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Evaluation of Antioxidant, Cholinesterase Inhibitory and Antimicrobial Properties of *Mentha longifolia* subsp. *noeana* and Its Secondary Metabolites*

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Abstract: The aim of the present study was to determine the chemical structures of the isolated compounds, the essential oil and fatty acid compositions of *Mentha longifolia* subsp. *noeana* with their biological activities. Ursolic acid (**1**), uvaol (**2**), stigmast-5-ene-3*β*-yl formate (**3**), stigmast-5-en-3-one (**4**), *β*-sitosterol (**5**), bis(2-ethylhexyl) benzene-1,2-dicarboxylate (**6**), hexacosyl (*E*)-ferulate (**7**) and 5-hydroxy-6,7,3',4'-tetramethoxy flavone (**8**) were obtained from the aerial parts. The compounds (**2-4**, **6**, **7**) were isolated for the first time from a *Mentha* species. Palmitic acid (40.8%) was the major component of the non-polar fraction obtained from the petroleum ether extract. Pulegone (32.3%) was the main constituent of the essential oil which exhibited strong butyrylcholinesterase inhibitory activity (77.36 ± 0.29%), moderate antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. The methanol extract showed 80% inhibition of lipid peroxidation, and the acetone extract possessed moderate DPPH free radical scavenging activity (60% inhibition) at 100 µg/mL.

Keywords: Lamiaceae; *M. longifolia* subsp. *noeana,* essential oil; antioxidant; anticholinesterase.

1. Introduction

The *Mentha* species (Lamiaceae family) widely distributed in Euroasia, Australia and South Africa have been grown in damp or wet places [1]. Some of them, especially *M.* x *piperita* L. (peppermint oil), *M. arvensis* L. (cornmint oil) and *M. spicata* L. (spearmint oil), have been cultivated in the world for their essential oils which have been used in food, beverage, confectionary, cosmetic and pharmaceutical industries. Their essential oils have been also used externally as antipruritic, astringent, antiseptic, antimicrobial, and rubefacient [2]. Since the mint essential oils have economic and medicinal values, their composition and biological activities have been investigated by many researchers [1]. The leaves, flowers and stems of the *Mentha* species have been used as carminative,

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antispasmodic, antiemetic, stimulant, analgesic, and emmenagogue in traditional medicine all around the world. Their leaves have been also consumed as herbal tea and spice [2]. The phytochemical investigations on the *Mentha* species revealed that they possessed flavonoids and their glycosides, phenolic compounds, triterpenoids, steroids, and lignans [3-6]. Numerous biological studies were also carried out on these species. Orhan et al. investigated the mutagenic and antimutagenic activities of luteolin derivatives obtained from *M. longifolia* subsp. *longifolia* [7]. Linarin isolated from the flower extract of *M. arvensis* exhibited selective dose dependent inhibitory activity on acetylcholinesterase [8]. Amzazi et al. indicated that *M. longifolia* extracts possessed potential anti-HIV agents [9].

Since some of the clinical effects of medicinal and edible plants are closely related to their antioxidant activity, the use of antioxidants in diet and as supplements may reduce the incidence of chronic diseases such as diabetes mellitus, cancer, and cardiovascular diseases. In addition, the antioxidants may be also relevant in slowing the progression of Alzheimer's disease which is frequently seen among elderly people all around the world [10]. For this reason, consumers have been more interested in natural antioxidants to protect their health. On the other hand, the resistance building by pathogenic microorganisms against the antibiotics is an important health problem, and researches on medicinal plants have been increased to find new antibacterial agents [10]. A literature survey showed that the essential oil possessed strong antimicrobial activity [11, 12].

In Turkey, the genus *Mentha* L. is represented by *M. aquatica* L., *M. arvensis* L., *M. longifolia* (L.) Hudson, *M. pulegium* L., *M. spicata* L., *M. suaveolens* Ehrh., and *M. x piperita* L. [13]. The infusion prepared from their leaves has been widely consumed as carminative, antiemetic, stimulant and flavoring, the essential oil from the leaves and flowering branches as antiseptic, antiemetic and refreshing in Turkish traditional medicine [14]. *M. longifolia* (L.) Hudson subsp. *noeana* (Boiss. Ex Briq.) Briq. has been found in southeastern Turkey and has been grown around the houses and fields as a snake repellent due to its pungent smell. A literature survey showed that there have been no previous biological and phytochemical reports on *M. longifolia* subsp. *noeana*. The aim of the present work was to evaluate the isolation and characterization of the constituents of *M. longifolia* subsp. *noeana* extracts (petroleum ether, acetone, and methanol), the chemical compositions of its essential oil and fatty acid with the antioxidant, anticholinesterase and antimicrobial activities.

2. Materials and Methods

2.1. Plant material

The aerial parts of *Mentha longifolia* (L.) Hudson subsp. *noeana* (Boiss. Ex Briq.) Briq. were collected from southeastern Turkey (Nusaybin-Mardin) in August 2008 by one of us (Dr. A. Ertaş), and identified by Yeter Yeşil (Department of Pharmaceutical Botany, Faculty of Pharmacy, Istanbul University). A voucher specimen was deposited in the Herbarium of Istanbul University (ISTE 83521).

2.2. Isolation of essential oil

The dried aerial parts of *M. longifolia* subsp. *noeana* were cut into small pieces and subjected to hydro distillation with water for 4 h, using a Clevenger-type apparatus to produce the essential oil which was dried over anhydrous sodium sulphate and stored at +4°C until required. A hundred milligram essential oil was obtained from 200 g of the dried aerial parts (yield 0.05% w/w).

2.3. GC/MS and GC-FID conditions (Essential oil)

The essential oil were diluted using CH_2Cl_2 (1:3 volume/volume) prior to GC/FID and GC/MS analysis. GC/FID performed using Thermo Electron Trace GC FID detector and GC/MS performed using same GC and Thermo Electron DSQ quadrupole for MS. A nonpolar Phenomenex DB5 fused silica column (30 m × 0.32 mm, 0.25 μm film thickness) was used with helium at 1 mL/min (20 psi) as a carrier gas. The GC oven temperature was kept at 60°C for 10 min and programmed to 280°C at a rate of 4°C/min and then kept constant at 280°C for 10 min. The split ratio was adjusted to 1:50, the

injection volume was 0.1 µL, and EI/MS was recorded at 70 eV ionization energy. The mass range was *m/z* 35–500 amu. Alkanes (C8-C24) were used as reference points in the calculation of Kovats Indices (KI) by the same conditions [15]. Identification of the compounds was based on comparing their retention times and mass spectra with those obtained from authentic samples and/or the NIST and Wiley spectra as well as data from the published literature. GC/FID and GC/MS were replicated three times (Mean RSD % <0.1).

2.4. Esterification of total fatty acids with GC/MS conditions

A hundred milligram of the sample was refluxed in 0.1M KOH solution in methanol (2 mL) for 1h. The solution was cooled and 5mL water was added. The aqueous mixture was neutralized with 0.5mL HCl solution and extracted with hexane:diethyl ether (1:1; 3.5 mL). The organic layer was separated and washed with water (10 mL), and dried over anhydrous Na_2SO_4. The organic solvent was removed under reduced pressure on a rotary evaporator to give fatty acid methyl esters [16-18].

The fatty acid methyl esters were analyzed using Thermo Electron Trace 2000 GC model gas chromatography and Thermo Electron DSQ quadrupole mass spectrometry. A nonpolar Phenomenex DB5 fused silica column (30 m × 0.32 mm, 0.25 µm film thickness) was used with helium at a 1 mL/min (20 psi) as a carrier gas, GC oven temperature was kept at 40°C for 5 min and programmed to 280°C at rate of 5°C/ min and kept constant at 280°C for 10 min. The split ratio was adjusted to 1:20 and the injection volume was 0.1 µL. EI/MS was taken at 70eV ionization energy. Mass range was from *m/z* 35-450 amu. Scan time was 0.5 sec with 0.1 interscan delay. The library search was carried out using NIST and Wiley GC/MS library, and TUBITAK-UME library. The relative percentage amount of the separated compounds was calculated from Total Ion Chromatography by the computerized integrator.

2.5. Extraction, fractionation and isolation

The dried and powdered aerial parts of *M. longifolia* subsp. *noeana* (1350 g) were sequentially macerated with petroleum ether (3 × 4 L), acetone (3 × 4 L) and methanol (3 × 4 L) for 24 h at room temperature. After filtration, the solvents were evaporated to dryness under vacuum. The petroleum ether extract (MLP) (8.9 g) was subjected to a silica gel column (2 × 100 cm) and eluted with petroleum ether (40 - 60°C) followed by a gradient of dichloromethane, and then methanol up to 100%. Similar fractions were combined with TLC control (Fractions A-E). Fraction A (2 g) was analyzed to determine its composition by GC/MS. Fraction B was further subjected to chromatography over Sephadex LH-20 eluted with hexane-dichloromethane (7:2, v/v) and final purifications were carried out on preparative TLC plates using the following solvent systems for; stigmast-5-ene-3β-yl formate (**3**, 9 mg) (petroleum ether:dichlorometane, 7:1), stigmast-5-en-3-one (**4**, 7 mg) (petroleum ether:dichlorometane, 7:1). Fraction C was also subjected to Sephadex LH-20 column eluted with hexane-dichloromethane (7:3, v/v) and final purifications were carried out on preparative TLC plates using the following solvent systems for; uvaol (**2**, 5 mg) ($CHCl_3$), β-sitosterol (**5**, 6 mg) (toluene:acetone:diethyl ether, 8:0.5:0.5). The acetone extract (28.5 g) was fractionated on a silica gel column (3.5 × 150 cm), eluted with petroleum ether, followed by a gradient of acetone, and then methanol up to 100%. Four fractions (Fractions A-D) were obtained after combining similar fractions with TLC control. After separating Fractions A and B on silica gel columns and preparative TLC, bis(2-ethylhexyl) benzene-1,2-dicarboxylate (**6**, 4 mg) (petroleum ether:dichloromethane, 5:3) and hexacosyl (*E*)-ferulate (**7**, 6 mg) (toluene:acetone, 3:1) were isolated. Ten grams of ursolic acid (**1**) were obtained from Fractions A-D of the acetone extract re-separating on silica gel columns and preparative TLC (toluene:diethyl ether, 1:1). The methanol extract (41.8 g) was fractionated on a silica gel column (5 × 150 cm) which was eluted petroleum ether, followed by a gradient of chloroform, and then methanol up to 100%. TLC analysis was used to combine similar fractions (Fractions A-D). Fraction A was subjected to a silica gel column, 5-hydroxy-6,7,3',4'-tetramethoxy flavone (**8**, 5 mg) (toluene:acetone, 2:1) was obtained using preparative TLC. TLC plates were visualized by spraying with cerium (IV) sulphate dissolved in 10% sulphuric acid following under UV light checking.

2.6. Determination of total phenolic and flavonoid contents of the extracts

2.6.1. Determination of total phenolic content

The concentrations of phenolic content in the crude extracts were expressed as micrograms of pyrocatechol equivalents (PEs) [19]. The solution (100 µL) of the samples in methanol was added to 4.6 mL of distilled water and 100 µL of Folin-Ciocalteu's Reagent, and mixed thoroughly. After 3 min, 300 µL sodium carbonate (2%) was added to the mixture and shaken intermittently for 2 h at room temperature. The absorbance was read at 760 nm. The concentration of phenolic compounds was calculated according to the following equation that was obtained from standard pyrocatechol graphic:

$$\text{Absorbance} = 0.0155 \text{ pyrocatechol } (\mu g) + 0.0259 \quad (R^2 = 0.9995)$$

2.6.2. Determination of total flavonoid content

Measurement of flavonoid content of the crude extracts was based on the method described by Moreno et al. with a slight modification and results were expressed as quercetin equivalents [20]. An aliquot of 1 mL of the solution (contains 1 mg of crude extract in methanol) was added to test tubes containing 0.1 mL of 10% aluminium nitrate, 0.1 mL of 1 M potassium acetate and 3.8 mL of methanol. After 40 min at room temperature, the absorbance was determined at 415 nm. The concentration of flavonoid compounds was calculated according to the following equation:

$$\text{Absorbance} = 0.1459 \text{ quercetin } (\mu g) - 0.1314 \quad (R^2 = 0.9977)$$

2.7. Antioxidant activity

2.7.1. *Determination of the antioxidant activity with the β-carotene bleaching method*

The antioxidant activity of the samples was evaluated using the *β*-carotene-linoleic acid test system with slight modifications [21]. *β*-Carotene (0.5 mg) in 1mL of chloroform was added to 25 µL of linoleic acid, and 200 mg of Tween-40 emulsifier mixture. After evaporation of chloroform under vacuum, 100 mL of distilled water saturated with oxygen, were added by vigorous shaking. Four milliliters of this mixture was transferred into different test tubes containing different concentrations of the samples. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a 96-well micro plate reader (BioTek Power Wave XS, USA). The emulsion system was incubated for 2 h at 50°C. A blank, devoid of *β*-carotene, was prepared for back ground subtraction. *α*-Tocopherol was used as a standard. The bleaching rate (R) of *β*-carotene was calculated according to the following equation:

$$R = \frac{\ln \frac{a}{b}}{t}$$

Where: ln=natural log, *a*=absorbance at time zero, *b*=absorbance at time *t* (120 min).

The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control, using following equation:

$$\text{AA (Inhibition\%)} = \frac{R_{Control} - R_{Sample}}{R_{Control}} \text{ x } 100$$

2.7.2. DPPH free radical scavenging activity

The free radical scavenging activity of the samples was determined by the DPPH assay described by Blois with slight modification [22]. 0.1 mM solution of DPPH in methanol was prepared

and 4 mL of this solution was added to 1 mL of sample solutions in methanol at different concentrations. Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated by using the following equation:

$$\text{DPPH Scavenging Effect (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \text{ x } 100$$

2.8. Anticholinesterase activity

Acetyl- and butyryl-cholinesterase inhibitory activities were measured by slightly modifying the spectrophotometric method developed by Ellman et al. [23]. Acetylthiocholine iodide and butyrylthiocholine iodide were used as substrates of the reaction and DTNB (5,5-dithio-bis(2-nitrobenzoic)acid) for the measurement of the anticholinesterase activity. All samples were dissolved in ethanol to prepare their stock solution at 4000 µg/mL concentration. Aliquots of 130 mL of 100 mM sodium phosphate buffer (pH 8.0), 10 µL of sample solution and 20 µL AChE (or BChE) solution were mixed and incubated for 15 min at 25ºC, and 10 µL of DTNB is added. The reaction was then initiated by the addition of 10 µL acetylthiocholine iodide (or butyrylthiocholine iodide). Final concentration of the tested solutions was 200 µg/mL. The hydrolysis of these substrates were monitored using a BioTek Power Wave XS by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine iodide or butyrylthiocholine iodide, at a wavelength of 412 nm. The experiments were carried out in triplicate. Galanthamine was used as a reference compound.

2.9. Antimicrobial activity

Gram-negative bacterial strains, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, Gram-positive bacterial strains, *Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* ATCC19615 and the fungi, *Candida albicans* ATCC10231 were used to determine the antimicrobial activity of the samples. Bacterial strains were cultured overnight in Nutrient Broth (NB) at 37°C with the exception of *C. albicans* cultured at 30°C. The microorganisms were purchased from Refik Saydam Sanitation Center, Turkey.

2.9.1 Antimicrobial screening

The antimicrobial activity of the essential oil, and petroleum ether, acetone and methanol extracts of the plant was tested by the paper disc diffusion method according to the National Committee for Clinical Laboratory Standards Guidelines [24] using 100 µL of suspension of the tested microorganisms, containing 10^8 colony forming units (CFU/mL) for inoculating the plates. Ten microliters of the essential oil and various concentrations of the extracts were loaded on the sterile filter paper discs (6 mm) and placed on the inoculated plates. The seeded plates were incubated at 37°C for 24 h and 30°C for 48 h for bacteria and fungi, respectively. Imipenem (IPM) and Nystatin (NYT) were used as positive controls for bacteria and fungi, respectively. All tests were performed in triplicate and the antimicrobial activity was expressed as diameter of inhibition zones (mm).

2.9.2. Determination of minimum inhibitory concentrations (MIC)

The minimal inhibitory concentration (MIC) was determined by a broth dilution method in test tubes according to the modified procedure of Kivanc & Akgul as follows: serial dilutions of the essential oil were made in a concentration range from 1.95 to 500 µg/mL [25]. Fifty microliters from each of various dilutions of the oils (dissolved in 10% DMSO) and 20 µL of the inoculum (containing

10^8 CFU/mL of live bacterial cells) were added to 5 mL of nutrient broth (NB) tubes. A positive (containing 20 µL inoculum and 5mL NB) and a negative control tubes (containing 50 µL of essential oil dissolved in 10% DMSO, 5 mL NB without inoculum) were prepared with samples. The contents of the tubes were mixed and the incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms. The experiment was carried out in triplicate.

2.10. Statistical analysis

The results of the antioxidant and anticholinesterase activity assays were mean ± SD of three parallel measurements. The statistical significance was estimated using a Student's *t*-test, p values < 0.05 were regarded as significant.

3. Results and Discussion

Eight known compounds consisting of two triterpenoids ursolic acid (**1**) and uvaol (**2**), three steroids stigmast-5-ene-3β-yl formate (**3**), stigmast-5-en-3-one (**4**) and β-sitosterol (**5**), two phenolic compounds bis(2-ethylhexyl) benzene-1,2-dicarboxylate (**6**) and hexacosyl (*E*)-ferulate (**7**), and a flavonoid 5-hydroxy-6,7,3',4'-tetramethoxy flavone (**8**), were isolated from the aerial parts of *M. longifolia* subsp. *noeana* (Figure 1) [26-32]. The structures of the compounds (**1–8**) were determined on the basis of spectroscopic evidence (UV, IR, ^{1}H- and ^{13}C-NMR (APT), HMQC, HMBC, Mass). Their spectral data were compared to those of the literature values. The compounds (**2-5**) were obtained from the petroleum ether extract, compounds (**1**, **6**, **7**) from the acetone extract, and compound (**8**) from the methanol extract. The compounds (**2-4**, **6**, **7**) were isolated for the first time from the *Mentha* species. In addition, 10 g ursolic acid (**1**) was obtained from the acetone extract (28.5 g), *M. longifolia* subsp. *noeana* could be considered as a natural source of ursolic acid.

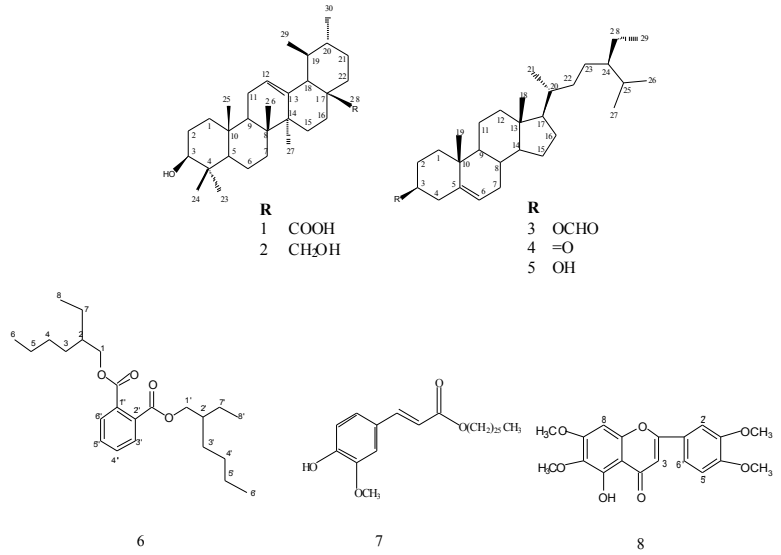

Figure 1. Chemical structures of the isolated compounds (**1-8**)

Total phenolic and flavonoid contents of the extracts were determined as pyrocatechol (PEs) and quercetin (QEs) equivalents, respectively. The phenolic contents of the petroleum ether (225.65 ± 3.42 µgPEs/mg extract), acetone (217.10 ± 1.82 µgPEs/mg extract) and methanol extracts (218.06 ± 3.19 µgPEs/mg extract) were higher than their flavonoid contents (33.54 ± 1.60, 29.10 ± 0.47, 24.08 ± 1.14 µgQEs/mg extract, respectively). In the present study, the antioxidant activity of the petroleum ether, acetone and methanol extracts, the isolated compounds (**1-8**) and the essential oil of *M.*

longifolia subsp. *noeana* was carried out using *β*-carotene bleaching method and DPPH free radical scavenging activity assay. Their inhibition of lipid peroxidation and DPPH free radical scavenging effect were determined at 10, 25, 50, and 100 µg/mL, and the results providing over 50% inhibition in these antioxidant assays were given in Figures 2 and 3, respectively. *α*-Tocopherol was used as a standard compound in each antioxidant assay. All extracts exhibited over 50% inhibition of lipid peroxidation at all concentrations, except for 10 µg/mL. The methanol extract showed the best activity among the tested samples at all concentrations and high antioxidant effect than *α*-tocopherol at 50 and 100 µg/mL.

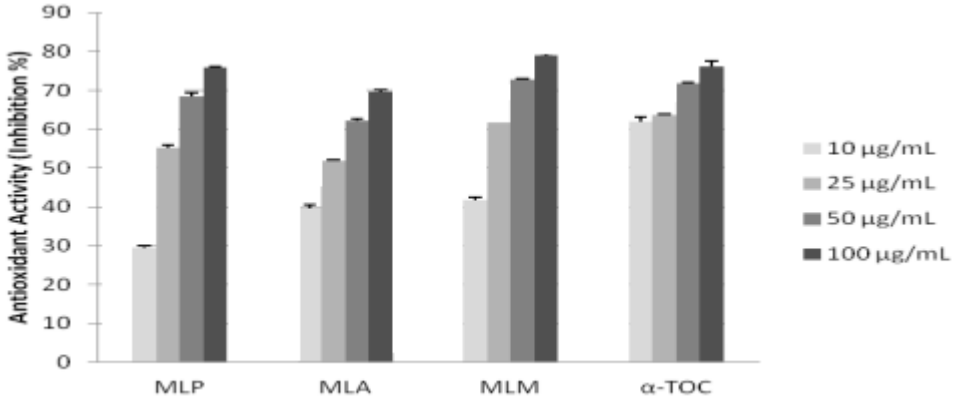

Figure 2. Inhibition (%) of lipid peroxidation of *M. longifolia* subsp. *noeana* extracts and *α*-Toc by *β*-carotene bleaching method. Values are means ± S.D., n = 3, $p < 0.05$, significantly different with Student's *t*-test.

In DPPH free radical scavenging activity assay, the petroleum ether and acetone extracts indicated almost the same weak scavenging effect at 10, 25, and 50 µg/mL, the acetone extract showed the best activity (60% inhibition) among the tested samples at 100 µg/mL. *α*-Tocopherol possessed 83% inhibition at the same concentration. The essential oil and isolated compounds (**1**-**8**) were found to be inactive in *β*-carotene bleaching method and DPPH free radical scavenging activity assay. Gulluce et al. also indicated that the inhibition of lipid peroxidation and DPPH free radical scavenging activity of the methanol extract prepared from the aerial parts of *M. longifolia* subsp. *longifolia* collected from northeastern Turkey was much better than the results of its essential oil [1].

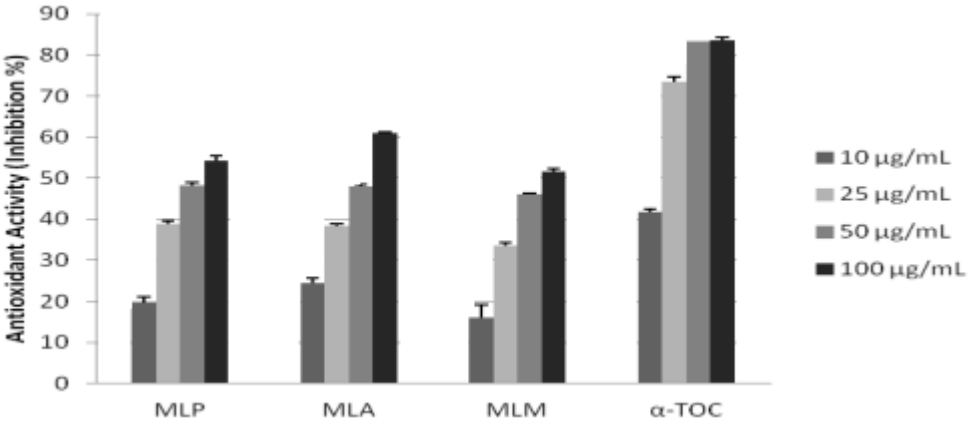

Figure 3. DPPH free radical scavenging activity of *M. longifolia* subsp. *noeana* extracts and *α*-Toc. Values are means ± S.D. n = 3, $p < 0.05$, significantly different with Student's *t*-test.

The main fatty acids of the non-polar fraction (Fraction A) obtained from the petroleum ether extract were determined as palmitic acid (40.8%), oleic acid (17.8%) and linoleic acid (15.3%) (Table 1). This is the first report on the fatty acid composition of *M. longifolia* subsp. *noeana*.

The chemical composition, antioxidant, anticholinesterase and antimicrobial activities of the essential oil obtained from the dried aerial parts of *M. longifolia* subsp. *noeana* were reported for the first time in this study. Twenty-three compounds (monoterpenes and sesquiterpenes) constituting 88.6% of the total oil were identified using GC/MS analysis (Table 2).

Table 1. Fatty acid composition of Fraction A.

Rt (min)[a]	Components[b]	% Composition
35.24	Myristic acid, methyl ester (C14:0)	2.0
37.91	Pentadecanoic acid, methyl ester (C15:0)	1.0
39.92	Palmitoleic acid, methyl ester (C16:1)	1.0
40.49	Palmitic acid, methyl ester (C16:0)	40.8
42.92	Heptadecanoic acid, methyl ester (C17:0)	t[c]
44.61	Linoleic acid, methyl ester (C18:2n6c)	15.3
44.73	Oleic acid, methyl ester (C18:1n9c)	17.8
44.85	Elaidic acid, methyl ester (C18:1n9t)	2.1
45.27	Stearic acid, methyl ester (C18:0)	9.0
49.67	Arachidic acid, methyl ester (C20:0)	4.2
51.75	Heneicosanoic acid, methyl ester (C21:0)	t
52.49	8,11,14-Eicosatrienoic acid (C20:3n6c)	5.0
53.74	Behenic acid, methyl ester (C22:0)	1.3
55.67	Tricosanoic acid, methyl ester (C23:0)	t
57.65	Lignoceric acid, methyl ester (C24:0)	t
	Total	99.5

[a]Retention time (as minute)
[b]Compounds listed in order of elution from a HP-5 MS column. A nonpolar Phenomenex DB-5 fused silica column
[c]Trace (< 0.6%)

The essential oil was rich in monoterpenes comprising mainly by oxygenated monoterpenes, pulegone (32.3%), followed by menthone (13.8%), isopulegole (9.7%), isopulegone (8.9%) and 1,8-cineole (8.0%). The percentages of sesquiterpenes in the essential oil of *M. longifolia* subsp. *noeana* was low, and caryophyllene oxide (3.3%) was the main sesquiterpene found in the essential oil. *cis*-Piperitone epoxide (18.4%), pulegone (15.5%), piperitenone oxide (14.7%), menthone (7.9%), isomenthone (6.6%), and carvone (4.9%) were found as the primary components in the essential oil of *M. longifolia* subsp. *longifolia* [1]. Piperitone oxide and piperitenone oxide or carvone and dihydrocarvone which were found high amounts in *M. longifolia* species were not present in *M. longifolia* subsp. *noeana*, but in this study dihydrocarveolacetate (2.7%) was isolated for the first time from the *Mentha* species [1].

The essential oil, petroleum ether, acetone and methanol extracts of *M. longifolia* subsp. *noeana* was tested against two Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*), two Gram-positive (*Staphylococcus aureus*, *Streptococcus pyogenes*) bacteria and a fungi (*Candida albicans*) using the disc diffusion method, followed by the measurement of minimal inhibitory concentrations (MIC) (Table 3). The essential oil exhibited moderate antimicrobial activity against *E. coli*, *S. aureus*, and *C. albicans*. The results indicated that the most sensitive microorganism was *C. albicans* (21.5 mm inhibition zone diameter and 3.9 µg/mL MIC value), and *S. pyogenes* was the most resistant bacteria with the smallest inhibition zone diameter (13.6 mm) and the highest MIC value (31.2 µg/mL). On the other hand, the petroleum ether, acetone and methanol extracts showed no antimicrobial activity even with 30 mg/mL concentration (their data not shown in Table 3). When the present results were compared with those of *M. longifolia* subsp. *longifolia*, there are similarities. Gulluce et al. indicated that the methanol extract from the aerial parts of *M. longifolia* subsp.

longifolia showed no antimicrobial activity, and the essential oil possessed strong antimicrobial activity against 15 bacteria, 14 fungi and a yeast species [1].

The anticholinesterase activity of the petroleum ether, acetone and methanol extracts, essential oil, and isolated compounds (**1**-**8**) was determined at 200 µg/mL, and galanthamine was used as a standard compound. The essential oil exhibited strong butyrylcholinesterase inhibitory activity (77.36 ± 0.29%), but it was found to be inactive against acetylcholinesterase. None of the extracts showed acetylcholinesterase inhibitory activity, the acetone (58.96 ± 1.72%) and methanol extracts (61.69 ± 5.00%) possessed almost the same inhibition against butyrylcholinesterase. Although the isolated compounds (**2**-**8**) exhibited no anticholinesterase activity, ursolic acid (**1**) indicated moderate acetyl- (55.08 ± 0.34%) and butyryl-cholinesterase inhibitory activities (68.12 ± 0.28%).

This report is the first phytochemical study on *M. longifolia* subsp. *noeana* indicated that further phytochemical and *in vitro* and *in vivo* biological studies should be carried out on these species to establish their other potential effect on human health together with our previous report [33].

Table 2. Chemical composition of the essential oil.

RI[a]	Rt (min)[b]	Constituents[c]	Composition%
939	9.50	α-Pinene	0.3
975	10.82	Sabinene	0.3
979	11.00	β-Pinene	0.6
991	11.27	β-Myrcene	t[d]
991	11.39	3-Octanol	0.4
1025	12.58	Cymene	t
1029	12.75	Limonene	0.9
1031	12.90	1,8-Cineole	8.0
1153	17.36	Menthone	13.8
1163	17.76	Isomenthone	2.6
1155	18.18	Isopulegone	8.9
1160	18.25	Isopulegol	9.7
1189	18.66	α-Terpineol	1.2
1237	20.45	Pulegone	32.3
1253	20.96	Piperitone	0.9
1253	21.95	Anethole	0.6
1344	22.89	Dihydrocarveolacetate	2.7
1175	23.97	Verbenone	0.6
1409	26.70	β-Caryophyllene	0.8
1432	27.10	α-Copaene	0.4
1438	27.40	Unidentified	9.2
1439	27.79	α-Humulene	t
1578	31.60	Spathulenol	0.3
1583	31.83	Caryophyllene oxide	3.3
		Total	97.8

[a] RI Retention indices (DB-5 column)
[b] Retention time (as minute)
[c] Compounds listed in order of elution from a HP-5 MS column. A nonpolar Phenomenex DB-5 fused silica column
[d] Trace (< 0.05%)

Table 3. Antimicrobial activity of the essential oil.

Test microorganisms	Essential oil		Standards	
	DD[a]	MIC[c]	DD[b]	MIC[c]
P. aeruginosa ATCC 27853	-	-	12 (IPM)	2 (IPM)
S. pyogenes ATCC19615	13.6	31.2	39.5 (IPM)	0.007 (IPM)
E. coli ATCC 25922	14.5	15.6	19 (IPM)	0.062 (IPM)
S. aureus ATCC 25923	15.5	7.8	27.5 (IPM)	0.007 (IPM)
C.albicans ATCC10231	21.5	3.9	25 (NYT)	0.25 (NYT)

-: No inhibition zone and/or MIC value measured.
[a] DD: Inhibition zone in diameter (mm) around the discs (6 mm) impregnated with 10 µL of essential oil.
[b] DD: Diameter of disc diffusion (mm); IPM= Imipenem (10 µg/disc); NYT = Nystatin (30 µg/disc).
[c] MIC: Minimal inhibitory concentrations as (µg/mL)

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