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Mentha longifolia Essential Oil from AlUla Region, Saudi Arabia: Chemical Composition and Classification

Ebtihaj O. Alnasri [1] & Heba A. Sindi [2]

[1]*Department of Food and Nutrition, Faculty of College of Human Sciences and Design, King Abdulaziz University, Jeddah, Saudi Arabia.* ealnassri@kau.edu.sa

[2]*Department of Food and Nutrition, Faculty of College of Human Sciences and Design, King Abdulaziz University, Jeddah, Saudi Arabia.* hasindi@kau.edu.sa

Abstract. this study investigated the chemical composition and biological activities of *Mentha longifolia* (L.) essential oil from the AlUla region using gas chromatography-mass spectrometry (GC-MS) analysis and identified 73 compounds with three predominant chemotypes: piperitone epoxide (37.96%), 18-Cineole (16.37%), and menthone (10.01%). Total phenolic content analysis revealed 240.168 ± 2.1 mg gallic acid/mg/mL, while antioxidant activity showed a DPPH IC_{50} value of 30.66 µg/ml. antimicrobial assessment using agar well diffusion demonstrated a stronger efficacy against Gram-positive bacteria than against Gram-negative bacteria. Principal component analysis (PCA) and agglomerative hierarchical clustering (AHC) identified four distinct chemotypes among the samples. Thus, given its significant antimicrobial and antioxidant properties, these findings established the potential of *M. longifolia* essential oil from the AlUla region as a natural preservative in food packaging applications.

Keywords**:** *Mentha longifolia* (L.); (2,2-diphenyl-2-picrylhydrazyl free radical (DPPH); Antibacterial activity; Piperitone epoxide principal, component analysis (PCA), agglomerative hierarchical clustering (AHC)

Introduction

The AlUla region in Al-Madinah, located on the Arabian Peninsula, has great historical and agricultural significance. It has received considerable attention in Saudi Arabia's Vision 2030 according to Saad (Saad, 2023). The region is known for being a significant source of various crops and medicinal plants (Pavan, 2024), with medicinal plants constituting about 12% of the overall floral species in the Arab Peninsula. There are about 300 species of medicinal plants belonging to 72 families in the region (Farouk et al., 2021). One such plant, *Mentha longifolia* (L.) Hudson *M. longifolia*, a member of the *Lamiaceae* family, is extensively grown in the Madinah region, as demonstrated by Ghaleb Dailah & Singh (Ghaleb Dailah, 2022; Singh et al., 2020), also known as wild mint, is traditionally used on its own or with other herbs to create refreshing drinks, often in the form of tea. It is also added to different food dishes to enhance their aroma and flavor (Abdelhalim & Hanrahan, 2021). Furthermore, (Moshrefi Araghi et al.,2019) have been confirmed

that mint is utilized in traditional medicine to treat influenza, cough, inflammatory diseases, and digestive disorders.

The aroma and flavor of these plants are attributed to their essential oils (EO), which are colorless liquids primarily composed of aromatic and volatile compounds (Bhavaniramya et al., 2019). Several previous studies have shown that essential oils have diverse applications in medicine, perfumery, cosmetics, and food preservation (Ranjbar et al., 2023) and is considered a promising natural substitute for synthetic additives (Prakash et al., 2024) such as acetic acid, malic acid, lactic acid, benzoic acid, and sorbic acid, which are commonly used as food preservers (Fan et al., 2023; Kaur et al., 2023; Tirloni et al., 2022).

Essential oils preserve food owing to their potent antioxidant and antibacterial properties. These characteristics help manage food spoilage, prevent contamination by foodborne pathogens, and extend food shelf-life (14, 15). Some common essential oils used in food packaging include rosemary (Yang et al., 2023), peppermint (Talebi et al., 2018), cinnamon (Zhou et al., 2022), oregano (Zhang et al., 2024), thyme (Peixoto et al., 2023), and cumin (Sharafati Chaleshtori et al., 2016). Numerous studies supported that natural products such as essential oils are valuable for fresh meat, butter, and fish preservation (Papadochristopoulos et al., 2021; Talebi et al., 2018). Although synthetic antibacterial agents are increasingly being used, they pose serious health risks when consumed in excessive amounts. Therefore, essential oils could be an excellent alternative to conventional food additives as they help protect consumers' health, preserve food's nutritional value, and prevent contamination (Bhavaniramya et al., 2019; Paudel et al., 2022).

To date, no studies have analyzed the biological activity of *M. longifolia* cultivated in the AlUla region as a natural preservative for food packaging. Nonetheless, the effectiveness of the biological activity of oil is contingent on its chemical composition, which can vary across geographic regions. *M. longifolia* demonstrates diverse characteristics and levels of essential oils, even within a particular ecological zone and genotype. Studying the similarities and dissimilarities of oil compounds in different locations is imperative. Statistical techniques such as PCA and agglomerative hierarchical clustering (AHC) are also essential according to, (Hassanpouraghdam et al., 2022) PCA helps to identify and visualize similarities and graphical arrangements of various chemical variables, and this technology also helps interpret data effectively.

Therefore, the present study aimed to analyze the chemical composition of *M. longifolia* essential oil grown in AlUla region to explain the volatile constituents using GC/MS. Essential oils (Eos) extract by hydro-distillation method and determine the antioxidant activity using the DPPH method, total phenolic content, inhibitory activity against some microorganisms using agar well diffusion, and compare the result *M. longifolia* essential oil chemical composition from AlUla region investigated in this work with the results reported in the literature using PCA and AHC.

Methods

2.1. Plant material

During the spring of 2023, fresh green plants of *Mentha longifolia* L. were collected from the AlUla region (26° 36' 31" N 37° 55' 23" E) in the Al Madinah Region of Saudi Arabia. The fresh leaves were dried under shade at room temperature for five days to prepare the plant material for further processing. After drying, the leaves were ground into a fine powder using a grinding machine (Fordeal 2000 w grinder, China) and sieved.

Figure 1: Google Maps image representing the location of the Alula region in Saudi Arabia

2.2 Chemicals and Microorganisms

Anhydrous sodium sulfate (Na_2SO_3), (2,2-diphenyl-1-picrylhydrazyl free radical (DPPH), Folin–Ciocalteu phenol reagent (FCR), methanol, gallic acid, sodium carbonate (Na_2Co_3), ascorbic acid, and nutrient agar were purchased from (Sigma Aldrich, Germany). *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* were provided by the Faculty of Pharmacy at King Abdulaziz University (Jeddah, Saudi Arabia).

2.3 *Mentha longifolia (L.) Essential Oil Extraction*

The method involved dissolving 30 g of ground *M. longifolia* leaves in 300 mL of distilled water in a 500 mL flask blend and then subjected to a Clevenger apparatus for 3 h, following the procedure outlined in a study (Amri et al., 2022). With some modifications, the oil was purified using anhydrous sodium sulfate and stored at four °C until further analysis.

2.4 *Gas Chromatography-Mass Spectrometry (GC/ MS)*

Following a specific protocol, (Benomari et al., 2023) essential oils were analyzed using gas chromatography with a flame ionization detector (GC-FID) on a Thermo Scientific TRACE 1300 Series system, paired with a TSQ 8000 Evo Triple Quadrupole GC-MS/MS. The analysis utilized an HP-5 MS capillary column (0.25 mm x 30 m, 0.25 µm film thickness) and a flame ionization detector. The injector was set to 250°C, and the detector to 280°C. The oven temperature program began at 50°C for 1 minute, increased at 5°C/min to 250°C, and held for 4 minutes. Essential oil

samples diluted 1:100 in hexane were injected in splitless mode at 1 µL. Nitrogen was used as the carrier gas at a 1.2 mL/min flow rate. Component identification was achieved by comparing the retention times and mass fragmentation patterns with those of the NIST library, with a detailed recording of the results.

2.5 *Determination of Total phenols of Mentha longifolia (L.) Essential Oil*

Total polyphenol content was assessed using the Folin–Ciocalteu method, with slight modifications to the standard procedure (Michiu et al., 2022). A stock solution of gallic acid was prepared by dissolving 10 mg in ethanol (10 ml) and completing up to 100 ml with water to produce a standard curve concentration ranging from 50 to 500 mg/L. The reaction solution was shielded from light and was incubated at room temperature for one hour. 50 µl of the essential oil extract was diluted 50 times using a solvent mixture of 50% ethanol and 50% water. Then, 50 µl of this diluted extract was combined with 200 µl of Folin–Ciocalteu's reagent and left to react for 1 min. Subsequently, 2.5 ml of 7% w/v sodium carbonate solution was added to the reaction mixture. The solution was protected from light with foil paper and incubated at room temperature for one hour. Subsequently, the absorbance was measured at a wavelength of 765 nm using an ethanol blank as a reference using a Thermo Scientific GENESYS 10S UV-Vis Spectrophotometer. The measured optical density (OD) was compared with a standard curve. The results are expressed as milligrams of gallic acid equivalents (GAE) per gram of dried weight. The samples were analyzed in triplicate.

2.6 Determination Mentha longifolia (L.) Essential Oil Antioxidant Activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl radical) assay was used to measure the antioxidant capacity of the essential oil *(M. longifolia)* according to a previously described method. (Nayan R. Bhalodia et al., 2010) . DPPH (1.9 mg) was dissolved in 50 ml of methanol/water (40/10; v/v). Samples were prepared at 10, 20, 30,40, and 50 ug/ml concentrations. DPPH (1ml, 0.1 mM) was added to (50 ul) *M. longifolia* essential oil. A synthetic antioxidant (1 mg) of Ascorbic acid dissolved in water (100 ml) was then prepared at concentrations ranging from 1.25 –10 ug/ml. From each concentration, (50ul) was mixed with (2ml) DPPH and kept in the dark at room temperature for 30 min. The absorbance was read at a wavelength of 517 nm using a GENESYS 10S UV-Vis Spectrophotometer (Thermo Scientific). The radical-scavenging activity (%) was determined using the following formula:

$$radical - scavenging\ (\%) = \frac{(absorbance\ of\ the\ control - absorbance\ of\ the\ sample)x100}{absorbance\ of\ the\ control}$$

Where A control = Absorbance of DPPH.

The extracts' radical scavenging activity was quantified using the IC_{50} value (µg/mL). IC_{50} (Inhibitory Concentration 50) was determined by plotting concentration against % inhibition and calculating the concentration required to scavenge 50% free radicals.

2.7 *Determination Mentha longifolia (L.) Essential Oil Antibacterial Activity*

The antibacterial activity of *M. longifolia* essential oil against three foodborne pathogenic bacteria (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis*, and *Escherichia coli* ATCC25922) was evaluated using the agar-well diffusion method, as described previously (Asdagh & Pirsa, 2020). *M. longifolia* essential oil (20 µl) was poured into an agar well 5 mm in diameter after a nutrient agar medium agar was swabbed with 100 µL of an inoculum containing 10^5 CFU/ml of the tested bacteria. Agar plates were then incubated at 37 °C for 24 h, allowing the bacteria to grow under optimal conditions. After incubation, the zone of inhibition of the clear area around the well, where bacterial growth was inhibited, was measured with a plastic ruler. An antibiotic e.g. Ciprofloxacin 5ug/ml was used as a positive control.

2.8 *Comparative analysis of the chemical composition of Mentha longifolia (L.) Essential Oil Kingdom Saudi Arabia (KSA) oil of the same variety from various origins*

The chemical compositions of the essential oil of *M. longifolia* leaves from KSA and the seven origins are listed in Table 1. GC/MS analysis of *M. longifolia* crucial oils from the eight origins identified a total of (12) compounds (Table 1), calculated from the experimental peak area in the literature. These compounds are divided into (3 families: hydrocarbon monoterpenes, oxygenated monoterpenes, and sesquiterpenes.

Table 1. Chemical comparison *of Mentha longifolia* EOs from the literature of different world locations

Compounds Families	Compounds	Kingdom of Saudi Arabia (KSA)	Senegal	Iran	Turkey	India	Bosnia and Herzegovina	Pakistan	Algeria
Hydrocarbon monoterpenes	α-Pinene	1.92	0.6	8.92	0	0.2	0.78	0.76	0.39
Oxygenated monoterpenes	Sabinene	0	0.9	13.94	0	0.11	0.47	0	0
	1,8-Cineole	16.37	11.4	37.16	0	0	12.03	2	0
	Menthone	10.01	21.2	0	0	0	0	0	2.76
	α-terpineol	4.88	1.4	0	1.52	0.33	0.91	1.17	0.38
	Pulegone	0.35	42.4	6.14	1.49	0	0	0	31.85
	Piperitone	0.01	0.5	0	28.32	0	0	0	0.65
	Piperitone epoxide	37.96	0	0	0	53.83	0	0	0
	Piperitenone	1.06	1.1	0	1.98	0	1.98	24.9	3.72
	Piperitenone oxide	0.15	0	0	11.81	0	4.81	28.3	0
Hydrocarbon sesquiterpenes	Germacrene D	0.06	0	0	0	1.46	0.16	8.16	0
	Caryophyllene oxide	0	0.1	0	4.19	0.48	0	3.92	0
-	References	present study	(Diop et al., 2016)	(Ranjbar et al., 2023)	(SEZEN et al., 2023)	(Singh et al., 2020)	(Nikšić et al., 2012)	(Iqbal et al., 2013)	(Benabdallah et al., 2016)

2.8.1 *Principal Component Analysis for the Main Compounds of Mentha longifolia Essential Oils from the Eight Origins Studied*

Analysis of the links between the chemical composition of the essential oil and the taxonomy of the eight origins was carried out by Principal Component Analysis (PCA). Only the discriminating variables were considered. This analysis was based on 12 major chemical components of essential oil. These compounds were α-pinene, sabinene, 1,8-sineole, menthone, α-terpineol, pulegone, piperitone, piperitone epoxide, piperitenone, piperitenone oxide, germacrene D, caryophyllene oxide. The results of the PCA were used to study and visualize correlations between variables based on chemical similarities and differences.

2.8.2 *Agglomerative Hierarchical Clustering (AHC)*

Agglomerative hierarchical clustering (AHC) also revealed the presence of four clusters, which were separated according to dissimilarity indices (Figure 4). Based on the Euclidean distance between groups, (AHC) indicated four clusters (from C1 to C4, Figure 4, the arrangement abovementioned), identified by their essential oil chemotypes with dissimilarity ≤11.

2.9 *Statistical analysis*

The chemical composition of the essential oils was determined by principal component analysis (PCA) and agglomerative hierarchical clustering (AHC). Combining the two approaches makes it possible to determine the similarities and differences among essential oils collected from different locations and any potential chemical compositional variations. It was carried out using XLSTAT version 2015 software, according to (Hung et al., 2020).

Results and Discussion

3.1. Chemical Composition Analysis of Mentha longifolia (L.) Essential Oil using GC-MS

Table 2 listed the constituents of essential oils extracted from *M. longifolia* (L.). Gas Chromatography-Mass Spectrometry (GC-MS) analysis showed 73 distinct compounds within the essential oil.

Table 2. Chemical Composition Analysis of *Mentha longifolia (L.)* Essential Oil using GC-MS

No[a]	Compounds	Chemical formula	RT [b]	LRI [c]	LRI lit [d]	Id. Met. [d]	Relative peak area %
1	Tricyclene	$C_{10}H_{16}$	8.16	913	913	MS, LRI	0.03
2	α- Thujene	$C_{10}H_{16}$	8.26	924	924	MS, LRI	0.05
3	α-Pinene	$C_{10}H_{16}$	8.47	932	932	MS, LRI, Co	1.92
4	α-Fenchene	$C_{10}H_{16}$	8.68	950	950	MS, LRI	0.01
5	Camphene	$C_{10}H_{16}$	8.92	952	952	MS, LRI	1.19
6	Sabinene	$C_{10}H_{16}$	9.22	970	970	MS, LRI	Tr
7	β-Pinene	$C_{10}H_{16}$	9.57	976	976	MS, LRI, Co	1.77
8	d-Oten-3-ol	C8H16O	9.72	978	978	MS, LRI	2.73
9	β-Myrcene	$C_{10}H_{16}$	9.87	989	989	MS, LRI	Tr
10	3-Octanol	$C_8H_{18}O$	10.03	996	996	MS, LRI	1.28

No[a]	Compounds	Chemical formula	RT [b]	LRI [c]	LRI lit [d]	Id. Met. [d]	Relative peak area %
11	Mentha-1(7),8-diene	$C_{10}H_{16}$	10.43	1001	1001	MS, LRI	0.05
12	p-Cymene	$C_{10}H_{14}$	11.12	1024	1024	MS, LRI	0.06
13	1,8-Cineole	$C_{10}H_{18}O$	11.4	1030	1030	MS, LRI, Co	16.37
14	(Z)-b-Ocimene	$C_{10}H_{16}$	11.45	1037	1037	MS, LRI	Tr
15	(E)-b-Ocimene	$C_{10}H_{16}$	11.69	1047	1047	MS, LRI	0.02
16	g-Terpinene	$C_{10}H_{16}$	12.05	1057	1057	MS, LRI, Co	0.07
17	(Z)-Sabinene hydrate	$C_{10}H_{18}O$	12.43	1067	1067	MS, LRI	0.17
18	(Z)-p-Mentha-2,en-1-ol	$C_{10}H_{18}O$	13.36	1119	1119	MS, LRI	0.31
19	3-Octyl acetate	$C_{10}H_{20}O_2$	14.13	1124	1124	MS, LRI	0.14
20	(E)-p-Mentha-2,en-1-ol	$C_{10}H_{16}O$	14.64	1138	1138	MS, LRI	0.50
21	Camphor	$C_{10}H_{16}O$	14.81	1146	1146	MS, LRI, Co	0.12
22	Menthone	$C_{10}H_{18}O$	15	1158	1158	MS, LRI	10.01
23	Terpinen-4-ol	$C_{10}H_{18}O$	15.25	1167	1167	MS, LRI, Co	0.76
24	Menthol	$C_{10}H_{20}O$	15.6	1187	1187	MS, LRI	8.14
25	Neoisomenthol	$C_{10}H_{20}O$	15.8	1188	1188	MS, LRI	0.41
26	α-terpineol	$C_{10}H_{18}O$	16	1190	1190	MS, LRI, Co	4.88
27	(Z)- Carveol	$C_{10}H_{16}O$	16.61	1227	1227	MS, LRI	0.14
28	*Pulegone	$C_{10}H_{16}O$	16.88	1238	1238	MS, LRI	0.35
29	Piperitone	$C_{10}H_{16}O$	17.12	1254	1254	MS, LRI	0.01
30	Piperitone epoxide	$C_{10}H_{16}O_2$	17.61	1255	1255	MS, LRI	37.96
31	(Z)- Carvone oxide	$C_{10}H_{16}O$	17.93	1266	1266	MS, LRI	0.45
32	(E)-Carvone oxide	$C_{10}H_{14}O_2$	18.25	1273	1273	MS, LRI	0.12
33	Neomenthyl acetate	$C_{12}H_{22}O_2$	18.4	1275	1275	MS, LRI	Tr
34	Bornyl acetate	$C_{12}H_{20}O_2$	18.75	1288	1288	MS, LRI	0.27
35	Dihydroedulan I	$C_{13}H_{22}O$	18.82	1295	1295	MS, LRI	0.20
36	Menthyl acetate	$C_{12}H_{22}O_2$	19.32	1296	1296	MS, LRI	0.06
37	Iso mentyl acetate	$C_{12}H_{22}O_2$	19.87	1307	1307	MS, LRI	0.04
38	Dihydrocarveol acetate	$C_{12}H_{20}O_2$	19.7	1328	1328	MS, LRI	0.04
39	(E)-Carvyl acetate	$C_{12}H_{18}O_2$	20.2	1338	1338	MS, LRI	3.53
40	Piperitenone	$C_{10}H_{14}O$	21.56	1339	1339	MS, LRI	1.06
41	(Z)-Carvyl acetate	$C_{12}H_{18}O_2$	22.33	1363	1363	MS, LRI	1.41
42	Piperitenone oxide	$C_{10}H_{14}O_2$	22.7	1365	1365	MS, LRI	0.15
43	a-Bourbenene	$C_{15}H_{24}$	22.71	1376	1376	MS, LRI	0.14
44	b-Bourbenone	$C_{15}H_{24}$	22.95	1383	1383	MS, LRI	0.08
45	b-Elemene	$C_{15}H_{24}$	23.24	1390	1390	MS, LRI	0.11
46	a-Gurgujene	$C_{15}H_{24}$	23.41	1413	1413	MS, LRI	0.07
47	(E)-Caryophyllene	$C_{15}H_{24}$	23.88	1417	1417	MS, LRI, Co	0.14
48	b-Copaene	$C_{15}H_{24}$	24.67	1426	1426	MS, LRI	0.31
49	b-Gurgujene	$C_{15}H_{24}$	24.84	1430	1430	MS, LRI	0.05
50	(Z) -Muurola-3,5-diene	$C_{15}H_{24}$	25.24	1444	1444	MS, LRI	0.03
51	a-Humulene	$C_{15}H_{24}$	25.76	1450	1450	MS, LRI	0.02
52	E-b-Farnesene	$C_{15}H_{24}$	26.35	1457	1457	MS, LRI	0.45
53	Alloaromandendrene	$C_{15}H_{24}$	26.86	1458	1458	MS, LRI	Tr
54	(Z)-Cadina-1 (6), 4-diene	$C_{15}H_{24}$	27	1460	1460	MS, LRI	Tr
55	(Z)-Muurola-4 (14),5-diene	$C_{15}H_{24}$	27.13	1463	1463	MS, LRI	0.14
56	(E)-Cadina-1 (6), 4-diene	$C_{15}H_{24}$	27.7	1475	1475	MS, LRI	1.33
57	Germacrene D	$C_{15}H_{24}$	28.04	1479	1479	MS, LRI	0.06
58	Bicyclogermacrene	$C_{15}H_{24}$	28.71	1494	1494	MS, LRI	0.03
59	a-Muurolene	$C_{15}H_{24}$	29.31	1498	1498	MS, LRI	Tr
60	b-Bisabolene	$C_{15}H_{24}$	33.39	1501	1501	MS, LRI	0.04
61	GermacreneA	$C_{15}H_{24}$	34.24	1504	1504	MS, LRI	Tr
62	g-Cadinene	$C_{15}H_{24}$	35.69	1511	1511	MS, LRI	Tr

No[a]	Compounds	Chemical formula	RT [b]	LRI [c]	LRI lit [d]	Id. Met. [d]	Relative peak area %
63	(E)-Calamenene	$C_{15}H_{22}$	37.21	1522	1522	MS, LRI	0.11
64	d-Cadinene	$C_{15}H_{24}$	39	1522	1522	MS, LRI	Tr
65	a-Cadinene	$C_{15}H_{24}$	39.61	1535	1535	MS, LRI	Tr
66	Spathulenol	$C_{15}H_{24}O$	40.15	1574	1574	MS, LRI	Tr
67	Germacrene-D-4-ol	$C_{15}H_{26}O$	43.84	1575	1575	MS, LRI	Tr
68	Caryophyllene oxide	$C_{15}H_{24}O$	43.98	1578	1578	MS, LRI	Tr
69	Glubulol	$C_{15}H_{26}O$	46.87	1589	1589	MS, LRI	Tr
70	Viridiflorol	$C_{15}H_{26}O$	47.93	1593	1593	MS, LRI	Tr
71	epi-a-Cadinol	$C_{15}H_{26}O$	49.68	1641	1641	MS, LRI	Tr
72	a-Cadinol	$C_{15}H_{26}O$	50.45	1650	1650	MS, LRI	Tr
73	(Z,Z,)-Farnesol	$C_{15}H_{26}O$	53.8	1693	1693	MS, LRI	Tr
	Total identified %		98.93				
	Monoterpene hydrocarbons		5.18				
	Oxygenated monoterpenes		80.12				
	Sesquiterpene hydrocarbons		3.26				
	Oxygenated Sesquiterpenes		5.56				
	Others		4.81				

[a] In order of elution on the ZB5 column, [b] Compounds revealed based on RI and MS[b] Retention time according to GC/Ms analysis [c] Linear retention indices from Adam J.P. (2007), [d] Identification method: MS: Comparison with mass spectra from NIST 2.0; LRI: linear retention indices based on C8–C25 alkanes; Co-injection of pure standards, [e] Traces (% < 0.05 in FID and identified by GC/MS).

Table 2 provided a comprehensive list of the compounds found in the sample, their chemical properties, and identification methods. The compounds belong to various classes, including monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes. The most abundant class of compounds is the oxygenated monoterpenes, which comprise 80.12% of the identified compounds. This class includes compounds such as 1,8-cineole (16.37%), menthone (10.01%), and menthol (8.14%). The second most abundant class is the monoterpene hydrocarbons, which account for 5.18% of the identified compounds. This class includes compounds such as α-Pinene (1.92%), β-pinene (1.77%), and camphene (1.19%). The sesquiterpene hydrocarbons and oxygenated sesquiterpenes comprise 3.26% and 5.56% of the identified compounds. The most abundant compounds in these classes include (E)-caryophyllene (0.14%) and caryophyllene oxide (Tr). The "Others" category, which accounts for 4.81% of the total identified compounds, includes compounds that do not fit into the previous categories, such as 3-Octanol (1.28%) and 3-Octyl acetate (0.14%).

The essential oil exhibited a predominance of monoterpenes, with oxygenated monoterpenes constituting the majority (80.12%), followed by Oxygenated Sesquiterpenes (5.56%) and Monoterpene Hydrocarbons (5.18%). This composition closely resembled that of the essential oil extracted from *M. longifolia* in the Albaha Area of Southern Saudi Arabia, which also contained a high concentration of monoterpenes (54.30%), primarily oxygenated monoterpenes (30.40%), and notable levels of sesquiterpene hydrocarbons (26.08%) (Burham et al., 2019, 2019). Similarly, the essential oil analyzed in Pakistan demonstrated a composition dominated by oxygenated monoterpenes (67.24%), followed by sesquiterpene hydrocarbons (17.19%), monoterpene

hydrocarbons (7.31%), and oxygenated sesquiterpenes (5.05%) (Iqbal et al., 2013). These differences in the composition of EOs between different geographic locations may be attributed mainly to soil type and environmental conditions.

The major compounds found in our study were piperitone epoxide (37.96%), 1,8-cineole (16.37%), and menthone (10.01%). The results of two studies (Farouk et al., 2021) conducted in Al Madinah Al Munawaroh showed some minor variations from our findings where pulegone (40.7%), (38.42%), and 1,8-cineole (33.4%), (15.60%), menthone (2.4%), and (13.20%). In similar results, *M. longifolia* collected from a different location in Saudi Arabia showed that the major constituents were pulegone (11.92- 62.54%) and menthone (7.84-34.13%) (Ibrahim et al., 2017) .

According to Table 2, our results are consistent with those of the same species grown in Tajikistan, which has significant concentrations of piperitone epoxide (7.8% - 77.6%), piperitenone oxide (1.5% - 49.1%), pulegone (0.3-5.4%) (Sharopov & Sulaymonova, 2012). Scientific evidence has shown that several environmental factors, including salt, food, temperature, and cultivar, significantly influence the metabolism and synthesis of essential oils. These components are crucial in influencing the type and composition of essential oils produced by various plants (Aqeel et al., 2023). Therefore, the results of the literature studies appear to be slightly different from those of the current study.

Our results show the top five compounds of *M. longifolia (L.)* essential oil. These compounds are commonly used in various applications, including aromatherapy, pharmaceuticals, cosmetics, and the food industry.

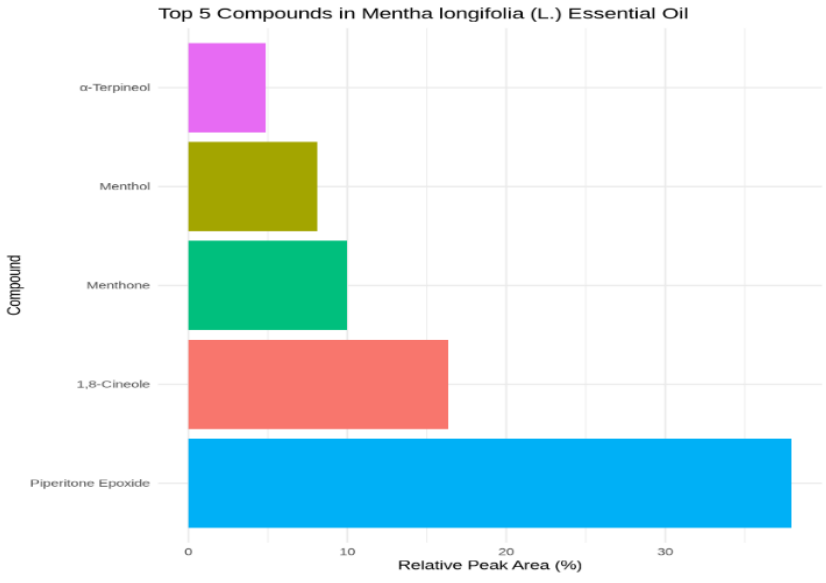

Figure 2. Top 5 compounds of *Mentha longifolia* essential oil

This plot highlights the dominance of piperitone epoxide, followed by 1,8-cineole, menthone, menthol, and α-Terpineol in the essential oil. piperitone epoxide is the most abundant compound,

producing 37.96% of the essential oil. It is primarily known for its antimicrobial and anti-inflammatory properties, according to Ali *et al.* (Ali, Elgat, EL-Hefny, et al., 2021). Also, 1,8-cineole (16.37%) is notable for its anti-inflammatory analgesic and antimicrobial properties (Hoch et al., 2023). Moreover, Hilfiger *et al*.(Hilfiger et al., 2021) explained menthone (10.01%) and menthol (8.14%) both have analgesic and anti-inflammatory properties. α-Terpineol (4.88%) is recognized for its antimicrobial, antioxidant, and anti-inflammatory properties.

3.2. *Antioxidant Activity DPPH and Total Phenol Contents for Mentha longifolia (L.) Essential Oil*

The antioxidant capacity of essential oils (EOs) is primarily based on their chemical composition. EOs with phenolic compounds exhibit significant antioxidant properties. Some monoterpene components, such as linalool, 1,8-cineole, and menthone, play a vital role in the antioxidant properties of EOs, as shown in Table (3).

The antioxidant capacity of the oil *M. longifolia* from AlUla was evaluated through DPPH radical scavenging activity in (Table 3). The results show that *M. longifolia (L.)* has high antioxidant activity (DPPH IC_{50} is 30.66 µg/mL) and a relatively high total phenolic content (240.168 mg GAE/g DW). This aligns with the general understanding that higher phenolic content often correlates with higher antioxidant activity. Ascorbic acid has a slightly lower DPPH IC_{50} value (4.77 ug/mL), indicating higher antioxidant activity than *M. longifolia* (L.). This outcome was lower than the earlier report [12], which demonstrated that the tested sample exhibited a high level of antioxidant activity. A study [37] discovered that pulegone, an oxygenated monoterpene with a ketone group, negatively affects antioxidant activity. Another investigation corroborated this finding [3] conducted in Al Madinah Al-Munawara, Saudi Arabia, which analyzed the volatile oil of *M. longifolia* and reported significantly lower free radical scavenging activity. The high concentration of pulegone (38.42%) in their sample was identified as the cause of this reduced activity. In contrast, our study found a low pulegone concentration (0.35%), as shown in Table 2, and our results indicated that the tested sample possesses high antioxidant activity. The significant difference in pulegone concentration could be attributed to variations in environmental factors (e.g., soil, climate), plant chemotypes, extraction methods, or storage conditions, all of which can influence the chemical composition of essential oils

Table 3. Antioxidant Activity DPPH assay and Total Phenol Contents for *Mentha longifolia* (L.) Essential Oil Comparison to the Synthetic Antioxidant ascorbic acid

Compounds	(DPPH) [a] IC_{50} (ug/mL)	Total Phenolic Content mg GAE/g DW [a]
Mentha longifolia (L.)	30.66 ± 0.09	240.168 ± 2.1
Ascorbic acid	4.77 ± 0.69	-

Tafrihi *et al.* (Tafrihi et al., 2021) Point out that Mentha species contain high amount of polyphenols. The result in Table 3 showed a total phenolic content of (240.168 ± 2.1mg) mg/g DW, which is higher than the results of the extract's total phenolic content in the study of Ahmed *et al.* (Ahmed et al., 2015). Yaghini *et al*. (Yaghini et al., 2021) demonstrated that variations in the phenolic content of medicinal plants can be attributed to various factors, including the genetic structure and phenological stage of the harvested plants and the extraction method used.

3.3 *Antibacterial Inhibition of Mentha longifolia (L.) Essential Oil*

Natural products have become an important source for developing antibacterial agents in the food and pharmaceutical industries due to their low toxicity, minimal side effects, and ability to prevent the development of bacterial resistance (Liu et al., 2022). Several studies (Ali, Elgat, El-hefny, et al., 2021; Tafrihi et al., 2021) have shown that *Mentha* essential oil has antibacterial properties against pathogen bacteria, including Gram-negative and Gram-positive strains. Some notable examples are *Pseudomonas aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Streptococcus aureus*.

Table 4. Antibacterial Inhibition of *Mentha longifolia* (L.) Essential Oils Against Tested Bacterial Strains Comparison to the antibiotic (Ciprofloxacin).

Bacterial species	Essential oil (Diameter in mm)	The control (Ciprofloxacin) (Diameter in mm)
Staphylococcus aureus	24.7±1.5	26.5± 0.7
Bacillus subtilis	22.5± 2.5	24.5± 0.4
Escherichia coli	12.5 ±0.57	29.5± 0.7

The results are presented as mean (n = 3) ± standard deviation.

Table 4 shows the antibacterial inhibition of *Mentha longifolia (L.)* essential oil against bacterial Strains compared to the control (Ciprofloxacin). The essential oil is most effective against *Staphylococcus aureus*, followed by *Bacillus subtilis*, and least effective against *Escherichia coli*. Meanwhile, Ciprofloxacin control is more effective than the essential oil against all three bacterial strains, with higher mean inhibition zones and relatively lower standard deviations.

According to the studies (Hooper & Jacoby, 2016; Keyhani et al., 2024) the difference in antibacterial effectiveness between Mentha longifolia essential oil and Ciprofloxacin can be attributed to their distinct modes of action. Ciprofloxacin, a synthetic antibiotic, specifically targets bacterial DNA gyrase and topoisomerase IV, enzymes essential for DNA replication, transcription, and repair. Ciprofloxacin disrupts bacterial DNA processes by inhibiting these enzymes, leading to cell death. It is a highly purified compound with standardized potency, resulting in consistent and potent activity. In contrast, *Mentha longifolia* essential oil is a natural product composed of a complex mixture of compounds, which act through multiple mechanisms, such as disrupting cell membrane integrity, interfering with membrane proteins, and causing leakage of cellular contents. Its composition can vary depending on environmental factors, leading to variability in its

antibacterial activity. This explains why Ciprofloxacin is more effective and consistent across all bacterial strains, while the essential oil shows variable effectiveness.

The mean inhibition zone of 24.7 mm indicates that the essential oil is quite effective against *Staphylococcus aureus*. The standard deviation of 1.5 mm suggests that the results are relatively consistent. In comparison, the antibiotic exhibits a slightly higher average inhibition zone of 26.5 mm, indicating it is somewhat more effective than the essential oil. The standard deviation of 0.7 mm suggests highly consistent results for the control. The p-value is 0.1124, which is insignificant. This indicates that the difference between the essential oil and antibiotic control treatments is not statistically significant for *Staphylococcus aureus*.

The essential oil showed effectiveness against *Bacillus subtilis*, with a mean inhibition zone of 22.5 mm. However, the standard deviation of 2.5 mm indicates some variability in the results. The antibiotic exhibits a slightly higher mean inhibition zone of 24.5 mm, with a standard deviation of 4 mm, suggesting more variability in the results than the essential oil. The p-value is 0.5880. This indicates that the difference between the essential oil and antibiotic treatments is not statistically significant for *Bacillus subtilis*.

The essential oil shows limited effectiveness against *Escherichia coli*, with a mean inhibition zone of 12.5 mm. The standard deviation of 0.57 mm indicates consistent results. In contrast, the antibiotic demonstrates high effectiveness against *Escherichia coli*, with a mean inhibition zone of 29.5 mm. The standard deviation of 0.7 mm suggests high consistency. This indicates that the difference between the essential oil and control treatments is statistically significant ($p < 0.05$) for *Escherichia coli.* Similarly, Abdel-Hameed *et al***.** (Abdel-Hameed et al., 2018) agree with our findings that *M. longifolia* essential oil exhibits a slightly higher inhibition zone in G-positive than G-negative bacteria. In contrast, disagreement with our results was found in a report by Burham et al. (Burham et al., 2019) from southern Saudi Arabia showed strong antibacterial activity of *Mentha* essential oil *against Escherichia coli*, with an inhibition zone of 24 mm.

3.4 *The comparative analysis of the chemical composition of Mentha longifolia essential oil from the Kingdom of Saudi Arabia (KSA) to the seven origins*

A comparative study of the chemical composition of the essential oils of *Mentha longifolia* from the Kingdom of Saudi Arabia (KSA) to the seven origins revealed similarities and differences. The results in Table 4 show that the (KSA), Senegal, Iran, Turkey, India, Bosnia and Herzegovina, Pakistan, and Algeria contain 10, 9, 4, 6,6,7,7, and 4 compounds, respectively.

The essential oil of *Mentha longifolia* (KSA) contains a high concentration of compounds 1,8-cineole piperitone epoxide and menthone. Table 1 indicates that menthone and piperenone oxide are the most abundant compounds in Senegal. In Iran, α-pinene, sabinene, and 1,8-cineole exhibited notable presence. In Turkey, the primary compounds identified in sample analysis were piperone and piperenone oxide. Research conducted in India has identified piperone epoxide as the primary

compound. Based on the data shown in Table 1, it is evident that Bosnia and Herzegovina are characterized by two primary compounds, specifically 1,8-cineole and piperitenone oxide. Additionally, the table presents the significant compounds found in Pakistan, namely piperenone oxide, piperenone, and germacrene D. Algeria exhibits the presence of the compounds pulegone and piperitenone.

Consequently, these findings demonstrate a range of chemical compositions in the essential oils derived from *Mentha longifolia* leaves throughout the eight tested locations. Various plant species in studies (Mohammedi et al., 2020; Porrello et al., 2024) have been documented to exhibit variability in the chemical composition of the essential oils of rosmarinus, *Crithmum maritimum* and *Ruta Montana* L.

The essential oils from the eight origins exhibited hydrocarbon monoterpene concentrations ranging from 1.92 to 8.92%, with Iran and KSA having the highest levels, excluding Turkey. The majority of hydrocarbon monoterpenes consisted of α-pinene, accounting for 8.92%. Oxygenated monoterpenes were the second most prevalent family in all the regions. Senegal and India exhibited the most significant proportions of content. The Oxygenated monoterpene family is distinguished by piperone epoxide (53.83%) in India and Pulegone (42.4%) in Senegal.

The presence of the hydrocarbon sesquiterpene family in essential oils is observed in eight regions, except Algeria, with amounts ranging from 0.06% to 8.16%. The predominant hydrocarbon sesquiterpene in Pakistan is Germacrene D (8.16%), while Caryophyllene oxide accounts for 4.19% in Turkey. The essential oils acquired from these eight origins exhibited a significant presence of oxygenated monoterpenes. This finding was associated with the outcomes reported previously (Gowda, 2023; Singh et al., 2020). The chemical composition of these essential oils can generally be attributed to several factors, such as geographical location.

3.4.1 *Principal Component Analysis (PCA)*

Figure 3 shows the distribution of essential oil compounds and their association with countries. The biplot effectively groups essential oil compounds and their associated countries based on similarities in chemical properties and geographical origins. The principal components help in understanding the significant variations among these compounds.

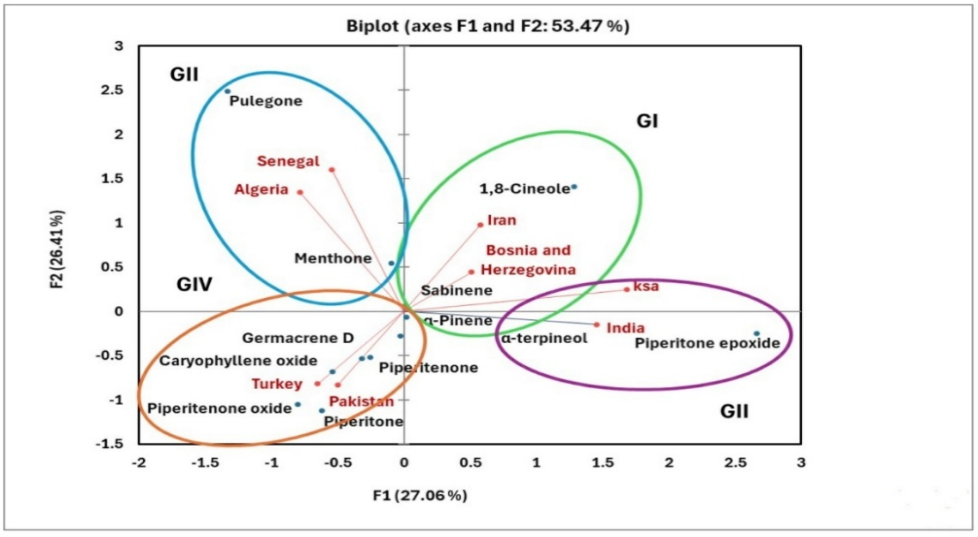

Figure 3. Principal compoDasnent biplot of F1 and F2 scores and loadings indicating the correlation of chemical components of *Mentha longifolia* essential oils from origins in the world

GI, formed by Iran and Bosnia, is characterized by the presence of α-pinene, sabinene, and 1,8-cineole. GII, formed by Senegal and Algeria, is characterized by the presence of (menthone and pulegone. G III, composed by KSA and India, is characterized by the presence of (α-terpineol and piperitone epoxide). GIV, formed by Turkey and Pakistan, is characterized by the presence of (germacrene D, caryophyllene oxide, piperenone oxide, piperenone, and piperitone). These components explained 27.06 % and 26.41 % of the total variance. The PCA score diagram represented 53.47% of the total variance in the dataset. The plane formed by the F1 and F2 axes provides a correlation between variables.

The F1 axis (27.06% of the variance) was mainly constructed by weak positive correlation between α-pinene (0.0436), sabinene (0.0436), 1,8-cineole, α-terpineol, and piperitone epoxide, with strong negative correlations between menthone, germacrene D (0.741), caryophyllene oxide, piperitenone, piperitenone oxide (0.741), and piperitone (-0.785). F1 describes variance related to the type of aroma or chemical structure, as it separates compounds with minty and woody aromas.

The F2 axis (26.41% of the variance) was mainly constructed by the moderate positive, positive correlation between germacrene D (0.478), piperitenone oxide (0.478), α-terpineol (0.403), and piperitone (0.403), with strong negative correlations between α-pinene (-0.881) and sabinene (-0.881). F2 describes variance related to the solubility, volatility, or other physical properties of the compounds, which influence their use in different products.

The strongest positive correlation is between Senegal and Algeria with a correlation coefficient of approximately 0.8883. Another strong positive correlation is between KSA and India with a

correlation coefficient of approximately 0.8879. Iran, Bosnia, and Herzegovina also show a strong positive correlation with a coefficient of approximately 0.8089. These pairs indicate that these countries' relative abundances of essential oil compounds are highly correlated

3.4.2 *Agglomerative Hierarchical Clustering (AHC)*

The dendrogram provides a hierarchical view of how various essential oil compounds are grouped based on their similarities and dissimilarities. The way compounds are grouped provides insights into their chemical similarities, which can be crucial for applications like flavoring, fragrance, or therapeutic uses in essential oils. The scale from 0 to 30 on the vertical axis of the dendrogram provides a quantitative measure of the dissimilarity between essential oil compounds. Lower values indicate higher similarity, while higher values indicate greater dissimilarity. This scale helps in understanding the relationships and differences between the compounds. The dendrogram is divided into four main groups labeled GI, GII, GIII, and GIV, each representing a cluster of compounds**.**

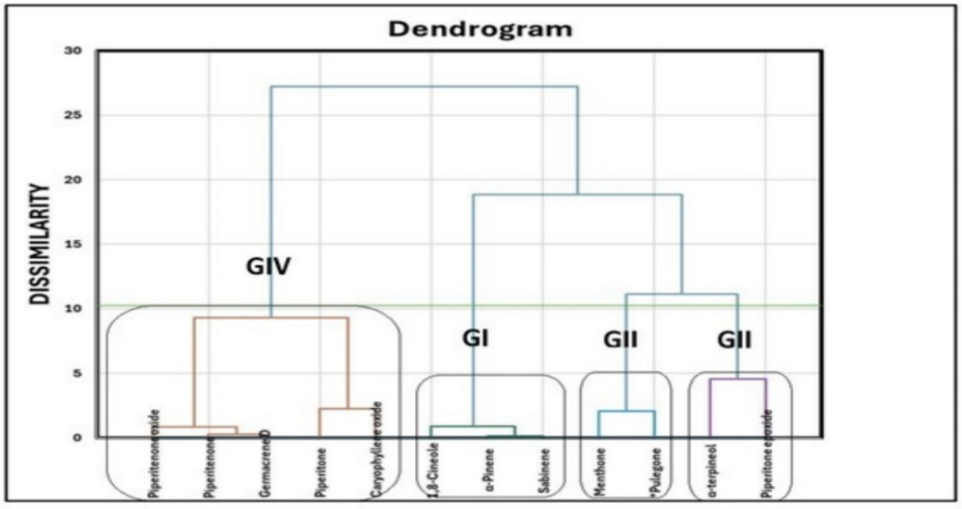

Figure 4. The vertical axis represents the level of dissimilarity between clusters. The scale ranges from 0 to 30. The horizontal axis lists various essential oil compounds.

GI includes compounds that cluster at a dissimilarity level below 10, which are grouped at a lower dissimilarity level, indicating they are more like each other. GII clustering at a dissimilarity level of around 5. This cluster is further divided into subgroups, showing more significant distinctions in similarity among these compounds. GIII is similar to GII, indicating a possible sub-grouping within the cluster consisting of two compounds. GIV, joining at higher dissimilarity levels around 10-15, suggesting they are less similar to the compounds in other groups.

Compounds that join at lower values on the scale are more similar to each other. For example, compounds in Group GI join at a dissimilarity level below 10, indicating they are quite similar.

Compounds that join at moderate values on the scale have moderate dissimilarity. For example, compounds in Group GIV join at a dissimilarity level around 10-15, indicating they have some differences but are not highly dissimilar. Compounds that join at higher values on the scale are less similar to each other. For example, the dissimilarity between Group GI and Group GIV is around 20, indicating significant differences between these groups.

The chemometric analysis results demonstrated that (AHC) findings were consistent with the results of (PCA) based on the qualitative characteristics of volatile oil. These findings led to classifying *Mentha longifolia* essential oil cultivars into four primary categories. Specific cultivars exhibit elevated levels of compounds, which could be further employed for prospective applications.

Conclusions

This study investigates the chemical composition and biological properties of *Mentha longifolia* L. essential oil from the AlUla region of Saudi Arabia. Gas chromatography-mass spectrometry (GC-MS) analysis revealed the predominant compounds to be piperitone epoxide (37.96%), 1,8-cineole (16.37%), and menthone (10.01%). The essential oil demonstrated remarkable antioxidant activity, primarily attributed to its high piperitone epoxide content and unique chemical profile. The antibacterial assessment showed significant efficacy, particularly against Gram-positive bacteria, due to bioactive oxygenated monoterpenes, including α-pinene and 1,8-cineole. Principal Component Analysis (PCA) and Agglomerative Hierarchical Clustering (AHC) identified four distinct chemotypes, highlighting the oil's chemical diversity. Comparative analysis with *M. longifolia* essential oils from different geographical locations revealed significant variations in chemical composition, suggesting the influence of environmental and genetic factors on the oil's properties. The findings demonstrate the potential of AlUla-sourced *M. longifolia* essential oil as a natural preservative in food packaging applications, owing to its strong antibacterial and antioxidant properties.

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زيت النعناع العطري من منطقة العلا، المملكة العربية السعودية: التركيب الكيميائي والتصنيف

ابتهاج عبيد الناصري[1]، هبه عباس سندي[2]

[2،1]كلية علوم الانسان والتصاميم، جامعة الملك عبد العزيز

ealnassri@kau.edu.sa[1]
hasindi@kau.edu.sa[2]

مستخلص. بحثت هذه الدراسة في التركيب الكيميائي والأنشطة البيولوجية لزيت النعناع العطري الطويل الأوراق (الحبق) من منطقة العلا باستخدام تحليل كروماتوغرافيا الغاز، والذي حدد 73 مركبًا كيميائيا: إيبوكسيد البيبيريتون (37.96٪)، 18–سينول (16.37٪)، والمينثون (10.01٪). وكشف تحليل محتوى الفينول الكلي باستخدام طريقة فولين عن 240.168 ± 2.1 ملغ من حمض الغاليك / ملغ / مل من الزيت العطري، بينما أظهر النشاط المضاد للأكسدة قيمة DPPH IC_{50} تبلغ 30.66 ميكروجرام / مل. كما أظهر الزيت العطري تأثيرات مضادة للبكتيريا كبيرة ضد كل من البكتيريا إيجابية الجرام وسلبية الجرام، وخاصة الإشريكية القولونية والمكورات العنقودية الذهبية. وقد نُسب النشاط المضاد للبكتيريا إلى تركيبة التربين الغنية بالزيت العطري، وخاصة أحاديات التربين المؤكسدة مثل إيبوكسيد البيبيريتون، و1،8–سينول، والمينثون. وقد حدد تحليل المكونات الأساسية والتصنيف الهرمي التراكمي أربعة أنماط كيميائية مميزة بين العينات. ونظراً لخصائصه المضادة للميكروبات ومضادات الأكسدة الهامة، فإن هذه النتائج أوضحت دور زيت النعناع العطري من منطقة العلا يمكن استخدامه كمادة حافظة طبيعية في تطبيقات تغليف الأغذية.

الكلمات المفتاحية: النعناع الطويل الأوراق؛ (2,2– ثنائي فينيل–2– بيكريل هيدرازيل الجذور الحرة (DPPH)؛ النشاط المضاد للبكتيريا؛ إيبوكسيد البيبيريتون، تحليل المكونات (PCA)، التصنيف الهرمي التراكمي (AHC)، العلا