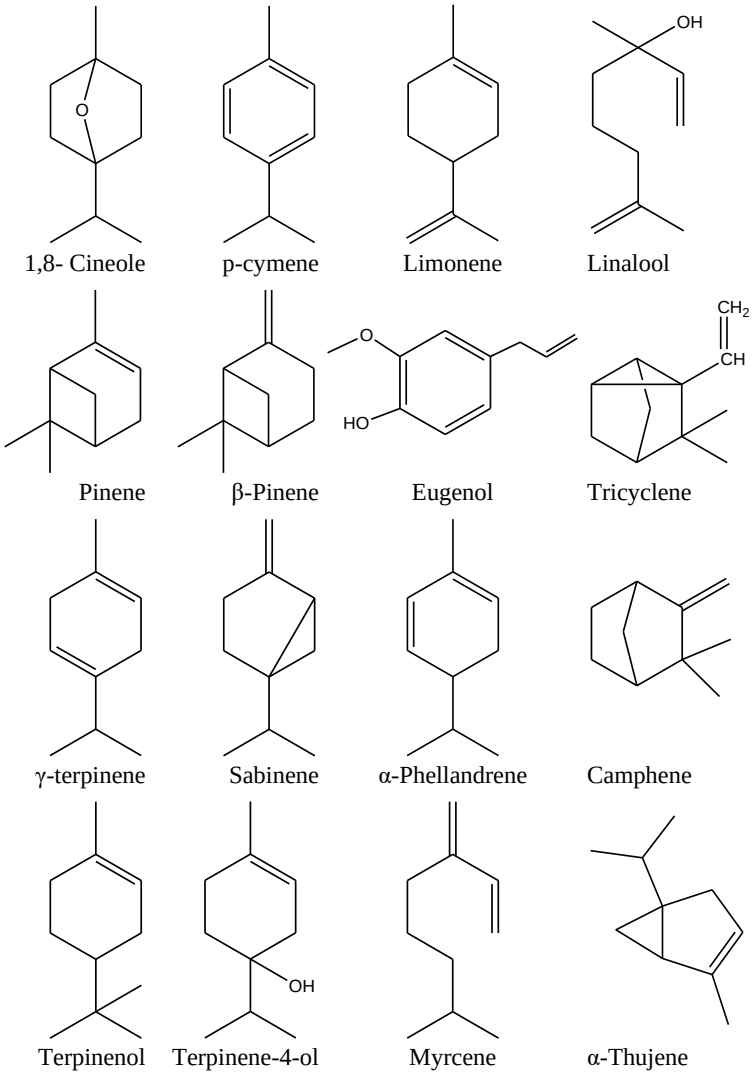
Figure 2. Compounds are identified in the essential oil of the leaves of *L. nobilis* (Chahal *et al.*, 2017).

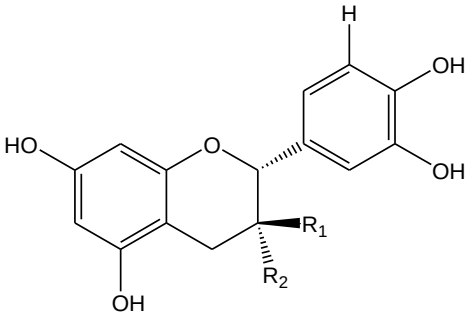


3.2.1. *Flavonoids*

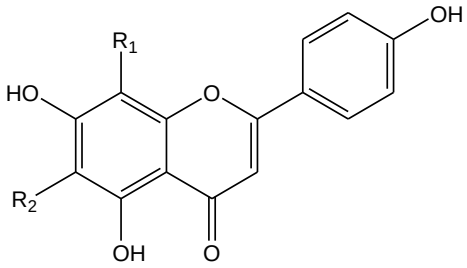
Flavonoids are a group of more than 6000 naturally occurring compounds that are nearly universal in vascular plants (Erlund, 2004). They are pigments responsible for the yellow, orange, and red colorings of different plant organs (Havsteen, 2002). Flavonoids are polyphenols, and therefore, their antioxidant activity depends on the reactivity of hydroxyl substituents in hydrogen atom transfer reactions.

Flavonoids are very effective scavengers of the most oxidizing molecules, including singlet oxygen and various free radicals involved in several diseases (Bravo, 1998). Flavonoids have positive effects on human health. Studies on flavonoid derivatives have shown a wide range of antibacterial, antiviral, anti-inflammatory, anticancer, and antiallergic activities (Di Carlo *et al.*, 1999; Hossain *et al.*, 2012; Montoro *et al.*, 2005). Flavonoids are the main phenolic constituents in extracts of noble bay leaves. Flavones and flavonols are the main flavonoids in leaf extracts. Apigenin, kaempferol, quercetin, and their glycosides are also frequently present in leaves (Alejo-Armijo *et al.*, 2017) (Figure 3).

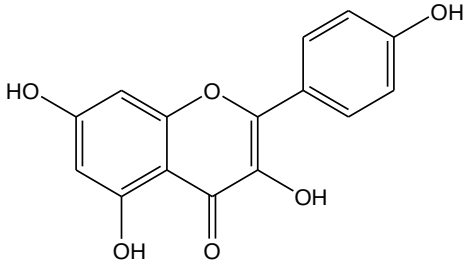
Figure 3. Some flavonoids of *L. nobilis* leaves (Konovalov & Alieva, 2019).



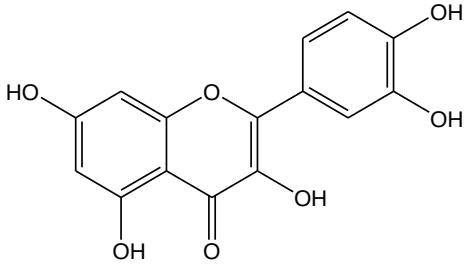
(-)-Epicatechin: R_1=H, R_2=OH, R_3=H
(-)-Epigallocatechin: R_1=H, R_2=OH, R_3=OH
(+)-Catechin: R_1=OH, R_2=H, R_3=H
(+)-Gallocatechin: R_1=OH, R_2=H, R_3=OH



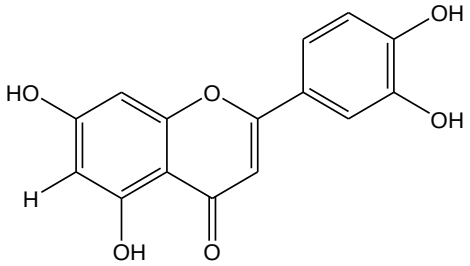
Apigenin: R_1=H, R_2=H
Apigenin 8-C-glucoside: R_1=glucoside, R_2=H
Apigenin-6,8-di-C-hexoside: R_1=hexoside, R_2=hexoside
Apigenin-6-C-glucoside: R_1=H, R_2=glucoside



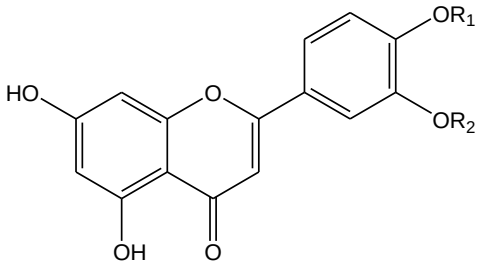
Kaempferol



Quercetin and derivatives (Q-3-O-rutinoside, Q-3-O-rhamnopyranoside, Q-3-O-glucopyranoside, …)



Luteolin



Hesperetin: R_1=CH_3, R_2=OH
Naringenin: R_1=H, R_2=H

Table 3. Phenolic compounds of *L. nobilis* leaves.

Compound	Content	Extraction method	Collection area	Reference
Phenolic acids				
Gallic acid	24.8 mg/g	Maceration (0.5g/70% ethanol at 70 °C for 15 min)	Bulgarian Monastery, Greece	(Stefanova *et al.*, 2020)
	4.5 / 10.5 / 4.8 μg/g	CRE / MAE / UAE (CRE: 40ml 50% ethanol for 30min; MAE: 50% ethanol, 80 °C, for 10 min and 400 W; UAE: (70% ethanol, 50% amplitude for 10 min)	Zagreb, Croatia	(Dobroslavić *et al.*, 2021)
	1.4 mg/g	Maceration (50% ethanol)	/	(Papageorgiou *et al.*, 2008)
	1420 μg/g	Ultra-Turrax extraction (30g/300 ethanol at 10 000 rpm for 4 min)	Semi-arid region, Algeria	(Bourebaba *et al.*, 2021)
Rosmarinic acid	47.5 mg/g	Maceration (0.5g/70% ethanol at 70 °C for 15 min)	Bulgarian Monastery, Greece	(Stefanova *et al.*, 2020)
	5.3 / 12.5 / 14.4 μg/g	CRE / MAE / UAE (CRE: 40ml 50% ethanol for 30min; MAE: 50% ethanol, 80 °C, for 10 min and 400 W; UAE: (70% ethanol, 50% amplitude for 10 min)	Zagreb, Croatia	(Dobroslavić *et al.*, 2021)
	0.1-0.2 mg/g	Maceration (50% ethanol)	/	(Papageorgiou *et al.*, 2008)
Chlorogenic acid	243.9 mg/g	Maceration (0.5g/70% ethanol at 70 °C for 15 min)	Bulgarian Monastery, Greece	(Stefanova *et al.*, 2020)
	3.8 / 3.8 / 3.9 μg/g	CRE / MAE / UAE (CRE: 40ml 50% ethanol for 30min; MAE: 50% ethanol, 80 °C, for 10 min and 400 W; UAE: (70% ethanol, 50% amplitude for 10 min)	Zagreb, Croatia	(Dobroslavić *et al.*, 2021)
	210 μg/g	Ultra-Turrax extraction (30g/300 ethanol at 10 000 rpm for 4 min)	Semi-arid region, Algeria	(Bourebaba *et al.*, 2021)
Caffeic acid	586.1 mg/g	Maceration (0.5g/70% ethanol at 70 °C for 15 min)	Bulgarian Monastery, Greece	(Stefanova *et al.*, 2020)
	25.5 / 343.1 / 207.3 μg/g	CRE / MAE / UAE (CRE: 40ml 50% ethanol for 30min; MAE: 50% ethanol, 80 °C, for 10 min and 400 W; UAE: (70% ethanol, 50% amplitude for 10 min)	Zagreb, Croatia	(Dobroslavić *et al.*, 2021)
p-Coumaric acid	151.1 mg/g	Maceration (0.5g/70% ethanol at 70 °C for 15 min)	Bulgarian Monastery, Greece	(Stefanova *et al.*, 2020)

	14 / 8.3 / 8.2 µg/g	CRE / MAE / UAE (CRE: 40ml 50% ethanol for 30min; MAE: 50% ethanol, 80 °C, for 10 min and 400 W; UAE: (70% ethanol, 50% amplitude for 10 min)	Zagreb, Croatia	(Dobroslavić *et al.*, 2021)
Ferulic acid	300.1 mg/g	Maceration (0.5g/70% ethanol at 70 °C for 15 min)	Bulgarian Monastery, Greece	(Stefanova *et al.*, 2020)
	94.4 / 7.8 / 11 µg/g	CRE / MAE / UAE (CRE: 40ml 50% ethanol for 30min; MAE: 50% ethanol, 80 °C, for 10 min and 400 W; UAE: (70% ethanol, 50% amplitude for 10 min)	Zagreb, Croatia	(Dobroslavić *et al.*, 2021)
Syringic acid	242 mg/g	Maceration (0.5g/70% ethanol at 70 °C for 15 min)	Bulgarian Monastery, Greece	(Stefanova *et al.*, 2020)
	0.3 / 0.4 / 0.6 µg/g	CRE / MAE / UAE (CRE: 40ml 50% ethanol for 30min; MAE: 50% ethanol, 80 °C, for 10 min and 400 W; UAE: (70% ethanol, 50% amplitude for 10 min)	Zagreb, Croatia	(Dobroslavić *et al.*, 2021)
Sinapic acid	607.7 mg/g	Maceration (0.5g/70% ethanol at 70 °C for 15 min)	Bulgarian Monastery, Greece	(Stefanova *et al.*, 2020)
Cinnamic acid	135 mg/g			
3,4-dihydroxybenzoic acid hexoside	17.5 / 28.9 / 24.9 µg/g	CRE / MAE / UAE (CRE: 40ml 50% ethanol for 30min; MAE: 50% ethanol, 80 °C, for 10 min and 400 W; UAE: (70% ethanol, 50% amplitude for 10 min)	Zagreb, Croatia	(Dobroslavić *et al.*, 2021)
Protocatehuic acid	28 / 35.4 / 20.4 µg/g			
p-hydroxybenzoic acid	7.2 / 10.2 / 13 µg/g			
Hydroxybenzoic acid	0.45-5 mg/g	Maceration (50% ethanol)	/	(Papageorgiou *et al.*, 2008)
Vanillic acid	0.45-5 mg/g			
Dicaffeoylquinic acid	160 µg/g	Ultra-Turrax extraction (30g/300 ethanol at 10 000 rpm for 4 min)	Semi-arid region, Algeria	(Bourebaba *et al.*, 2021)
3-5-Dicaffeoylquinic acid	110 µg/g			
Flavonoids				
Myricetin	124.5 mg/g	Maceration (0.5g/70% ethanol at 70 °C for 15 min)	Bulgarian Monastery, Greece	(Stefanova *et al.*, 2020)
	6.5 / 7.3 / 7.8 µg/g	CRE / MAE / UAE (CRE: 40ml 50% ethanol for 30min; MAE: 50% ethanol, 80 °C, for 10 min and 400 W; UAE: (70% ethanol, 50% amplitude for 10 min)	Zagreb, Croatia	(Dobroslavić *et al.*, 2021)
Quercetin	48.9 mg/g	Maceration (0.5g/70% ethanol at 70 °C for 15 min)	Bulgarian Monastery, Greece	(Stefanova *et al.*, 2020)
	30 µg/g	Ultra-Turrax extraction (30g/300 ethanol at 10 000 rpm for 4 min)	Semi-arid region, Algeria	(Bourebaba *et al.*, 2021)
Luteolin	4.8 mg/g	Maceration (0.5g/70% ethanol at 70 °C for 15 min)	Bulgarian Monastery, Greece	(Stefanova *et al.*, 2020)

	38 / 71.7 / 113.6 μg/g	CRE / MAE / UAE (CRE: 40ml 50% ethanol for 30min; MAE: 50% ethanol, 80 °C, for 10 min and 400 W; UAE: (70% ethanol, 50% amplitude for 10 min)	Zagreb, Croatia	(Dobroslavić *et al.*, 2021)
	0.2-4.5 mg/g	/	Maceration (50% ethanol)	(Papageorgiou *et al.*, 2008)
	268.6 mg/g	Maceration (0.5g/70% ethanol at 70 °C for 15 min)	Bulgarian Monastery, Greece	(Stefanova *et al.*, 2020)
Apigenin	6.5 / 37.4 / 85.2 μg/g	CRE / MAE / UAE (CRE: 40ml 50% ethanol for 30min; MAE: 50% ethanol, 80 °C, for 10 min and 400 W; UAE: (70% ethanol, 50% amplitude for 10 min)	Zagreb, Croatia	(Dobroslavić *et al.*, 2021)
Kaempferol	122.2 mg/g	Maceration (0.5g/70% ethanol at 70 °C for 15 min)	Bulgarian Monastery, Greece	(Stefanova *et al.*, 2020)
	217.4 mg/g			
Rutin	280.7 / 982.1 / 231.4 μg/g	CRE / MAE / UAE (CRE: 40ml 50% ethanol for 30min; MAE: 50% ethanol, 80 °C, for 10 min and 400 W; UAE: (70% ethanol, 50% amplitude for 10 min)	Zagreb, Croatia	(Dobroslavić *et al.*, 2021)
	929.4 mg/g	Maceration (50% ethanol)	/	(Lu *et al.*, 2011)
Hyperoside	141.8 mg/g	Maceration (0.5g/70% ethanol at 70 °C for 15 min)	Bulgarian Monastery, Greece	(Stefanova *et al.*, 2020)
(Epi)catechin-hexoside	3.92 mg/g	Maceration (1 g/30 mL methanol for 1h)	Castro Daire, Portugal	(Dias *et al.*, 2014)
(+)-Gallocatechin	5.97 mg/g			
Catechin	0.76 mg/g			
	0.58 mg/g	Maceration (50% ethanol)	/	(Nasukhova *et al.*, 2019)
	330 μg/g	Ultra-Turrax extraction (30g/300 ethanol at 10 000 rpm for 4 min)	Semi-arid region, Algeria	(Bourebaba *et al.*, 2021)
	723.7 / 126.2 / 198.8 μg/g	CRE / MAE / UAE (CRE: 40ml 50% ethanol for 30min; MAE: 50% ethanol, 80 °C, for 10 min and 400 W; UAE: (70% ethanol, 50% amplitude for 10 min)	Zagreb, Croatia	(Dobroslavić *et al.*, 2021)
	22.9 mg/g	Infusion (25g/200ml boiling water)	Sardinia, Italy	(Dall'Acqua *et al.*, 2009)
(-)-Epicatechin	15.69 mg/g	Maceration (1 g/30 mL methanol for 1h)	Castro Daire, Portugal	(Dias *et al.*, 2014)
	3.44 mg/g	Maceration (50% ethanol)	/	(Nasukhova *et al.*, 2019)
	9510 μg/g	Ultra-Turrax extraction (30g/300 ethanol at 10 000 rpm for 4 min)	Semi-arid region, Algeria	(Bourebaba *et al.*, 2021)

	711.7 / 136.5 / 139 µg/g	CRE / MAE / UAE (CRE: 40ml 50% ethanol for 30min; MAE: 50% ethanol, 80 °C, for 10 min and 400 W; UAE: (70% ethanol, 50% amplitude for 10 min)	Zagreb, Croatia	(Dobroslavić *et al.*, 2021)
Luteolin 6-C-glucoside	1.35 mg/g	Maceration (1 g/30 mL methanol for 1h)	Castro Daire, Portugal	(Dias *et al.*, 2014)
Apigenin 8-C-glucoside	0.99 mg/g			
2"-*O*-Rhamnosyl-C-hexosyl-apigenin	0.56 mg/g			
Quercetin 3-*O*-rutinoside	1.58 mg/g			
Apigenin 6-C-glucoside	1.61 mg/g			
Quercetin 3-*O*-glucoside	4.32 mg/g			
Quercetin *O*-hexoside	4.99 mg/g			
Kaempferol 3-*O*-rutinoside	1.63 mg/g			
Quercetin *O*-pentoside	1.56 mg/g			
Kaempferol 3-*O*-glucoside	1.89 mg/g			
Isorhamnetin *O*-rutinoside	3.13 mg/g			
Quercetin *O*-rhamnoside	4.62 mg/g			
Isorhamnetin *O*-hexoside	1.29 mg/g			
Kaempferol *O*-pentoside	0.67 mg/g			
Isorhamnetin *O*-pentoside	0.22 mg/g			
Kaempferol *O*-hexoside	1.83 mg/g			
Isorhamnetin *O*-rhamnoside	0.03 mg/g			
kaempferol-3-*O*-glucopyranoside	23 mg/g	Infusion (25g/200ml boiling water)	Sardinia, Italy	(Dall'Acqua *et al.*, 2009)
kaempferol-3-*O*-rhamnopyranoside	28 mg/g			
kaempferol-3-*O*-(2’’,4’’-di-E-*p*-coumaroyl)-rhamnoside	22.9 mg/g			
kaempferol-3-Oarabinopyranoside	16 mg/g			
kaempferol-3- *O*-[6-*O*-(rhamnopyranosyl)glucopyranoside]	2.8 mg/g			

Quercetin-3-*O*-glucopyranoside	38 mg/g			
Quercetin-3-*O*-rhamnopyranoside	21 mg/g			
Quercetin-3-*O*-[6-*O*-(rhamnopyranosyl) glucopyranoside]	15.5 mg/g			
3'-methoxyquercetin-3-*O*-[6-*O*-(rhamnopyranosyl)glucopyranoside]	12.2 mg/g			
3'-methoxyquercetin-3-Oglucopyranoside	20 mg/g			
2''-Rhamnosylisovitexin	13.4 mg/g			
Kaempferol-3-*O*-deoxyhexoside	1.4 / 0.6 / 1 µg/g	CRE / MAE / UAE (CRE: 40ml 50% ethanol for 30min; MAE: 50% ethanol, 80 °C, for 10 min and 400 W; UAE: (70% ethanol, 50% amplitude for 10 min)	Zagreb, Croatia	(Dobroslavić *et al.*, 2021)
Quercetin-3-glucoside	513.4 / 1027.4 / 918.3 µg/g			
Kaempferol-3-rutinoside	241.7 / 57.8 / 75.2 µg/g			
Quercetin-3-pentoside	283.2 / 86.2 / 54.3 µg/g			
Kaempferol-3-*O*-hexoside	1116.3 / 187.3 / 142.5 µg/g			
Isorhamnetin-3-hexoside	405.6 / 251 / 216.2 µg/g			
Quercetin-3-rhamnoside	127.4 / 143.4 / 399.6 µg/g			
Kaempferol-3-*O*-pentoside	439 / 83.7 / 79.2 µg/g			
Epicatechin gallate	1 / 4.5 / 2.6 µg/g			
Epigallocatechin gallate	4.9 / 2.2 / 0.8 µg/g			
Luteolin-6-C-glucoside	21 / 52.3 / 40.4 µg/g			
Apigenin-6-C-(*O*-deoxyhexosyl)-hexoside	0.9 / 1.3 / 1 µg/g			

Gallocatechin	900 µg/g	Ultra-Turrax extraction (30g/300 ethanol at 10 000 rpm for 4 min)	Semi-arid region, Algeria	(Bourebaba *et al.*, 2021)
Quercetagetin dimethyl ether/isomer	40 µg/g			
Quercetagetin trimethyl ether/isomer	50 µg/g			
Kaempferol rutinoside/isomer	1450 µg/g			
Quercetin methyl ether/isomer	30 µg/g			
Quercetin dimethyl ether/isomer	80 µg/g			
Procyanidin dimer	1.92 mg/g	Maceration (1 g/30 mL methanol for 1h)	Castro Daire, Portugal	(Dias *et al.*, 2014)
	16.97 mg/g	Maceration (50% ethanol)	/	(Nasukhova *et al.*, 2019)
Cinnamtannin B-1	2.3 mg/g	Infusion (25g/200ml boiling water)	Sardinia, Italy	(Dall'Acqua *et al.*, 2009)
Procyanidin trimer	1.25 mg/g	Maceration (1 g/30 mL methanol for 1h)	Castro Daire, Portugal	(Dias *et al.*, 2014)
	1.29 mg/g			
	203.3 / 77.2 / - µg/g	CRE / MAE / UAE (CRE: 40ml 50% ethanol for 30min; MAE: 50% ethanol, 80 °C, for 10 min and 400 W; UAE: (70% ethanol, 50% amplitude for 10 min)	Zagreb, Croatia	(Dobroslavić *et al.*, 2021)
Trimeric proanthocyanidins 1	1.24 mg/g	Maceration (50% ethanol)	/	(Nasukhova *et al.*, 2019)
Trimeric proanthocyanidins 2	5.05 mg/g			
Procyanidin tetramer	3.54 mg/g	Maceration (1 g/30 mL methanol for 1h)	Castro Daire, Portugal	(Dias *et al.*, 2014)
	1.16 mg/g	Maceration (50% ethanol)	/	(Nasukhova *et al.*, 2019)

CRE: conventional heat-reflux extraction, MAE: microwave-assisted extraction, UAE: ultrasound-assisted extraction.

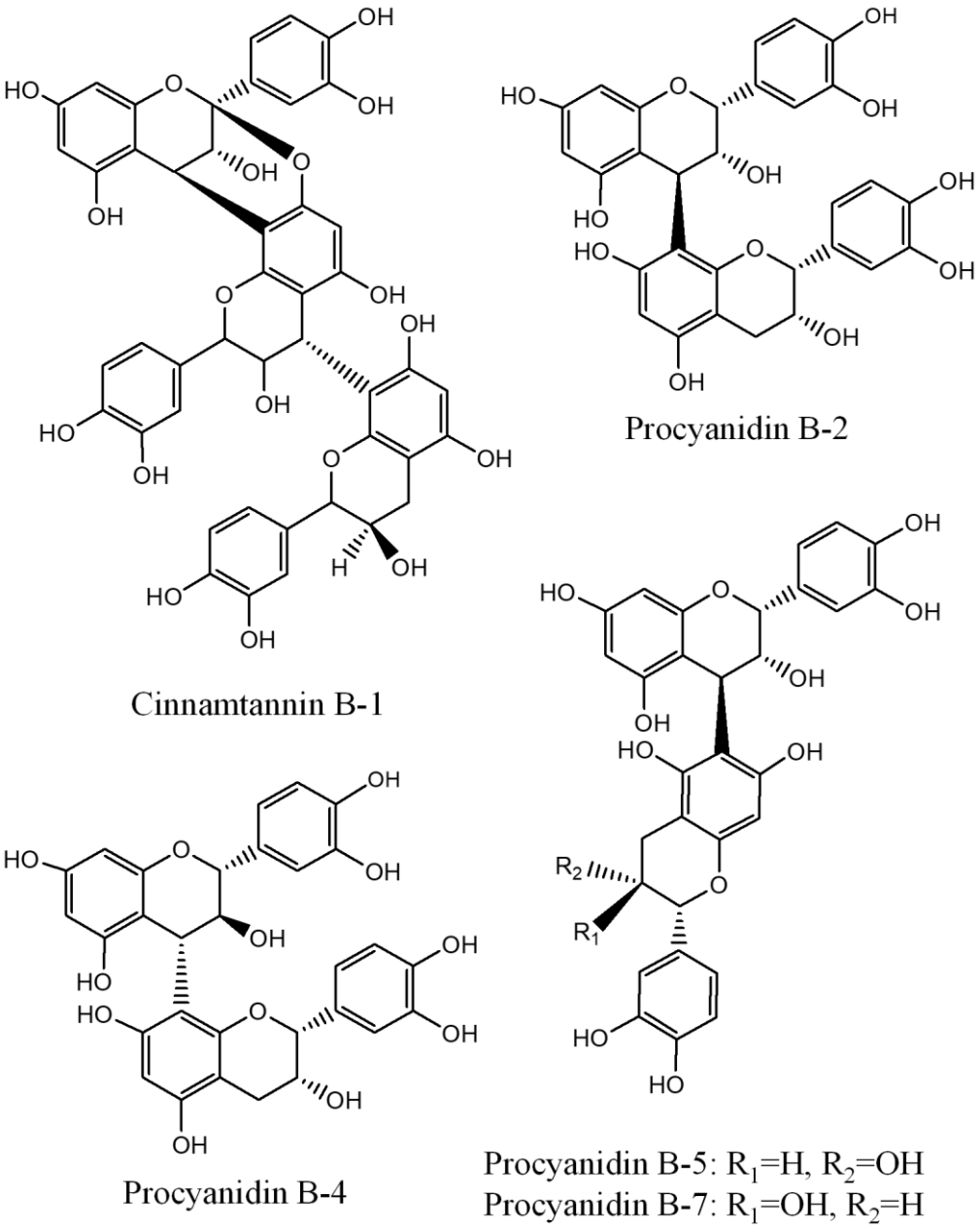
3.2.2. *Proanthocyanidins*

Proanthocyanidins, also called condensed tannins, are oligomers and polymers of flavans linked by specific bonds. These secondary metabolites have significant antioxidant activity. They are widespread in some foods and dietary supplements. The proanthocyanidin composition of bay leaves was studied by Vinha *et al.* (2015). They reported that dimeric proanthocyanidins were most abundant in this part (Figure 4).

3.2.3. *Other phenolic compounds*

Other phenolic compounds detected in bay leaves are structural derivatives of caffeic and coumaric acids. Frequently, these compounds are esters of these acids with quinic or 3,4-dihydroxy phenyl lactic acids. Four lignans could also be isolated from a hydroalcoholic extract of bay leaves (Alejo-Armijo *et al.*, 2017).

Figure 4. Some proanthocyanidins of *L. nobilis* leaves (Konovalov & Alieva, 2019).

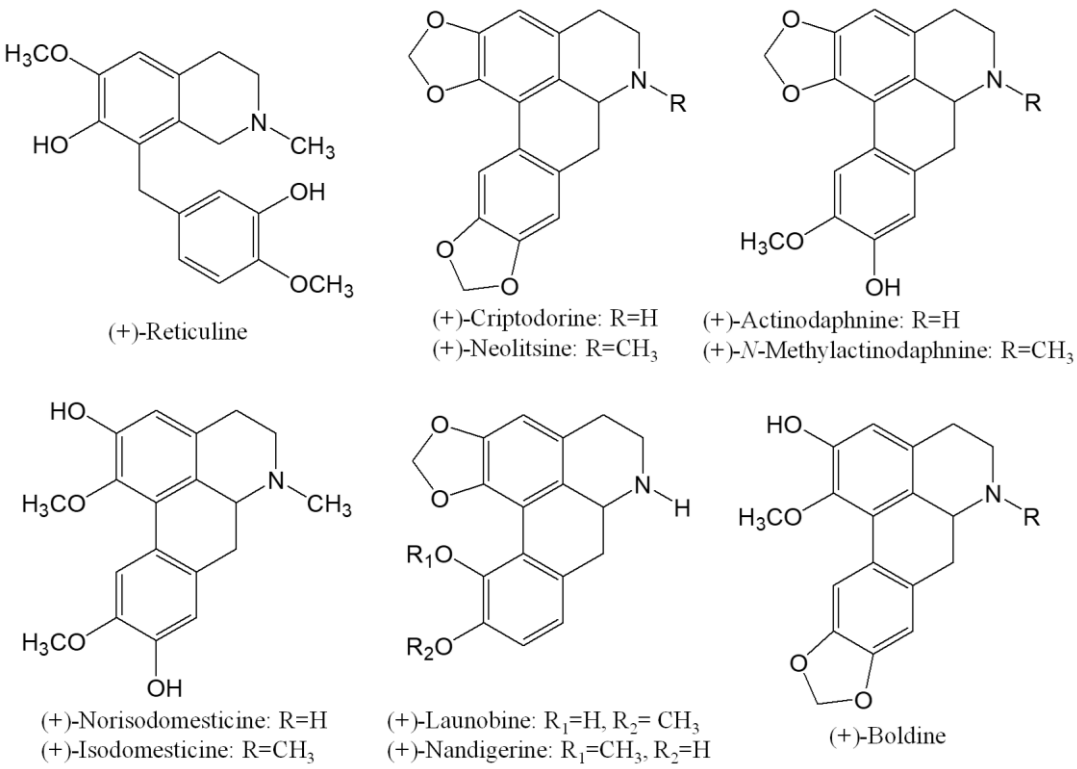


3.3. Alkaloids

Alkaloids are a group of secondary metabolites, overwhelmingly of plant origin. Chemically, alkaloids are nitrogen-based molecules, most often heterocyclic. Over ten thousand alkaloids have been isolated from plants. Alkaloids have a wide range of biological activities, including antioxidant, antibacterial, and anticancer activities (Hassan *et al.*, 2013; Nithya *et al.*, 2016).

Ten alkaloids (1-10) have been isolated from noble bay leaves, of which five are noraporphins (2, 4, 6, 8, 9), four are aporphins (3, 5, 7, 10), and one only (1) is an alkaloid of the benzyl-tetrahydro-isoquinoline type (Figure 5). Compound 9 has also been isolated from roots, and is found 1, 4, and 8 in the bark of stems and roots (Pech & Bruneton, 1982). Launobin (8) has also been obtained from noble laurel wood. Noraporphin 4 is the major alkaloid of bay leaves (Pech & Bruneton, 1982).

Figure 5. Alkaloids of *L. nobilis* leaves (Alejo-Armijo *et al.*, 2017).



4. TRADITIONAL USES and THERAPEUTIC EFFECTS

In addition to its well-known culinary virtues, noble laurel also exhibits medicinal properties, known since ancient times. The leaf is traded internationally and its production is estimated at over 2,000 tonnes per year. Türkiye is the world's largest producer and exporter with around two-thirds of international trade. The leaf is used in many food products as well as in toiletries and medicines (Ravindran, 2017).

4.1. Medicinal Uses

Traditionally, bay leaves have been used in herbal medicine against several diseases such as rheumatism, sprains, indigestion, and earaches. The leaves having anti-diarrheal, anti-inflammatory, and anti-diabetic activity are used for improving the response of the immune system. These leaves contain numerous molecules, which are responsible for anti-inflammatory activity, inhibiting alcohol absorption, and which can enhance the activity of glutathione S-transferase in the liver (Fang *et al.*, 2005).

Many components of the essential oil of noble laurels such as eugenol, methyl eugenol, and pinene have anticonvulsant activity (Sayyah *et al.*, 2002). The essential oil of this leaf also has analgesic activities (Barla *et al.*, 2007). Different research has revealed that bay leaves can also be used to treat diabetes and migraine (Aljamal, 2010; Fang *et al.*, 2005; Mirbadal Zadeh & Shirdel, 2011). Fresh leaves are used to treat blood dysentery, inflammation, and congestion of the kidneys. They are also used to treat arthritis, headaches, fungal diseases, anorexia, colds, cataracts, diarrhea, and colonic ulcer (Parthasarathy *et al.*, 2008).

Laurel leaves are effective against many infections caused by fungi, viruses, bacteria, and protozoa. They are also useful for inhibiting the growth of cancer cells and for fevers, coughs, flu, colds, bronchitis, and asthma. Laurel juice is an effective drug for soothing sore eyes and night blindness, usually caused by vitamin A deficiency (Hanif *et al.*, 2020).

4.2. Culinary Uses

Different parts of plants can be eaten as spices, including bark, flowers, leaves, roots, stems, and seeds. Spices can also be consumed in fresh, dried, and powdered forms (Raghavan & Orsat, 2007; Schweiggert *et al.*, 2007). Laurel leaves are mainly used to flavor several dishes, stews, soups, sauces, fish, meats, and drinks. As the fresh leaves are bitter, the leaves are usually dried before use. The dried and powdered leaves are used industrially in the manufacture of various foods (Elzebroek & Wind, 2008).

4.3. Cosmetic Uses

Laurel contains essential oil which can be obtained from the leaves by steam distillation; the oil is used in industry to scent candles, perfumes, creams, and soaps (Elzebroek & Wind, 2008). In Syria, it is a main component of the traditional and very old Aleppo soap, which also contains olive oil and caustic soda, and Salicornia ashes (Ballabio & Goetz, 2010).

4.4. Pharmacological Uses

Due to the presence of various antioxidant molecules in bay laurel, several biological and pharmacological activities have been reported by researchers, such as antioxidant, antibacterial, antifungal, antiviral, and insecticidal activities (Chahal *et al.*, 2017).

4.4.1. *Antioxidant activity*

L. nobilis leaves demonstrated strong antioxidant properties. Elmastaş *et al.* (2006) showed that freeze-dried extracts (water and ethanol) of bay leaves showed strong total antioxidant activity in the linoleic acid emulsion. The concentrations of 20, 40, and 60 µg/ml showed inhibition of 84.9, 95.7, 96.8 and 94.2, 97.7 and 98.6% of the lipid peroxidation of the emulsion of linoleic acid, respectively for aqueous and ethanolic extracts. The findings are comparable to 60 µg/ml of standard antioxidants including butylated hydroxyanisole, butylated hydroxytoluene, and α-tocopherol with 96.6, 99.1, and 76.9% inhibition of lipid peroxidation, respectively. The alkaloid extract obtained from bay leaves expressed a high antioxidant activity with an IC50 of 63.28 µg/ml higher than gallic acid (143.18 µg/ml) used as standard. The phenolic extract exhibited an antioxidant power with an IC50 of 317.57 µg/ml, less than that of alkaloids extract (Khaled Khodja *et al.*, 2021).

4.4.2. *Antidiabetic activity*

L. nobilis leaves are very beneficial to diabetic people; their extract can improve the blood glucose level and prevent the apparition of diabetes complications. The study carried out by Khan *et al.* (2009) on forty people with type 2 diabetes, given capsules containing 1, 2, or 3 g of ground bay leaves, showed a significant decrease in serum glucose level by 21-26% after 30 days of treatment. Another study realized *in vitro* showed that *L. nobilis* ethanolic extract improves insulin sensitivity by increasing insulin receptor substrate expression, and reduces

considerably the intracellular oxidative stress induced by chronic hyperglycemia. These biological activities are attributed to phenolic compounds, mainly gallic acid (Bourebaba *et al.*, 2021).

Some experiences realized *in vivo* reported also the possible ameliorative effect of bay leaves extracts on diabetic people; for example, the study conducted by Mohammed *et al.* (2021) on streptozotocin-induced diabetic rats showed that leaves extract of *L. nobilis*, 200 mg/kg of bay extract administered every day orally using the intragastric tube for 28 days, decreases significantly the blood glucose level and improves the regeneration of the pancreatic islets. Al Chalabi *et al.* (2020) reported that the polyphenols contained in the alcoholic extract of *L. nobilis* decrease significantly the fasting blood glucose level and improve insulin secretion in alloxan-induced diabetic rats.

On the other hand, the bioactive molecules contained in bay leaves can enhance significantly the post-prandial glucose level in diabetic patients. In this regard, the terpenes found in the aqueous and the methanol/acetone extract of bay leaves exert a potent protective effect against high-fat diet-induced type 2 diabetes in rats. These compounds can probably act via the inhibition of carbohydrate hydrolyzing enzymes such as α-amylase and α-glucosidase (Daher *et al.*, 2021). In addition, an *in vitro* study carried out by Duletić-Laušević *et al.* (2019) showed that the phenolic compounds extract of *L. nobilis* leaves inhibit significantly the α-glucosidase activity at levels ranging from 11-93% depending on the extraction solvent (acetone and methanol) and origin of leaves (commercial and cultivated), which can probably reduce the postprandial glycemia in diabetic people.

4.4.3. *Antihyperlipidemic activity*

Several experimental studies demonstrated the possible curative effect of bay leaf extracts and their isolated molecules on dyslipidemia. The daily oral administration for female rabbits of 100 mg/ml/kg of bay leaf crude, 50 mg/ml/kg of isolated flavonoids extract, or 12.5 mg/ml/kg of isolated glycosides extracts during 30 days demonstrated a significant decrease of total cholesterol, LDL-Cholesterol, and triglycerides levels AL-Samarrai *et al.* (2017). Another study showed that polyphenols contained in the alcoholic extract of *L. nobilis* decrease significantly the level of total cholesterol, LDL-Cholesterol, and triglycerides as well as increase the level of HDL-cholesterol in alloxan-induced diabetic rats using a dose of 200mg/kg administered orally during one month (Al Chalabi *et al.*, 2020).

The investigation conducted by Chbili *et al.* (2020) on 30 healthy volunteers showed that the consumption of *L. nobilis* tea (one intake a day for 10 days of the infusion of 5 g of dried bay leaves in 100 ml water) increases significantly the concentration of HDL cholesterol. Another investigation realized on 56 people with type 1 diabetes demonstrated that the consumption of bay leaves decreases risk factors for diabetes and reduced total cholesterol, LDL, and triglycerides but increases HDL levels (Aljamal, 2010).

4.4.4. *Hepatoprotective activity*

The crude extract of *L. nobilis* leaves showed good hepatoprotective activity against paracetamol's toxic effects on rat hepatocytes at a concentration of 40 µg/ml (Ayoub *et al.*, 2013). Similarly, an experimental study performed on rats showed that the methanol extract of *L. nobilis* at 400 mg/kg acts on the liver as a potent scavenger of free radicals to prevent the hepatotoxicity induced by paracetamol (Ravindran *et al.*, 2013). Another *in vivo* study carried out by Gasparyan *et al.* (2015) revealed that some phenolic compounds such as flavonoids and eugenol of *L. nobilis* protect hepatocytes of rats against metabolic and histological abnormalities induced by tetrachloromethane.

4.4.5. *Antimutagenic activity*

The noble laurel contains the antimutagen *p*-coumarate 3-kaempferol at a level of 20mg/100g. This compound acts against the dietary carcinogen 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) with an IC50 of 1.9µg. This value is close to those found for strong antimutagens like flavonols and flavones. The mutagenicity is due to a desmutagenic action that converts the metabolically activated form of Trp-P-2 to its carcinogenic form. The antimutagenicity effect of *p*-coumarate 3-kaempferol is due to the inhibition of metabolic activation of Trp-P-2 to its ultimate carcinogenic form and the kaempferyl moiety contributes particularly to this activity (Samejima *et al.*, 1998).

4.4.6. *Anticonvulsant activity*

The leaf essential oil of *L. nobilis*, which has been used as an antiepileptic remedy in Iranian traditional medicine, was evaluated for anticonvulsant activity against experimental seizures. The essential oil protected mice against tonic convulsions induced by maximal electroshock and especially by pentylenetetrazole. Components responsible for this effect may be methyleugenol, eugenol, and pinene present in the essential oil. At anticonvulsant doses (1.22–1.71 ml/kg), the essential oil produced sedation and motor impairment. This effect seems to be related in part to cineol, eugenol, and methyleugenol (Sayyah *et al.*, 2002).

4.4.7. *Immunostimulatory activity*

The immunostimulatory effects of noble laurel powder have been demonstrated on rainbow trout. Three groups of rainbow trout were fed experimental diets. After 21 days, nonspecific immune parameters such as phagocytosis in blood leukocytes, lysozymes, and protein levels were examined and showed immunostimulatory activity. The phagocytic activity of fish fed with a diet supplemented by 0.5 and %1 laurel was significantly higher than other groups (Bilen & Bulut, 2010).

4.4.8. *Analgesic and anti-inflammatory activities*

The essential oil of bay laurel has shown analgesic and anti-inflammatory activities in mice and rats. The essential oil exhibited a significant analgesic effect in the tail-flick and formalin tests, a dose-dependent anti-inflammatory effect in the formalin-induced edema, and a moderate sedative effect at the anti-inflammatory doses. The analgesic and anti-inflammatory effect of the essential oil was comparable to reference analgesics and non-steroid anti-inflammatory drugs: morphine and piroxicam (Sayyah *et al.*, 2003).

4.4.9. *Wound healing activity*

Many healing models have been used to estimate the healing activity of bay laurel, and many factors have been studied to assess wound healing. It revealed that the animals treated with *L. nobilis* had moderately higher rates of wound contraction and weight of the granulation tissue and significantly more hydroxyproline content than the control group. The histological study of the granulation tissue of treated animals with *L. nobilis* showed a larger number of inflammatory cells, and lesser collagen when compared with the control group of animals (Nayak *et al.*, 2006).

4.4.10. *Antibacterial activity*

Bay leaves expressed good antimicrobial activities that have been related to the presence of many classes of bioactive compounds. Indeed, the methanolic extract of bay laurel essential oil showed significant antibacterial activity *in vitro* against *Staphilococcus aureus, Enterococcus gallinarum, Haemophilus influenzae and Pseudomonas aeruginosa* strains, with respective minimum inhibitory concentrations (MIC) of 10, 6.5, 6, and 6 mg/ml (Ozcan *et al.*, 2010). Similarly, in another study, the extract of essential oils from *L. nobilis* leaves showed highly

effective antibactericidal activity against *Staphylococcus aureus*, *Staphylococcus intermedius*, and *Klebsiella pneumonia*, with minimum inhibitory concentrations ranging from 0.01 to 1 mg/ml (Derwich *et al.*, 2009).

L. nobilis essential oil expressed a significant antibactericidal and antistaphylococcal activity (MIC values ranging from 3.9 to 15.6 mg/m) and a strong biofilm inhibition effect of more than 70% obtained by a low sub-inhibitory concentration (1/16 MIC). The MTT (3- (4, 5-dimethyl-thiazol-2-yl)- 2,5-diphenyl-tetrazolium bromide) test reveals that these essential oils exhibited excellent antibiofilm activity with eradication percentages ranging from 79.6 to 95.2%. This oil could have a promising role in preventing oral infections (Merghni *et al.*, 2016).

Khaled Khodja *et al.* (2021) showed that the alkaloid extract (1 mg/ml) of bay leaves had strong antibacterial activity against the staphylococcal strains responsible for food poisoning with inhibition zones ranging from 12 to 22 mm. However, the extract of phenolic compounds (1 mg/ml) showed better activity against phytopathogenic strains associated with potato soft rot with inhibition zones varying from 12 to 17 mm. The experiments conducted by Berendika *et al.* (2022) demonstrated that the phenolic extract of laurel at 50 and 100 mg/kg positively affects the health of rats after intragastric treatment by increasing the number of colonies of lactobacilli and bifidobacteria and reducing the growth of pathogenic bacteria such as Enterobacteriaceae strains providing thus better health status for animals.

4.4.11. *Fungicidal activity*

Peixoto *et al.* (2017) demonstrated the antifungal potential of the chemically characterized essential oil of *L. nobilis* against *Candida spp*. The MIC and MFC (minimum fungicidal concentrations) values of the essential oil ranged from 250 to 500 mg/ml. MIC values increased in the presence of sorbitol (osmotic protector) and ergosterol, indicating that the essential oil may affect cell wall biosynthesis and membrane ion permeability. At 2 MIC, essential oil disrupted the initial adhesion of *C. albicans* biofilms and affected biofilm formation without difference compared to nystatin. The phytochemical analysis identified isoeugenol as the main compound (53.49%) in the sample. The antifungal activity of *L. nobilis* essential oil is probably due to the monoterpenes and sesquiterpenes present in its composition.

Most of the aromatic molecules that are active (phenols, geraniol) on bacteria are also active on micromycetes, but with longer treatment times. The antifungal activity of bay laurel was examined *in vitro* on seven strains of phytopathogenic fungi at different concentrations such as 50, 125, and 250 mg/mL. The greatest antifungal activity was obtained against the fungus *Botrytis cinerea* at a concentration of 250 mg/mL (Patrakar *et al.*, 2012).

4.4.12. *Antiviral activity*

The combination of 1,8-cineole and monoterpenol, present in the essential oil of *L. nobilis*, is very effective in treating low pathologies of viral origin. *L. nobilis* essential oil containing the constituents β-ocimene, 1,8-cineol, α-pinene, and β-pinene has been reported for its inhibitory activity *in vitro* against the replication of the SARS-CoV coronavirus and the HSV-1 virus by visually scoring of the virus-induced cytopathogenic effect post-infection. *L. nobilis* oil demonstrated good activity against SARS-CoV with an IC50 value of 120 µg/ml and a selectivity index (SI) of 4,16 (Patrakar *et al.*, 2012). Roviello and Roviello (2021) examined over forty compounds found in bay laurel and found that nine of them had significantly high affinity for the major SARS-CoV-2 protease Mpro. One of the most important targets in anti-COVID-19 therapeutic strategies. Among these ligands derived from laurel, lauruside 5 is considered to be the most promising candidate as a potentially effective inhibitor of Mpro. The combination of 1,8-cineole and monoterpenol, present in the essential oil of *L. nobilis*, is very effective in treating low ENT (ear, nose, throat) pathologies of viral origin (Franchomme *et al.*, 2001).

4.4.13. *Insecticidal activity*

L. nobilis leaf essential oil was tested for its insecticidal activity against *Tribolium castaneum* using five different concentrations ranging from 4-12 mg/g and the results showed that the polar fraction of essential oil was more active as compared to the non-polar fraction, and the insecticidal potential was found to be both concentration and time-dependent (Chahal *et al.*, 2016).

Jemâa *et al.* (2012) evaluated the essential oils of *L. nobilis* for their repellent and toxic activities against two major stored product pests: *Rhyzopertha dominica* and *Tribolium castaneum*. The results showed that *L. nobilis* essential oils were repellent and toxic for both pests. *L. nobilis* essential oil was effective and expressed a half repulsive dose (RD50) of 0.013 ml/cm^2 for *R. dominica* versus 0.045 ml/cm^2 for *T. castaneum*. The corresponding half lethal dose (LC50) of 68 ml/l of air and 172 ml/l of air, respectively, and this clearly justifies the interest in the effectiveness of noble laurel essential oils both as insecticides and repellents against stored product pests. Likewise, Erler *et al.* (2006) reported that the essential oils of noble laurel would have a repellent activity against the insects of the species *Culex pipiens*.

5. CONCLUSION

L. nobilis is an aromatic herb with relevant medicinal properties due to its important chemical composition and its potential therapeutic effects. Due to its wide use, the chemical composition and biological activity of the plant have been widely studied. Several pharmacological studies have scientifically demonstrated some of the known activities of traditional medicine, including overall antimicrobial and antioxidant properties. Screening of literature on bay showed that essential oil possesses a wide range of biologically active compounds. Bay leaf essential oil finds application with a lot of pharmacological activities such as antimicrobial, insecticidal, antioxidant, anticonvulsant, etc. The presence of diverse constituents in bay leaf essential oil may be responsible for wide spectrum of biological activities.

Laurel leaves, due to a wide range of structurally diverse bioactive molecules and their antioxidant, antimicrobial, anti-inflammatory, and other health beneficial properties, are an excellent base for the production of high-quality extracts with potential applications in the food, pharmaceutical, and cosmetic industries. Insights into the biopotential of laurel require new approaches in the production of plant extracts, and consequently, the use of advanced green techniques that allow the development of formulations and high value-added products with improved biological properties and actions.

Since *L. nobilis* leaves are rich in phenolic compounds, future research should focus on various techniques that would result in better extraction of these compounds. Studying the biological activities of bay polyphenols for application in functional foods and supplements is extremely important. The pharmacological activities mentioned in the present study present prospects for the future and open up new areas for the multidisciplinary research and development of sustainable, effective, and economical procedures that would make it possible to exploit to the maximum the great potential of the leaves of *L. nobilis* and their bioactive molecules.

From this study, we can conclude that the results examined in the review article will be useful to researchers looking for new bioactive molecules and therapeutic interests extracted from the leaves of *L. nobilis*. The isolated compounds will be considered in the future for more clinical evaluations. Considering the pharmacological potential of *L. nobilis* leaves, this condiment will certainly attract more attention in the future.

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Declaration of Conflicting Interests and Ethics

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

Authorship Contribution Statement

Yazid Khaled Khodja: Writing - original draft. **Mostapha Bachir-bey:** Writing - review & editing. **Messaoud Belmouhoub:** Writing the manuscript. **Rachid Ladjouzi:** Editing. **Farid Dahmoune:** Resources. **Bachra Khettal:** Supervision.

Orcid

Yazid Khaled Khodja https://orcid.org/0000-0001-8565-4991
Mostapha Bachir-bey https://orcid.org/0000-0002-9987-1505
Messaoud Belmouhoub https://orcid.org/0000-0003-1748-8914
Rachid Ladjouzi https://orcid.org/0000-0003-3178-5333
Farid Dahmoune https://orcid.org/0000-0001-6072-0411
Bachra Khettal https://orcid.org/0000-0001-6803-4109

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Research Article

The effect of extraction methods on the yields, chemical composition and antifungal activity of sawdust *Cedrus atlantica* Manetti essential oils

Chaimaa Bouyahia[1], Maria Benbouzid[1], Souad El Hajjaji[1], Miloudia Slaoui[2], Fatiha Bentata[3], Mustapha Labhilili[4], Abdelhakim Bouyahya[5,*]

[1]Laboratory of Spectroscopy, Molecular Modeling, Materials, Nanomaterials, Water and Environment, CERNE2D, Faculty of Sciences, Mohammed V University in Rabat, Av Ibn Battouta, BP1014, Agdal, Morocco
[2]Laboratory Energy Materials and Sustainable Development (EMDD), Center, Water Natural Resources Environment and Sustainable Development (CERNE2D), Ecole Supérieure de Technologie de Salé, Boulevard MohamedVI-Salé, Université Mohammed V in RABAT, Morocco
[3]Research Unit of Biotechnology, National Institute for Agricultural Research, Rabat, Morocco
[4]Research Unit of Aromatic and Medicinal Plants, National Institute for Agricultural Research, Rabat, Morocco
[5]BioPath, Faculty of Sciences, Mohammed V University in Rabat, Morocco

Abstract: The aim of this work is to determine the effect of the extraction method on the yield, the chemical composition, and the antifungal activity of cedarwood essential oils (EOs) from sawdust of Moroccan *Cedrus atlantica (C. atlantica)*. EOs were extracted by different methods: hydrodistillation, soxhlet, maceration, and ultrasound. The chemical composition was determined using gas chromatography mass spectrometry (GC-MS) analysis. The yields of EOs were 5.60%, 11.68%, 4.82% and 9.33% for hydrodistillation, soxhlet, maceration and ultrasound, respectively. GC-MS revealed a diversity of chemical compounds depending on extraction methods. Indeed, the main compound of EOs obtained by soxhlet, maceration, and ultrasound was copalic acid methyl ester by a rate of 28.41%, 20.24%, and 24.17%, respectively. However, β-himachalene (21.32%) followed by α-himachalene (9.40%), β-Copaen-4α-ol (7.71%) and longifolene (6.74%) are the main compounds of EO extracted by hydrodistillation. The antifungal activity of cedarwood EO was tested in vitro on two pathogenic fungi*: Fusarium culmorum* (*F. Culmorum)* and *Botrytis cineria (B. Cinerea)*. The minimum inhibitory concentration (MIC) was determined by successive dilutions of the stock solutions. The extracted EOs by soxhlet, maceration and ultrasound showed the important inhibitory effect against *B. cinerea* (MIC=1.25 mL/L). However, *F. culmorum* showed resistance towards all tested EOs. The finding of this study showed clearly that the volatile composition of EOs can be variable according to extraction. methods. Moreover, antifungal effects are depending on chemical composition of EOs but also to tested staris.

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1. INTRODUCTION

Medicinal plants are an important source of significant secondary metabolites with therapeutic potential (Al-Rimawi *et al*., 2020; Newman *et al*., 2020). The growing need for new treatments for a variety of health issues, as well as the emergence of multidrug-resistant bacteria, cancer chemotherapy resistance, adverse effects of commercial drugs, and economic pressures, have

*CONTACT: Abdelhakim Bouyahya ✉ a.bouyahya@um5r.ac.ma; souad.elhajjaji@um5.ac.ma ✉ Faculty of Sciences, Mohammed V University in Rabat, Morocco

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all contributed to an increase in interest in natural product chemistry research (Belkacem *et al*., 2021; Bouyahia *et al*.,2022). Eos are the volatile components of plants that are responsible for their aroma. They have biological properties, which include antimicrobial (Abers *et al*., 2021), anticancer (Fitsiou & Pappa, 2019), anti-inflammatory (Ribeiro *et al*., 2018, antiparasitic (Mancianti & Ebani, 2020), and antioxidant (Pateiro *et al*., 2018) activities.

We were interested in the species *C. atlantica* in the context of this work because it is an important forest tree species distributed in northern Africa and one of the most economically and ecologically important species in Morocco's Mediterranean mountains belonging to the Pinaceae family (Moukrim *et al*., 2020). Numerous pharmacological investigations have been reported that *C. atlantica* exhibited several activities such as anticancer (Huang *et al*., 2020; Hung *et al*., 2020), molluscicidal (Lahlou, 2003), antioxidant (Belkacem *et al*., 2021; Bouyahia *et al*., 2022), antimicrobial (Derwich *et al*., 2010a; Zrira *et al*., 2016), larvicidal (Zoubi *et al*., 2017), antiviral (Loizzo *et al*., 2008), insecticidal (Ainane *et al*., 2019; Emer *et al*., 2018), and antifungal (Bouchra *et al*., 2003; Fidah, 2016) effects.

Phytopathogenic fungi are the cause of several plant diseases that cause yield losses of up to 20% per year on average (Tasei, 1996). To compensate these losses, synthetic fungicides are the primary means of control. However, these chemicals exhibit serious biological side effects on ecosystems (Tasei, 1996; Yu *et al*., 2011). Furthermore, the use of natural bioactive compounds such as aromatic and medicinal plants is considered actually as a promising strategy (AMPs) (Benkherara *et al*., 2011).

In Morocco, growing awareness of the harmful effects of pesticides on the environment has prompted studies on the development of alternative methods such as organic farming and the use of botanical extracts and EOs in plant protection (El Guilli *et al*., 2009; Habybellah, 2006). The main methods used to obtain the EOs from the plant materials are hydrodistillation, maceration, steam distillation, expression and empyreumatic (or destructive) distillation. Hydrodistillation has been the most widely used method (Stahl-Biskup & Sáez, 2002). Ultrasound assisted extraction (Da Porto *et al*., 2009), Microwave-assisted extraction (Wang *et al*., 2010), and supercritical fluid extraction (Li *et al*., 2009) are examples of new approaches that have been used to improve extraction yield, reduce extraction time, and lower operational costs. Many studies on medicinal plants have found that harvest season, geographical origin and extraction method all have an impact on the chemical composition and functional activities of EOs (Govrin & Levine, 2000; Yesil *et al*., 2007).

However, no comparative work exposing the effect of different extraction methods on the chemical composition of EOs as well as the antifungal activity has been published to our knowledge. As a result, the purpose of this study was to investigate the effect of the extraction methods (hydrodistillation, soxhlet, maceration et ultrasound) on the yield, chemical composition and antifungal activity of EOs extracted from *C. atlantica* wood.

2. MATERIAL and METHODS

2.1. Plant material

The sawdust samples were collected at Azrou sawmill (Middle Atlas). Grinding was performed until a fine and homogeneous powder was obtained.

2.2. Essential oil extraction

2.2.1. *Extraction with an organic solvent*

The oils studied were extracted with an organic solvent at the rate of 3 tests to express the values of the yields relative to the dry matter. During each test, 100 g of the raw material was mixed with 700 mL of hexane. The EOs were extracted using the soxhlet, maceration and ultrasound.

- **Soxhlet**

A mass of cedar wood powder was placed in a porous cartridge which was introduced into a soxhlet extractor with a balloon in the base where the hexane was introduced for 6 h

- **Maceration**

A quantity of the powder of cedar wood was macerated in cold for 12 h, with the hexane which retains the chemical compounds we intend to extract. Once the time had elapsed, the mixture had been filtered through a filter paper and the maceration obtained was stored in a bottle.

- **Ultrasound**

Ultrasound-assisted extraction was performed with a powder sonicated in hexane for 10 minutes. After this treatment, the extract was filtered and conserved in a bottle.

The mixture collected by the different extraction methods mentioned above was subjected to vacuum pressure using a rotary evaporator, to separate the solvent and the EOs.

2.2.2. *Extraction by hydrodistillation*

A quantity of water and 100 g of sawdust were immersed in a balloon. installed at the Clevenger device for 6 hours. The mixture (oil and water) was separated by density difference. The recovered oil was placed in a small opaque bottle and stored at fridge.

2.3. GC–MS analysis

The constituents were identified using a gas chromatography coupled to a mass spectroscopy. Perkin Elmer Version ClarusTM GC-680 with Q-8 MS is the device. It is equipped with an auto-sampler which gives access to the automatic injection of samples into the injector and an HP-5MS type capillary column traversed by Helium gas. The mass spectrometer was powered by a source of electronic ionization SMART source which allowed to ionize and vaporize the different molecules as well as a quadrupole filter to separate the different ions according to their m/z ratio. The GC-MS system was controlled by a computer device accompanied by Turbomass TM software which allowed the programming of analysis methods as well as the qualitative and quantitative identification of the species detected.

The analysis time was 82 min with a gas flow rate of 1 mL / min. The ionization energy was 70 eV. The volume injected was 0.5 µL (10%of the EO was dissolvedwith 90% of hexane) at 280 °C. The oven temperature was programmed to start at 60°C for 1 minute, followed by a temperature gradient of 2°C/min up to 200°C, where it remained for 1 minute. Subsequently, the temperature was raised to 300°C with a ramp of 20°C/min and held for 5 minutes.

2.4. Fungal Material

B. cineria is a pathogenic ubiquitous and polyphagous ascomycete. This fungus is responsible for rots on a large number of economically important host plants in agriculture. It attacks more than 200 crop species worldwide (Govrin & Levine, 2000; Lecompte *et al*., 2013). *F. culmorum* is a fungus found in all cereal growing regions around the world that causes a destructive disease that not only reduces yield and grain quality but also contaminates grain with various mycotoxins (Kang *et al.*,2000). The two fungal strains studied, *F. culmorum* and *B. cineria*, came from the phytopathology laboratory of the INRA in Rabat. They were regularly transplanted and maintained on PDA (Potato Dextrose Agar) medium.

2.5. Antifungal Effects of Essential Oils

PDA medium with a concentration of 1.25 ml/L of each EO was prepared for the two fungi. After homogenization using 0.5 % of tween 80 as an emulsifier, both solutions and control medium were poured into 9 cm diameter Petri dishes and allowed to solidify. After 24 hours, a

mycelial fragment of 5 mm in diameter was placed in the center of each Petri dish. The plates were incubated at 20°C for 7 days. The diameter of the fungal colonies was measured daily. MIC is relative to the EO whose initial concentration completely controls the growth of the fungus. Culture media at concentrations of 0.624, 0.312 and 0.156 ml/L corresponding to dilutions of 1/2, 1/4 and 1/8 to stock solutions (1.25 ml/L) respectively, plus a control medium were prepared and poured into 9 cm diameter Petri dishes. After solidification, the dishes were inoculated and incubated at 20°C for 7 days. Each treatment was repeated 3 times, and the growth of the fungus was monitored daily in each dish.

To investigate the effect of the EOs on the two fungi, mycelial growth was plotted against the variation of radial mycelial growth with days of incubation. The reduction rate was calculated according to the equation:

$$\boldsymbol{I = (dt - dc/dt) \times 100}$$

Where: I = Rate of reduction of mycelial growth.

dt = Maximum diametrical growth (cm) of mycelium on control medium

dc = Maximum diametrical growth (cm) of mycelium on the culture medium at a certain concentration of EO.

According to Laib *et al.* (2012), the concentration of an EO is highly active with an inhibition between 75 and 100% and then the fungal strain is highly susceptible. The concentration of an EO is active when the inhibition is between 50 and 75% and the fungal strain is said to be sensitive; moderately active when it has an inhibition between 25 and 50% and the strain is said to be limited; and not very or not at all active when it has an inhibition between 0 and 25%; the strain is said to be little sensitive or resistant.

3. RESULTS and DISCUSSION

3.1. Yields of extraction

The extraction yield values ranged from 4.82 % for the maceration to 11.68 % for the soxhlet (Table 1). The yield for the EOs obtained by soxhlet and ultrasound methods was higher than that of the classical methods (hydrodistillation and maceration). This confirms the advantages of the new extraction methods in terms of yield. These yields provided by the sawdust are higher than those obtained by the needles (1.8%) (Derwich *et al*.,2010b), winged (2.6%) and wingless (3.6%) seeds (Rhafouri *et al*., 2014). The studies led by Aberchane *et al*. (2001); Fidah *et al*. (2016) on the cedarwood, originating from Azrou provided a yield did not exceed 3.41%. EOs yields are subject to fluctuations and variations that can be attributed not only to the extraction method or the part of the tree used, but also to the provenance or age and harvesting period (Duval, 2012).

Table 1. The extraction yield of the different extraction methods.

Extracts	Extraction time	Yield (%)
Hydrodistillation	6 h	5.60
Soxhlet	6 h	11.68
Maceration	12 h	4.82
Ultrasound	10 min	9.33

3.2. Chemical Composition of The EOs

The chemical composition of the extracted EOs of various methods is given in Table 2. These analyses allowed us to identify approximately 33 components, which represent about 76.19%, 81.14%, 72.84% and 74.03% of the total EOs composition for hydrodistillation, soxhlet, maceration and ultrasound respectively. The obtained results showed that the chemical

composition of the oils obtained by hexanic solvent is different to those extracted by hydrodistillation (Figure 1). The main compound found by soxhlet (Figure 2), maceration (Figure 3) and ultrasound (Figure 4) is: Copalic acid methyl ester (28.41%, 20.24%, and 24.17%, respectively). On the contrary, we find the major constituent one for the oil obtained by hydrodistillation being: β-himachalene (21.32%) followed by α-himachalene (9.40%). These differences can be due to the influence of the polarity of the solvent and the extraction method.

The chemical analysis obtained by hydrodistillation has constituents relatively similar to those of other cedarwood EO analyzed by Aberchane *et al*. (2001). They found that the major compounds are β-himachalene (39.72%), α-himachalene (15.78%) γ-himachalene (9.56%) and E-a-atlantone (9.15%). In addition, in a recent study realized by Jaouadi *et al*. (2016) about EO of *C. atlantica* (in the provinces of the Middle Atlas of Morocco in the forest of Itzer) approximately comparable to the composition of our EO, the main compounds identified were: β-himachalene (24.25%), α-himachalene (13.76%), methyl-1,4-cyclohexadiene (9.06%) trans-cadina-1(6),4-diene (7.65%), and 6-camphenol (7.44%).

Figure 1. GC-MS chromatogram of *C. atlantica* EO extracted by hydrodistillation.

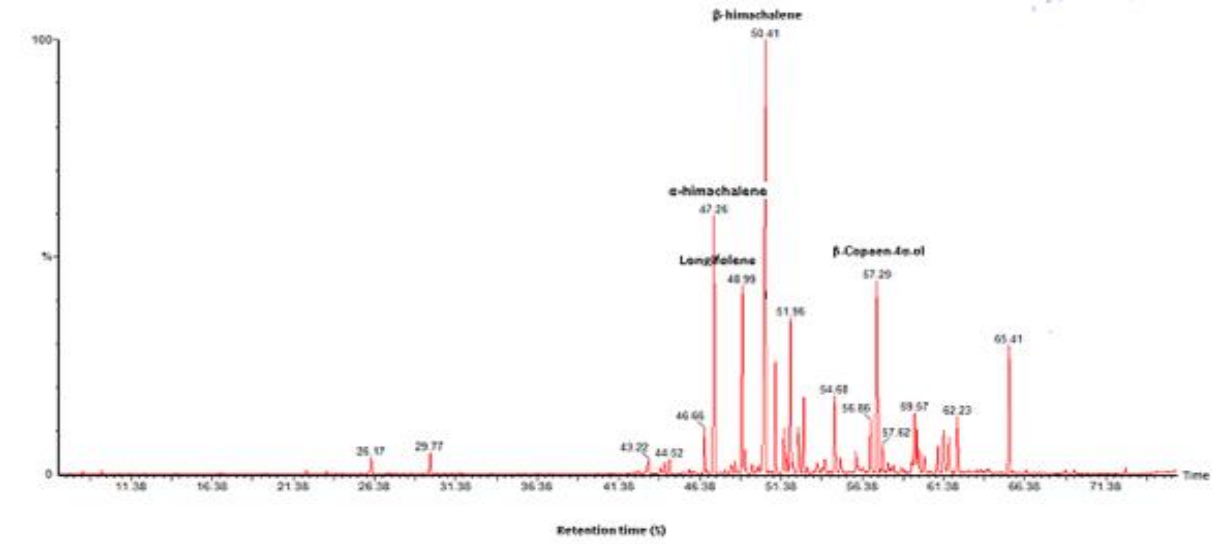


Figure 2. GC-MS chromatogram of *C. atlantica* EOextracted by soxhlet.

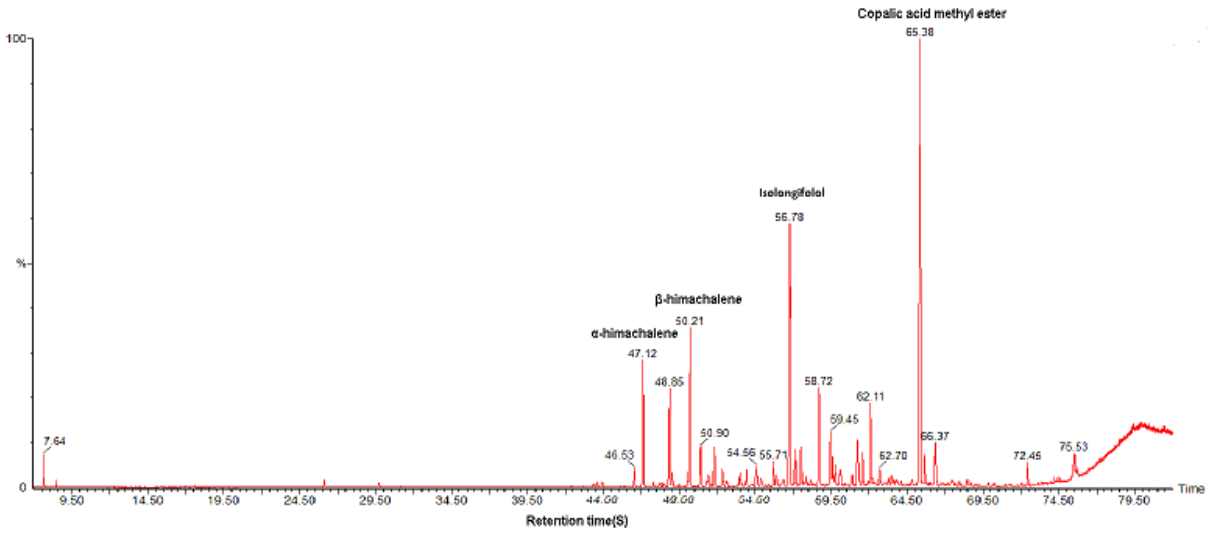


Figure 3. GC-MS chromatogram of *C. atlantica* EO extracted by maceration.

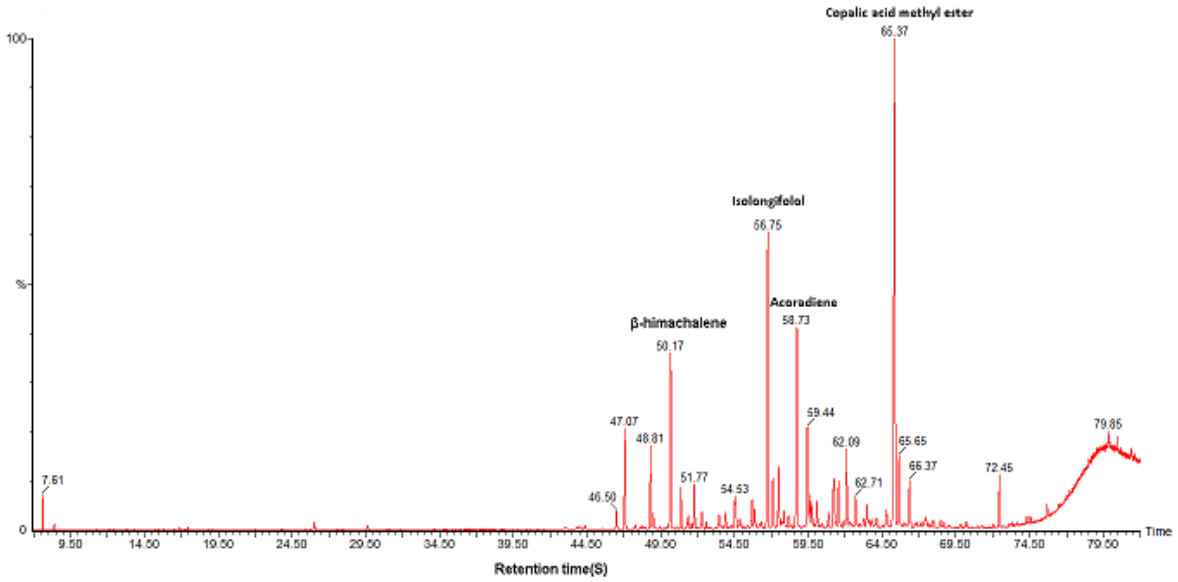


Figure 4. GC-MS chromatogram of *C. atlantica* EO extracted by ultrasound.

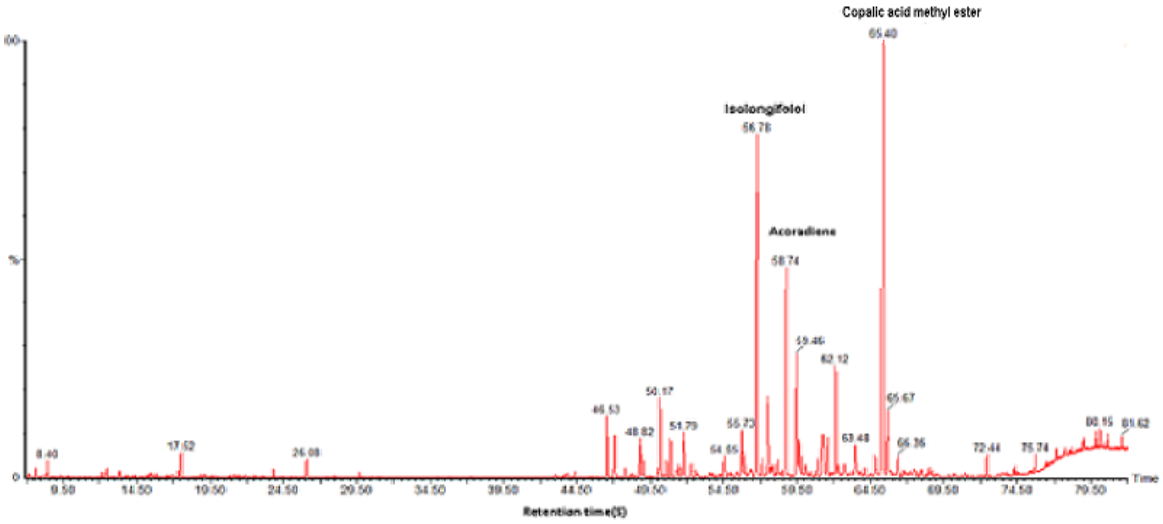


Table 2. Chemical composition of cedarwood EOs.

Compound	Hydrodistillation		Soxhlet		Maceration		Ultrasound	
	TR	%	TR	%	TR	%	TR	%
P-Cresol	22.18	0.12	nd	nd	nd	nd	Nd	nd
Endo-borneol	28.59	0.02	nd	nd	nd	nd	Nd	nd
Calarene	44.21	0.37	nd	nd	nd	nd	44.36	0.18
Farnesol	46.65	1.43	46.53	0.83	46.49	0.5	46.52	1.85
shouldα -himachalene	47.26	9.4	47.11	5.48	47.06	2.71	47.07	1.34
Longipinene	nd	nd	48.85	4.12	48.81	2.31	48.81	1.19
Longifolene	48.99	6.74	49.02	0.66	48.98	0.43	48.99	0.71
β-himachalene	50.41	21.32	50.20	7.3	50.17	5.14	50.17	2.52
1-Mesitylbuta-1.3-diene	51.03	3.77	50.89	1.79	50.86	1.12	50.87	1.22
δ-Cadinene	nd	nd	51.40	0.73	51.36	0.5	51.38	0.54
E-Calamenene	51.54	1.73	nd	nd	nd	nd	51.6	0.37
α-Calacorene	51.75	0.62	51.80	1.71	51.76	1.21	51.78	1.54
Androstenediol	55.02	0.66	nd	nd	nd	nd	55.03	0.07
Longiborneol	56.00	0.91	nd	nd	nd	nd	Nd	nd
Isolongifolol	56.86	2	56.77	12.99	56.74	9.25	56.78	13.65
β-Copaen-4α-ol	57.28	7.71	nd	nd	nd	nd	Nd	nd
α-Cubebene	57.62	0.9	nd	nd	nd	nd	57.67	0.16
γ-Gurjunene	58.28	0.24	nd	nd	nd	nd	Nd	nd
Acoradiene	58.78	0.28	58.72	4.79	58.73	6.74	58.74	8.58
β-humulene	59.57	1.83	59.45	2.35	59.44	2.82	59.46	4.05
Carveol	59.73	1.01	59.75	0.66	59.74	0.49	59.75	0.77
Ledane	59.87	0.65	nd	nd	nd	nd	Nd	nd
Cadalene	60.20	0.66	nd	nd	60.09	0.76	Nd	nd
Cedreanol	61.25	3.32	61.25	3.3	61.24	2.42	61.25	2.31
Curlone	61.37	3.69	nd	nd	nd	nd	Nd	nd
Tumerone	62.05	0.03	62.10	3.57	62.09	2.23	62.11	3.55
Tujopsene	62.22	1.95	nd	nd	nd	nd	62.31	0.34
Longipinane	62.77	0.04	62.70	0.83	62.7	0.96	62.71	0.49
β-cis-caryophyllene	63.4	0.11	63.49	0.44	63.46	0.64	63.47	1.3
Copaen-15-ol	64.86	0.07	nd	nd	64.79	0.72	64.8	0.9
Copalic acid methyl ester	65.40	4.54	65.38	28.41	65.37	20.24	65.4	24.17
Trans-Calamenene	65.59	0.07	65.64	1.18	65.64	1.75	65.66	2.02
Octasiloxane	nd	nd	nd	nd	79.85	9.9	79.85	0.21
Total identified		76.19%		81.14%		72.84%		74.03%

Table 2. *Continues.*

Sesquiterpene	54.37%	31.38%	27.09%	26.55%
Calarene	0.37	nd	nd	0.18
α-himachalene	9.4	5.48	2.71	1.34
Longipinene	nd	4.12	2.31	1.19
Longifolene	6.74	0.66	0.43	0.71
β-himachalene	21.32	7.3	5.14	2.52
1-Mesitylbuta-1.3-diene	3.77	1.79	1.12	1.22
δ-Cadinene	nd	0.73	0.5	0.54
E-Calamenene	1.73	nd	nd	0.37
α-Calacorene	0.62	1.71	1.21	1.54
α-Cubebene	0.9	nd	nd	0.16
γ -Gurjunene	0.24	nd	nd	nd
Acoradiene	0.28	4.79	6.74	8.58
β-humulene	1.83	2.35	2.82	4.05
Ledane	0.65	nd	nd	nd
Cadalene	0.66	nd	0.76	nd
Curlone	3.69	nd	nd	nd
Tujopsene	1.95	nd	nd	0.34
Longipinane	0.04	0.83	0.96	0.49
β-cis-caryophyllene	0.11	0.44	0.64	1.3
Trans-Calamenene	0.07	1.18	1.75	2.02
Alcohols	17.25%	17.78%	13.38%	19.55%
P-Cresol	0.12	nd	nd	nd
Endo-borneol	0.02	nd	nd	nd
Farnesol	1.43	0.83	0.5	1.85
Androstenediol	0.66	nd	nd	0.07
Longiborneol	0.91	nd	nd	
Isolongifolol	2	12.99	9.25	13.65
Carveol	1.01	0.66	0.49	0.77
β-Copaen-4α-ol	7.71	nd	nd	nd
Cedreanol	3.32	3.3	2.42	2.31
Copaen-15-ol	0.07	_	0.72	0.9
Tumerone	0.03%	3.57%	2.23%	3.55%
Copalic acid methyl ester	4.54%	28.41%	20.24%	24.17%
Octasiloxane	nd	nd	9.9%	0.21%

nd: not detected.

3.3. Antifungal Activity

3.3.1. *Fusarium culmorum*

Mycelial growth was slowed for the concentrations of 1.25mL/L, 0.624 mL/L, 0.312 mL/L, and 0.156 mL/L of *C. atlantica* EOs extracted by hydrodistillation, soxhlet, maceration and ulrasound (Table 3). At the stock concentration of 1.25 mL/L, *C. atlantica* EOs did not control the development of the fungus, but they significantly slowed its growth compared to the control (Figure 5).

Figure 5. Effects of stock concentrations (1.25 mL of the EO/L of culture medium) of *C. atlantica* EOs on the radial growth of *F. culmorum*.

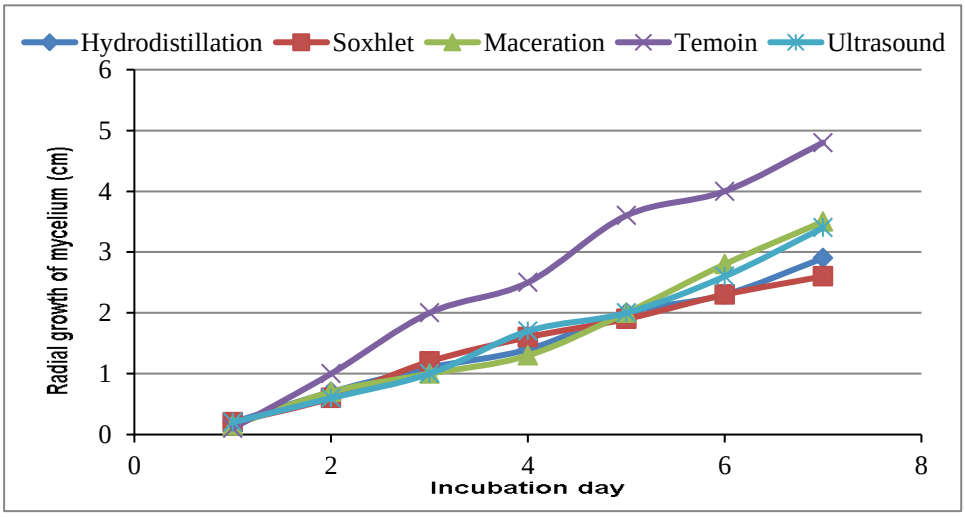


Figure 6. The effect of concentration (1. 25 mL/L) of EOs (H: oil extracted by hydrodistillation, S: oil extracted by soxhlet, M: oil extracted by maceration and U: oil extracted by ultrasound) on growth of *F. culmorum* after 7 days of incubation.

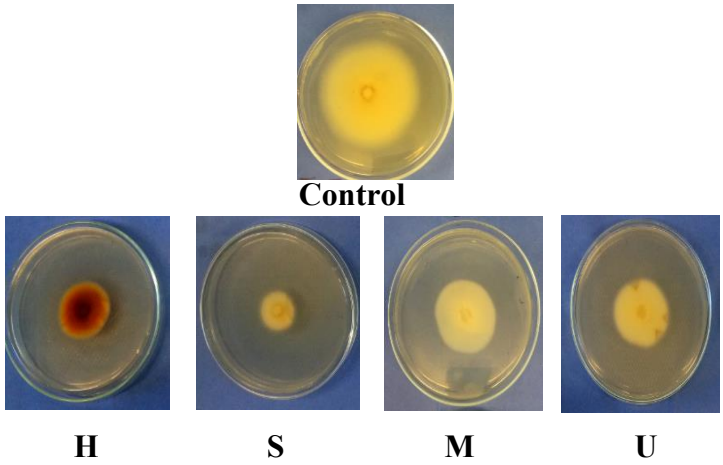


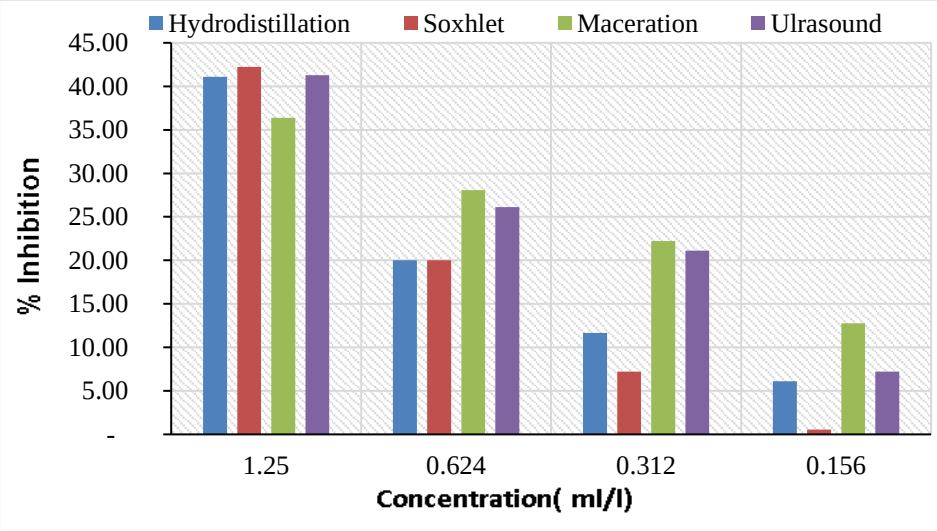
Figure 6 shows the effect of concentration (1. 25 mL/L) of EOs on growth of F. culmorum after 7 days of incubation. Our results confirmed other authors' works on the antifungal power of *C. atlantica* EOs. Indeed, Uwineza *et al*. (2016) found that the EO of *C. atlantica* bark did not control the development of the fungus, but it only inhibited the growth of *F. culmorum*. According to the formula 1, the mode of action for each concentration is presented in Figure 7. It confirms the fact that *F. culmorum* is limited to the highest concentration (1.25 mL/L) of *C. atlantica* EO.

Table 3. Average growth (in cm) of F. *culmorum* on culture medium PDA supplemented with different concentrations of cedar EOs.

F.Culmorum	Concentration (mL/L)	D1	D2	D3	D4	D5	D6	D7
Hydrodistillation	1.25	0.2	0.7	1.1	1.4	2	2.3	2.9
	0.624	0	0.5	1.5	2.1	2.9	3.3	4.1
	0.312	0	0.7	1.7	2.3	3.1	3.6	4.5
	0.156	0.1	0.9	1.8	2.5	3.1	3.8	4.7
Soxhlet	1.25	0.2	0.6	1.2	1.6	1.9	2.3	2.6
	0.624	0	0.4	1.5	2.1	2.9	3.5	4
	0.312	0	0.8	1.8	2.4	3.3	3.9	4.5
	0.156	0.1	1	1.9	2.5	3.6	4	4.8
Maceration	1.25	0.15	0.7	1	1.3	2	2.8	3.5
	0.624	0	0.3	1.1	1.6	2.15	3.5	4.3
	0.312	0	0.4	1.2	1.7	2.2	3.8	4.7
	0.156	0	0.7	1.5	2.5	2.3	3.9	4.8
Ultrasound	1.25	0	0 .3	0.7	1.4	2	2.9	3.3
	0.624	0	0.5	0.9	1.7	2.2	3.4	4.6
	0.312	0	0.6	1	1.8	2.3	3.8	4.7
	0.156	0	0.8	1.8	2.5	2.8	4	4.8
Control	0	0.1	1	2	2.5	3.6	4	4.8

D: Incubation day after inoculation

Figure 7. Reduction rate of radial growth of *F. culmorum* by different concentrations of *C. atlantica* EOs.



3.3.2. *Botrytis cinerea*

The stock solution (1.25 mL/L) of *C. atlantica* EOs extracted by hexanic solvent (soxhlet, maceration and ultrasound) completely inhibited mycelial growth, whereas the EO extracted by hydrodistillation slowed down the development of this fungus considerably but did not inhibit it completely (Figure 8). The successive dilutions 1/2, 1/4 and 1/8 were used for each PDA-EO mixture, corresponding to oil concentrations of 0.624 mL/L, 0.312 mL/L and 0.156 mL/L respectively. All these concentrations decreased the growth compared to the control (Table 4).

Figure 8. Effects of stock concentrations (1.25 ml/L) of EOs of *C. Atlantica* on the radial growth of *B. cinerea*.

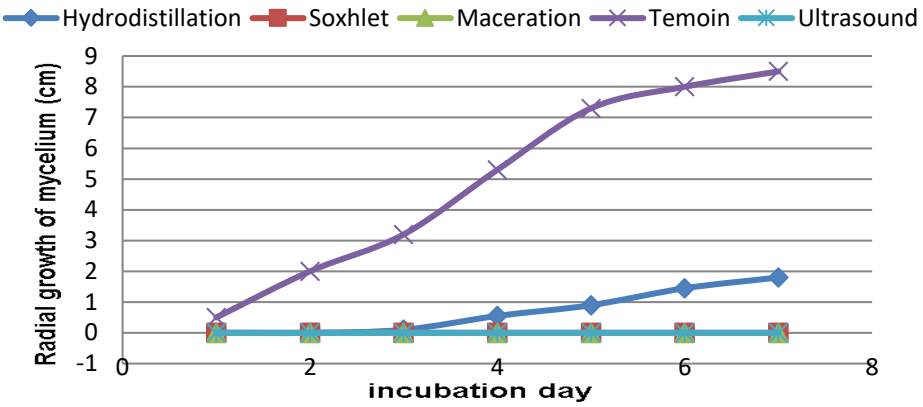


Figure 9. The effect of concentration (1.25 mL/L) of EOs (H: oil extracted by hydrodistillation, S: oil extracted by soxhlet, M: oil extracted by maceration and U: oil extracted by ultrasound) on growth of *B. cinerea* after 7 days of incubation.

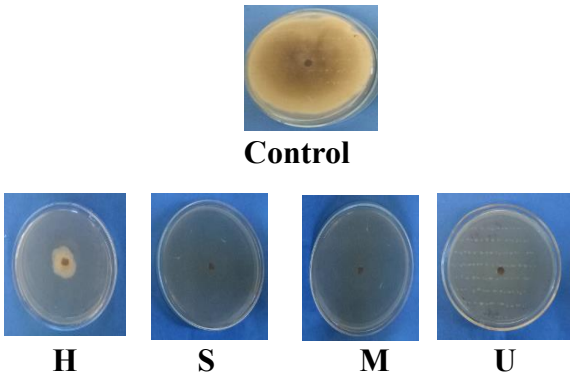


Table 4. Average growth (in cm) of *B.cinerea* on culture medium PDA with different concentrations of EOs of *C. atlantica*.

B. cinerea	Concentration (mL/L)	D1	D2	D3	D4	D5	D6	D7
Hydrodistillation	1.25	0	0	0.1	0.55	0.9	1.45	1.8
	0.62	0	0	1	1.5	1.7	2.5	3.1
	0.31	0	0.1	1.1	2	2.8	3	3.5
	0.15	0.1	0.8	3	5.3	7.2	8	8.5
Soxhlet	1.25	0	0	0	0	0	0	0
	0.62	0	0	0	0	0.2	0.8	1.1
	0.31	0	0.1	1.1	1.6	2.1	2.5	3.3
	0.15	0.3	1.3	2.7	5.3	6.5	8	8.5
Maceration	1.25	0	0	0	0	0	0	0
	0.62	0	0	0.9	1.9	2.9	3.3	3.8
	0.31	0	0.1	1	2.1	2.7	3.5	4.5
	0.15	0.1	0.5	1.1	3	4.4	5.3	6.2
Ultrasound	1.25	0	0	0	0	0	0	0
	0.62	0	0.1	1.3	2.3	3.4	3.8	4.1
	0.31	0.1	1.2	2.7	4.2	4.5	5	5.2
	0.15	0.2	1.5	3.2	4.4	4.8	5.2	5.9
Control	0	0.5	2	3.2	5.3	7.3	8	8.5

D: Incubation day after inoculation

Figure 10. Rate of reduction of radial growth of *B. cinerea* by different concentrations of *C. atlantica* EOs for the two extraction methods.

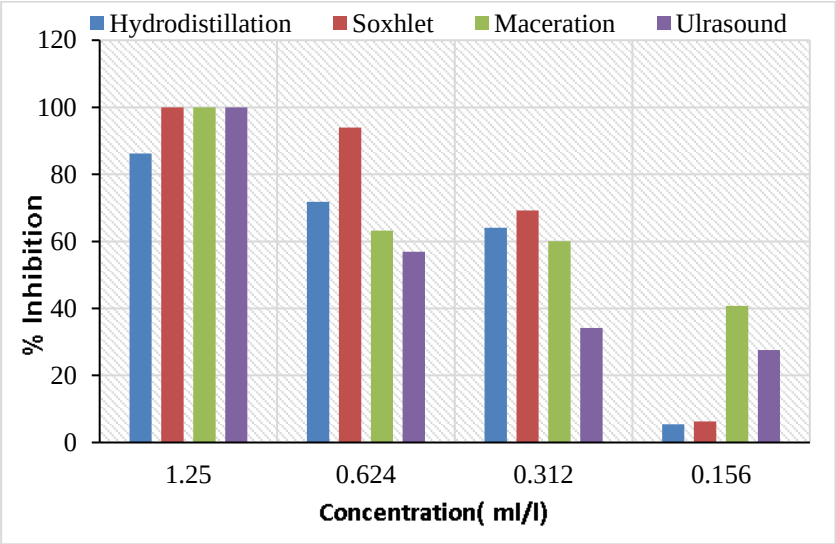


Figure 9 shows the effect of concentration (1.25 mL/L) of EOs on growth of B. cinerea after 7 days of incubation. The 1.25 mL/L concentration of *C. atlantica* EOs extracted by soxhlet. maceration and ultrasound (Figure 10) corresponds to the MIC for B. cinerea. The studies led by Chebli *et al*. (2004) of *C. atlantica* leaf oil showed inhibition of mycelial growth against *B. cinerea* at 250 ppm. Figure 6 shows that. *B. cinerea* is very sensitive to 1.25 mL/L of the *C. atlantica* EOs.

C. atlantica EOs extracted by soxhlet. maceration and ultrasound with hexanic solvent had stronger antifungal activity than EOs extracted with hydrodistillation. This is related to their majority compounds. This was confirmed with the studies by Nakamura *et al*. (2017). which showed that copalic acid has a greater antifungal property. The research condcuted by Rhafouri *et al*. (2014) showed the antifungal power of *C. atlantica* EO extracted by hydrodistillation with a concentration of 1/100 (v/v). and this allowed us to assume that for the EO extracted by hydrodistillation. Higher concentrations could give better results than those obtained in this study.

All these biological properties of EOs could well be attributed not only to their majority compounds (Bourkhiss *et al*., 2007; Kellouche & Soltani, 2004). But also to the synergistic effect of minority compounds (Bouzouita *et al*., 2008; Kordali *et al*., 2008).

4. CONCLUSION

The yields of EO extracted by hyrodistillation. soxhlet. maceration and ultrasound were 5.60%. 11.68%. 4.82%, and 9.33%, respectively. The main constituent of EOs obtained by hexanic solvent (soxhlet, maceration, and ultrasound) is copalic acid methyl ester (28.41%. 20.24% and 24.17% respectively). While, the major compound of the EO obtained by hydrodistillation is: β-himachalene (21.32 %), according to the results of the antifungal activity evaluations. the EOs of *C. atlantica* extracted from the hexanic solvent were the best against *B. cinerea* with the minimum fungicidal concentration of 1.25 mL/L. While for the same concentration of *C. atlantica* EO. it was found that *F. culmorum* slowed the development of the fungus without stopping it completely. Our study demonstrated the potential of *C. atlantica* EO as a conservative antifungal agent in vitro against two pathogens *B. cinerea and F. culmorum*.

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

Chaimaa Bouyahia and **Maria Benbouzid**: Investigation, Software, Resources, and Writing - original draft. **Souad El Hajjaji** and **Miloudia Slaoui**: Methodology, Supervision, and Validation. **Fatiha Bentata**, **Mustapha Labhilili**, and **Abdelhakim Bouyahya**: visualization, editing the original draft.

Orcid

Chaimaa Bouyahia https://orcid.org/0000-0003-4223-7201
Maria Benbouzid https://orcid.org/0000-0002-5318-9156
Souad El Hajjaji https://orcid.org/0000-0003-1467-704X
Miloudia Slaoui https://orcid.org/0000-0003-1737-0633
Fatiha Bentata https://orcid.org/0000-0002-0008-0050
Mustapha Labhilili https://orcid.org/0000-0002-4844-8096
Abdelhakim Bouyahya https://orcid.org/0000-0001-9317-1631

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Research Article

Chemical composition of essential oils from *Crocus ancyrensis* (Herbert) Maw Spreading In Çorum (Türkiye) Region

Hacer Dogan[1,*], **Omer Kayir**[1], **Erol Alver**[2], **Ibrahim Bilici**[2]

[1]Scientific, Technical, Application and Research Center, Hitit University, Çorum, Türkiye
[2]Chemical Engineering, Facultyof Engineering, HititUniversity, Çorum, Türkiye

Abstract: *Crocus ancyrensis* is a yellow-flowered *Crocus* species and is in the same family and genus with saffron. Although various studies have been conducted on the antioxidant capacity and essential oil content characterization of saffron (*Crocus sativus*), there is no literature information about the *C. ancyrensis* plant, which is known as *Crocus* among the people. The members of *Crocus* family contain many valuable components including antioxidants, phenolic compounds and essential oils. The essential oils obtained from this family is a complex mixture of more than 30 components, which are primarily terpenes and their derivatives. These mixtures are used in paint, medicine, and food applications especially in the cosmetics sector. In the study, the essential oils of the *Crocus ancyrensis* plant were extracted with the clevenger system and characterized by GC-MS analyses. As a result, 23 volatile components were identified. 2-Hexenal, 1-ethylbutyl Hydroperoxide, 2-nitro-Hexane, β-Isophorone, α-Isophorone, 2-Caren-10-al and Eugenol are found as the main components of *Crocus ancyrensis* plant extract. Due to the antioxidant, antimicrobial, antifungal, anticancer and odorant properties of some of the identified components, *C. ancyrensis* can be used as a medicinal aromatic plant in various fields, especially in the cosmetics and perfume industry.

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1. INTRODUCTION

Essential oils found in parts of plants such as leaves, stems, roots, and flowers contain complex compounds with strong odor and are easy to crystallize (Bakkali *et al.*, 2008; Chavez-Gonzalez *et al.*, 2016). The chemical contents vary based on the type of plants, the geography, climate, production methods, and the area where the plant is grown (De Martino *et al.*, 2015). They are frequently used in cosmetics, food, and pharmaceutical (Asil, 2021) industries due to their unique different properties such as scenting, flavoring, and antibacterial activity (Buckle, 2015). Different methods such as supercritical CO2 extraction (Donelian *et al.*, 2016), ultrasonic extraction (Asil, 2018) and microwave enhanced hydrodistillation can be used for essential oil extraction from herbal plants (Hamidi, 2016; Jain *et al.*, 2022)

*CONTACT: Hacer DOGAN hacerdogan@hitit.edu.tr Hitit University, Scientific, Technical, Application and Research Center, Çorum, Türkiye

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Crocus belongs to the Iridaceae family containing approximately 2050 species around the world (Goldblatt *et al.*, 2008). The most well-known and researched one is *Crocus sativus* L., which has a high commercial value, and is called saffron. *Crocus* species include more than 80 species (about 30 are cultivated) and Türkiye is one of the richest countries in terms of these growing species. Especially, endemic *Crocus* taxa are found about 61% in Türkiye (Ozhatay, 2002). It is known that saffron is widely used as an aphrodisiac, antispasmodic, expectorant (Recio *et al.*, 1995; Asil & Göktürk, 2021), as well as various pharmacological effects such as antitumor, antioxidant, antidepressant, anti-inflammatory, memory and learning enhancement, treatment of hepatic disorders, and reducing insulin resistance (Sanchez-Vioque *et al.*, 2012).

For this reason, many studies have focused on *Crocus sativus*. (Srivastava *et al.*, 2010; Samarghandian & Borji, 2014; Mollazadeh *et al.*, 2015; Mzabri *et al.*, 2019; Cid-Perez *et al.*, 2021; He *et al.*, 2021; Abu-Izneid *et al.*, 2022; Butnariu *et al.*, 2022; El Midaoui *et al.*, 2022).

Sayarer *et al.* (2015) analyzed *Crocus ancyrensis* and found that safranal, α-isophorone and β-isophorone are the main components (Sayarer, 2015). In another study, Küçük *et al.* (2019) revealed that chemical composition of *Crocus ancyrensis* from Eskişehir region, Türkiye is compatible with the findings in Sayarer's study and contained β-Isophorone (14.4%), heptanal (11.5%) and heneicosane (8.5%) (Küçük *et al.*, 2019). For this reason, they have reported that *Crocus ancyrensis* could be an alternative source to *Crocus sativus L.*, which contains safranal (77.9%), α-isophorone (13.5%), and β-isophorone (2.2%). Besides, it's found that alcohol and water extracts of *Crocus ancyrensis* showed an inhibitory effect on *Aeromonas hydrophila* bacterial fish pathogens (Turker *et al.*, 2009). On the other hand, Küçük *et al.* (2019) reported that local people used *Crocus ancyrensis* tea as a traditional medicine for abdominal pain and diuretic (Küçük *et al.*, 2019). However, there are limited number of studies on the *Crocus ancyrensis* extracts in the literature (Gunbatan *et al.*, 2016; Küçük *et al.*, 2019).

The aim of this study is to investigate the chemical composition of the essential oils obtained from the endemic *Crocus ancyrensis* plant known as "Ankara Çiğdemi" growing in Çorum region of Türkiye and to reveal its volatile components in detail for the first time.

2. MATERIAL and METHODS

2.1. Plant Materials

Crocus ancyrensis, which is shown in Figure 1, was collected from the region of Çorum location (40°40′26″ N, 34°48′15″ E) with the permission of the Republic of Türkiye, Ministry of Agriculture and Forestry. Collected herbs were identified by Dr. Bedrettin SELVİ from Tokat Gaziosmanpasa University, Faculty of Arts and Sciences, Department of Biology.

Figure 1. The picture of collected *Crocus ancryensis.*

2.2. Isolation of Essential Oils

Clevenger-type apparatus was used for the isolation of essential oils by hydro-distillation method. Air-dried aerial part (stigma, anthers, leaves, throat) of the plant material (30 g each) was added to beaker containing distilled water (200 mL). Distillation process was performed for 3 h and, enriched essential oils were dried over anhydrous sodium sulphate. The obtained essential oils were stored in the refrigerator at 4 °C for GC-MS analyses.

2.3. GC and GC-MS Analyses

GC MS analysis was carried out with Thermo Scientific- Trace GC Ultra system and TSQ Quantum XLS mass spectrometer. Additionally, TG-5MS apolar capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness) was used and 1.0 mL/minute high purity Helium (He) was selected as carrier gas. The injection temperature was adjusted to 250 °C. TG-5MS apolar capillary column was fixed from 50 to 120 °C at a rate of 3 °C/min, 120 to 220°C at a rate of 3 °C/min, held for 0.67 min, 220 to 250 at 5 °C/min, held for 5.0 min. TSQ Quantum XLS GC MS was set as the ionization energy at 70 eV, the ion source at 250 °C, and the transfer line temperature at 280 °C. Split/splitless (25:1 split) mode was used for the diluted samples (1/10 in acetone, v/v) of 1.0 µL. The results of Mass spectra of molecules were identified via library (WILEY and NIST) using the relative peak areas.

3. RESULTS

The analyses of the volatile components obtained through hydrodistillation method from the *Crocus* plant grown in Çorum region were accomplished by GC-MS and the results are given in Table 1. As a result of GC-MS analysis, 23 volatile components were determined in the plant extract (Figure 2). Among the substances obtained by GC-MS analysis, 2-Hexenal (22.27%), 1-ethylbutyl Hydroperoxide, (12.15%), 2-nitro-Hexane, (16.86%), β-Isophorone (7.76%), α-Isophorone (4.96%), 2-Caren-10-al (4.27%), and Eugenol (13.22%) were determined as the main components.

2-Hexenal, which was determined as the most dominant volatile component, is a simple long-chain unsaturated aldehyde naturally found in various vegetables and fruits. It is allowed to be used as a food additive by the US Food and Drug Administration (FDA), and has antimicrobial properties. It is colorless and sharp grass-smelling liquid and is mainly used in the perfume industry. However, its rapid evaporation limits its utilization in cosmetics (Lanciotti *et al.*, 2003; Joo *et al.*, 2012).

Eugenol, one of the components found in *Crocus ancryensis* extract, was detected as 13.27% by GC-MS analysis. Eugenol is a pale yellow volatile phenolic compound soluble in organic solvents and extracted from plants such as clove oil, nutmeg, cinnamon, basil and bay leaf (Marchese *et al.*, 2017; Nejad *et al.*, 2017). It has been reported to be effective in the treatment of skin infections, skin lesions, and inflammatory disorders (Kamatou *et al.*, 2012; Nejad *et al.*, 2017). In addition, there are studies showing that it can be used in the treatment of various diseases including leukemia, colon and melanoma cancers (Kim *et al.*, 2003; Ghosh *et al.*, 2005; Jaganathan *et al.*, 2011). Eugenol, the main component of clove oil, is widely used in dentistry due to its anesthetic and analgesic effects (Pramod *et al.*, 2010). Due to its antimicrobial and antioxidant properties, it is used in the pharmaceutical and cosmetic industry as well as its use as protective agent in food industry (Turker *et al.*, 2009; Woranuch & Yoksan, 2013). Although it has a wide use area, there are literature studies indicating that excessive use of eugenol has a toxic effect (Basch *et al.*, 2008; Kamatou *et al.*, 2012).

There are not enough studies in the literature on the biological activities of 2-nitro-Hexane, which was found to be 16.86% in essential oil obtained from *Crocus ancryensis*. Gafar *et al.* (2013) stated that this compound, which they detected in *Cucumis melo Linn* seed extracts,

could be an industrial-based material (Gafar *et al.*, 2013). In addition, K J Abdulla *et al.* (2019) found 2-nitro-hexane as a degradation product in GC-MS analyzes in their study on the biodegradation of crude oil using local bacterial isolates (Abdulla *et al.*, 2019).

There is no literature on the biological activity of 1-ethylbutyl hydroperoxide, which was detected as 12.15% among the volatile components of the plant by GC-MS analysis (Meenakshi *et al.*, 2012; Padmashree *et al.*, 2018). In the meantime, Padmashree M., *et al.*, (2018) found as 5.85% 1-ethylbutyl hydroperoxide in ethanol extracts of Ipomoea staphylina leaves (Padmashree *et al.*, 2018).

While Ozcelik *et al.* (2020) detected as 7.39% and 14.93% hydroperoxide1-ethylbutyl, respectively, in the methanol extracts obtained from onion and garlic wastes (Ozcelik *et al.*, 2020), M., Al-Owaisi *et al.* (2014) found as 4.39% hydroperoxide1-ethylbutyl in the crude extracts of Moringa peregrina (Forssk.) Fiori leaves (Al-Owaisi *et al.*, 2014).

α-Isophorone and β-Isophorone, unsaturated cyclic ketones, were found to be 4.96% and 7.76%, respectively, in the plant extract as a result of GC-MS analysis. Isophorone is a colorless liquid with mint-like odor (Kataoka *et al.*, 2007; Ershova *et al.*, 2018) and derivatives are considered among the most important aromatic compounds of *Crocus sativus* (Panighel *et al.*, 2014; Condurso *et al.*, 2017). It has been reported to contribute to the aroma of honey, thyme, citrus, rosemary, and lavender (Graikou *et al.*, 2022). 1,8-cineole and camphor, which are structurally related to isophorones, show biological activity, anticancer, and antioxidant properties against some microorganisms (Vuuren & Viljoen, 2007; Kiran *et al.*, 2013; He *et al.*, 2021). Kuran İ *et al.* (2013) obtained two Isophorone derivatives from alternaria fungi and stated that these were effective against some microorganisms (He *et al.*, 2021). In addition, Isophorone has found application in various areas in industry such as paints, varnishes, printing inks, oils, waxes, pesticides, and solvent of natural and synthetic resins (Kataoka *et al.*, 2007; Panighel *et al.*, 2014).

It was determined that 2-Caren-10-al was found in the *Crocus* extract at a ratio of 4.27%. Abushama *et al.* (2013) reported that *Cuminum cyminum* L., which has a high ratio of 2-Caren-10-al among its volatile components, can be used as a potential antimicrobial and antitumor agent. However, they stated that more fractionation, isolation and chemical analysis should be done to identify the chemical compounds responsible for its bioactivity (Abushama *et al.*, 2013). Ghasemi *et al.* (2019) reported that 2 Caren-10-al, which is found in high amounts in the structure of *Cuminum cyminum* has an important contribution to the its antifungal activity (Ghasemi *et al.*, 2019).

Figure 2. GC-MS Chromatogram of Essential oil obtained from *Crocus* plant.

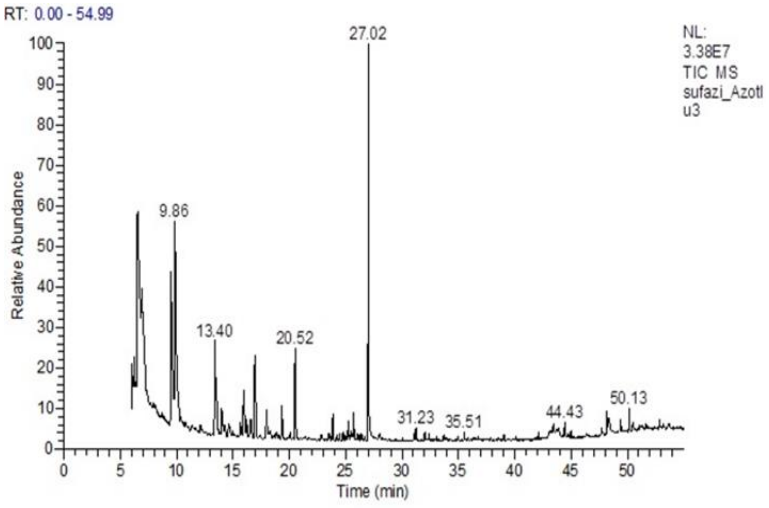

Table 1. Essential oil constituents of *Crocus ancyrensis*.

No	RT	Compound Name	Area %	RRI
1	6.56	2-Hexenal	22.27	845
2	9.53	1-ethylbutyl Hydroperoxide	12.15	921
3	9.89	2-nitro-Hexane	16.86	935
4	10.81	Nonanal	0.28	1081
5	13.41	β-Isophorone	7.76	1097
6	14.03	Tridecane	2.10	1117
7	14.64	1-ethyl-2-methyl-Benzene,	1.17	1124
8	15.67	3,3,5,5-Tetramethylcyclohexanol	0.78	1245
9	15.97	Tetradecane	3.18	1286
10	16.22	Undecane	0.88	1294
11	16.59	Isomenthol	1.11	1334
12	16.96	α-Isophorone	4.96	1534
13	17.98	2,6,6-Trimethyl-2-cyclohexene-1,4-dione (4-oxoisophorone)	1.96	1612
14	18.84	1,3,4-trimethyl-3-Cyclohexene-1-carboxaldehyde	0.62	1674
15	19.35	3-Caren-10-al	1.73	1697
16	20.08	α-Terpineol	0.51	1701
17	20.52	2-Caren-10-al	4.27	1724
18	24.76	2,6,10-trimethyl-Tetradecane	0.26	1784
19	25.72	Hexadecane	1.07	1811
20	27.02	Eugenol	13.22	1901
21	43.69	Heptacosane	0.60	2307
22	48.16	Tricosane	0.79	2181
23	48.33	9-Octadecenamide	1.47	2228

RRI: Relative retention indices. % Calculated from data (calculated according to *n*-alkanes).

4. DISCUSSION and CONCLUSION

In this study, essential oils of *Crocus ancyrensis* collected from Çorum region were extracted by hydrodistillation method and characterized by GC MS. As a result, 23 components were determined in the plant extract. Of these components, 2-Hexenal (22.27%), 1-ethylbutyl Hydroperoxide (12.15%), 2-nitro-Hexane, (16.86%), β-Isophorone (7.76%), α-Isophorone (4.96%), 2-Caren-10-al (4.27%) and Eugenol (13.27) can be considered as main components. It has been reported in the literature that some components of the essential oils showed different biological activities. By conducting more detailed research, the biological activities of the essential oils obtained from *Crocus ancyrensis* such as antimicrobial, antifungal, antioxidant and anticancer properties can be revealed and evaluated for industrial applications such as food, cosmetic, and pharmaceutical applications.

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Declaration of Conflicting Interests and Ethics

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors confirm that there were no ethical in preparing this manuscript. The datasets generated and/or

analyzed during the current study are available from the corresponding author on reasonable request. All authors consent to participating in this work. All authors consent to publishing this work. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Hacer Dogan: Analysis, Investigation, Resources, Writing -review & editing. **Omer Kayir:** Analysis, Investigation, Resources, Writing -review & editing. **Erol Alver:** Supervision, collecting, Methodology, Investigation, Resources, Writing -review & editing. **Ibrahim Bilici:** Supervision, collecting, Methodology, Investigation, Resources, Writing

Orcid

Hacer Dogan https://orcid.org/0000-0002-4091-9033
Omer Kayir https://orcid.org/0000-0001-5790-8739
Erol Alver https://orcid.org/0000-0002-6010-6910
Ibrahim Bilici https://orcid.org/0000-0001-8151-6911

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