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Antioxidant and Biological Activities of *Mentha longifolia*, L. Extracts

Hassan, R. A.; S. T. Abotaleb; H. B. Hamed and M. A. Sharaf- Eldeen*

Agric. Biochem. Dept, Fac. of Agric.,Mansoura Univ., Mansoura,Egypt.

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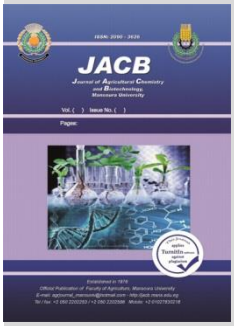

ABSTRACT

Lamiaceae is one of the large plant families used to evaluate some secondary metabolites such as terpenoids and phenolic compounds. *Mentha longifolia* (*M. longifolia*) is one of genus *Mentha* which includes 25 to 30 species. The plant was extracted with methanol to obtain methanol extract as crud extract. Methanol extract was fractionated with four different solvents graduated in polarity i.e pet ether, methylene chloride, ethyl acetate and butanol to obtain pet ether, methylene chloride, ethyl acetate and butanol fractions . The obtained methanol and its fractional extracts were tested for their total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity using scavenging activity of DPPH radical method. Antimicrobial activity of the plant methanolic extract against two Gram-positive bacterial isolates, two Gram-negative bacterial isolates, one isolate of yeast and one fungal isolate was performed. Results showed that the highest concentration of TPC was 228.36 mg GAE/g for ethyl acetate fraction, while the lowest one was observed for pet. ether fraction (154.56 mg GAE/g). On the other hand, TFC was ranged from 293.86 to 153.39 mg QE/g dw for pet. ether and butanol fractions, respectively. The highest antioxidant activity was found for ethyl acetate fraction i.e with lower IC_{50} = 107.01µg/ml.

Keywords: Antioxidant; Microbial isolates; *Mentha longifolia* L.; Plant extracts.

INTRODUCTION

Medicinal plants have a great role in providing health and treatment as well as disease prevention in human communities (Changizi-Ashtiyani *et al* 2013). Medicinal herbs are very rich in secondary metabolites which show physiological effects on the function of mammalian tissues in health and disease conditions (Taheri *et al* 2012).

Mentha longifolia (L) (horsemint in English) belongs to the genus *Mentha*, *Lamiaceae* family and is associated with medicinal and aromatic herbs. It is widespread throughout the Mediterranean region, Central and Northern Europe, Asia Minor and Africa (Janković 1974). Traditionally, *M. longifolia* has been used for the treatment of diarrhea, dysentery and stomachache and alsocardiac diseases (Haq *et al*., 2011).

Mentha spp. have been used as a folk remedy for treatment of nausea, bronchitis, flatulence, anorexia, ulcerative colitis and liver complaints due to its anti-inflammatory, carminative, antiemetic, diaphoretic, antispasmodic, analgesic and stimulant. In addition, Leaves, flowers and the stems of *Mentha* spp. are often used in herbal tea or as additives in merchant spice mix for numerous foods to exhibit aroma and flavor. (Mikaili *et al* 2013). *M. longifolia* has many uses in foodstuff, flavours, beverages, cosmetics and folks medicine (Kanatt *et al*., 2007).

Aerial part of the *M. longifolia* containing essential oils that has medicinal effects. This essential oil was reported to have fungicidal, antioxidant, anti-inflammatory and antimicrobial efficiency (Mkaddem *et al*., 2009).

M. longifolia leaves are used usually to prepare tea beverage for coughs, colds, stomach cramps, asthma, flatulence, indigestion and headaches.

Furthermore, wild mint externally has been used to remedy cuts and swollen glands. (Edris *et al*. 2003) .

Microbial pathogens in food may cause spoilage and contribute to foodborne disease incidence, and the emergence of multidrug resistant bacteria - such as Staphylococcus aureus (S. aureus), Escherichia coli (E. coli), and Pseudomonas aeruginosa (P. aeruginosa) - has increased rapidly, causing the increase of morbidity and mortality (Miladi *et al* 2016).

In recent decades, many spices have been applied to treat infectious diseases or protect food because they were experimentally proved to possess antimicrobial activities against pathogenic and spoilage fungi and bacteria .Moreover, the secondary metabolites of these spices are known as antimicrobial agents (Arora and Kaur 1999).

The aim of this work is to study the TPC and TFC of the investigated sample. In addition the research includes study the antioxidant activity of plant extracts. The effect of the plant extract on pathogenic bacteria, yeast and fungi was also investigated.

MATERIALS AND METHODS

Plant material :

Fresh leaves of *M. longifolia* were obtained from local farms in march 2018 and left for air drying in the shade, then ground using a blender to powder and stored in well-closed containers in refrigerator till analysis.

Plant extracts :

The dried powder leaves were soaked in appropriate volume of pure methanol and kept at room temperature (25°C) over night. The extract were then filtered and the residues were re-extracted twice by soaking in methanol. The combined extracts were evaporated under vacuum using rotary evaporator at 40°C . The crude

* **Corresponding author.**
E-mail address: Mostafa_sharaf8@yahoo.com
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extract was preserved in sterilized glass under refrigerated conditions till further use.

Fractionation of methanolic extract :

Methanol extract of plant leaves under study was fractionated by using four solvents have increasing polarity i.e pet ether, ethylene chloride (CH_2Cl_2), ethyl acetate ($C_2H_5COOCH_3$) and butanol (C_4H_9OH). The extraction process was carried out three times for each solvent, then the solvents were removed using rotary evaporator. The obtained fractions were kept in refrigerator till use.

Total phenolic content :

Total phenolic of plant extracts were estimated as indicated by Singleton *et al* (1999). Gallic acid was chosen as a reference with concentration ranged from 0.025 to 0.5 mg/mL. One mL of each extract containing (0.3 mg/mL), 9 mL dist. H_2O, 1 mL of Folin-Ciocalteu's reagent and 1 mL (7% wt /v) Na_2CO_3. The solution was left for 90 mins. at Lab. condition, then at 765 nm, the absorbance was determined and phenolics content were expressed as mg GAE / g dw extract.

Total flavonoids content :

Total flavonoids content of *M. longifolia* extracts were estimated by $ALCL_3$ colorimetric method as mentioned by Lin and Tang (2007). Each extract (1.o ml) was added to 0.1 mL of (10% w /v) $ALCL_3$, 0.1 mL of (1 M potassium acetate) and 2.8 mL of dist. H_2O. The mix was left for 40 mins. at Lab. temperature, the absorbance was estimated at 415 nm. Quercetin (Q) was utilized as a reference between 0.005 to 0.1 mg/mL and the flavonoids were expressed as mg QE/ g dw extract.

Antioxidant Activity Assay:

scavenging activity of DPPH radical:

The free-radical scavenging activity was measured using 2,2 diphenyl-1-picrylhydrazyl (DPPH) method described by Moon and Terao, (1998) with some modification. Different volumes of each extract (0.3 mg/mL) was added, separately to solution of DPPH to make up a total volume of 2.0 mL. After standing for 15 mins at room temperature, the absorbance was evaluated at517nm using UV–Vis spectrophotometer. High absorbance of the reaction mixture indicated low free radical scavenging activity. Butylated hydroxyl toluene (BHT) was utilized as a favorable control. Radical inhibition of free radical by extract was studied as follows:

Antiradical activity (%) = ($A_{control} - A_{sample}$) / $A_{control}$ ×100.

Where: **$A_{control}$ = Absorbance of control**

A_{sample} = Absorbance of sample

The IC_{50} value, defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% and was calculated based on linear regression of plots of the percentage antiradical activity against the concentration of the tested compounds (Nahak and Sahu,2010). The experiment was carried out in three times and the results are recorded as average values.

Antimicrobial activity:

1- Microbial media and plant extracts sterilization:

Potato Dextrose Agar (PDA) and nutrient Agar (NA) media **(Oxoid, 2006)**, were prepared for fungal and bacterial growth, respectively. These ready-made media were purchased and sterilized in autoclave at 121°C for 15 min. The plant extract were sterilized by micro filter (Flowpore D 0.2 µm, Made in Germany).

2- Microbial strains and maintenance:

Six microbial species were kindly taken from Agric. Microbiology Dept., Fac. of Agric., Damietta University, Damietta, Egypt. These microorganisms included bacteria, yeast and fungi, which are: *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae* and *Aspergillus flavus*. The fungal or bacterial strains were kept on PDA or NA media, respectively, at 5°C untill use. The microbial strains (bacteria, the first four strains or yeast and fungi, the last two strains) were sub-cultured on new slants of NA or PDA media and kept at 37 or 25°C for 2 or 5 days, respectively.

3- Cultivation methods and antimicrobial activity determination:

All microbial strains were grown on NA slant at 37 °C for one day. Five ml of sterilized saline solution (0.9% Na Cl) was added to each slants. The antimicrobial activity was determined using well diffusion methods on Petri dishes containing about 20 ml of NA media (El-Kadi *et al*., 2018). All plates were inoculated with the suitable microbial strains by using a sterile cotton swab. Subsequently, three small wells of 6.3 mm in diameter was done by a sterilized cork borer. Each well was filled up with 30, 60 or 100 µl of *M. longifolia* extract. All plates were incubated at 37 and 25°C according the microbes. Inhibition zones which appeared around the well were carefully measured after one or 6 days according the microbes using a digital Vernier caliper (El-Fadaly *et al*., 2018). The mean value of three replicates was calculated.

The assessment of antimicrobial agents = A-B (mm).

Where: A : the diameter of complete clear zone (mm) .

B : the diameter of cork borer (6.35) (Azzaz *et al*., 2017).

RESULTS AND DISCUSSION

1- Total Phenolic Content (TPC) :

Total phenolic content (as mg GAE/ g) are given in Table 1. These results demonstrate the presence of natural antioxidant phenolic compounds in all these extracts.

The obtained results illustrated that methanol extract, butanol, ethyl acetate, methylene chloride and pet. ether fractions contained average values of 189.8, 171.26, 228.36, 190.26 and 154.56 mg GAE/g dw extract , respectively.

Table 1.Total phenolic of *M. longifolia* extracts:

Methanol extract and its fractions	Phenolic content as mg GAE/g dw extract
Pet. ether	154.56
Methylene chloride	190.26
Ethyl acetate	228.36
Methanol	189.8
Butanol	171.26

It was clear that, ethyl acetate fraction has the highest content of total phenolic, (228.36 mg GAE/g). While, pet. ether fraction has the lowest one of total phenolic, (154.56mg GAE/g). On the other hand, methylene chloride fraction, methanol extract and butanol fraction are containing medium values of total phenolic with average values of 190.26, 189.8 and 171.26 mg GAE/g, respectively.

These results are higher than those of other workers. For instance, Al-Ali *et al*., (2015) gave average value of 113.8 mg GAE/ g dw for *M. longifolia* methanol

extract. On the other hand, our results are lower than those mentioned by Al-Okbi *et al* (2015), who reported that the ethanolic extract of this plant gave average value of 303.2 mg GAE / g dw .

2- Total flavonoid Content (TFC) :

Flavonoids are a large group of phenolic compounds consists of anthocyanin, flavonols and flavanols.

Total flavonoids content as shown in Table (2) are ranged from 153.39 to 293.86 mg QE/g dw for butanol and pet. ether fractions, respectively. While, ethyl acetate fraction, methanol extract and methylene chloride fraction contained average values of 182.73, 207.43 and 236.23 mg QE/g for total flavonoid compounds, respectively.

The obtained data in Table (2) showed that methanol extract gave 207.4 3 mg QUE/ g dw for total flavonoids. These results are higher than those of Al-Ali *et al* (2015) , who gave average value of 106.7 mg QUE /g dw for TFC of *M. longifolia* methanolic extract.

Table 2. Total flavonoids in *M. longifolia* extracts:

Methanol extract and its fractions	Flavonoid content (mg QE/g dw extract)
Pet. ether	293.86
methylene chloride	236.23
Ethyl acetate	182.73
Methanol	207.43
Butanol	153.39

3- scavenging activity of DPPH radical in *M. longifolia* extracts :

The antioxidant activity of the tested extracts are estimated using DPPH radical scavenging activity. The antioxidants scavenging activities for DPPH are attributed to their hydrogen-donating abilities (Biswas *et al*., 2010) Vitamin C is used as a reference antioxidant compound (Table 3).

An antioxidant needed to decrease the initial DPPH concentration by 50% (IC_{50}) is a parameter used to measure the antioxidant activity (Sanchez *et al*., 1998). The lower IC_{50} pointed to the higher antioxidant activity.

Ethyl acetate fraction was the most active antioxidant (IC_{50}, 107.01), in comparison with the rest extracts which gave average values of IC_{50} 109.89, 115.68, 124.19 and 136.83 µg/mL for pet. ether, butanol, methylene chloride and methanol extracts , respectively.

The obtained results for IC_{50} value of *M. longifolia* methanol extract is 136.83 µg/ml, this value is lower than that mentioned by Bahadori *et al*. (2018) who gave average value of 162.08 µg/ml of the same parameter of *M. longifolia* methanol extract. In the opposite trend, Hajlaoui *et al*. (2009), found that IC_{50} value for M. *longifolia* methanol extract was 20 µg/ml .

Table 3 . Antioxidant activity of *M. longifolia* extracts:

extracts	IC_{50} (µg/ml)
Pet. ether	109.89
methylene chloride	124.19
Ethyl acetate	107.01
Methanol	136.83
Butanol	115.68

Inhibition percentage of antioxidant activity (AOA) :

Results in Table(4) showed that *M. longifolia* leaves extracts had variable inhibition percentage values for antioxidant activity(AOA).

The inhibition percent was calculated from the following equation:

$$\% \text{ inhibition} = (A_{control} - A_{sample})/A_{control} \times 100.$$

The antioxidant inhibition percentage of methanol extract, butanol, ethyl acetate, methylene chloride and pet. ether fractions at doses of 50, 100 and 200 ppm are shown in Table(4).

The obtained data showed that using of the concentration of 200 µg/ml for different extracts was more effective than the others. Ethyl acetate fraction had the higher value of inhibition percentage followed by butanol, methylene chloride fractions, methanol extract**,** and finally with pet. ether fraction in average values of 80.19, 76.61, 73.35, 72.20 and 10.54 %, respectively.

Table 4. Inhibition percentage of antioxidant activity (AOA) induced by *M. longifolia* leaves extracts.

Conc. (µg/ml)	Pet. ether fraction	methylene chloride fraction	Ethyl acetate fraction	Methanol extract	Butanol fraction
50	1.78	24.21	32.90	18.84	30.35
100	1.91	52.58	68.37	38.01	5853
200	10.54	73.35	80.19	72.20	76.61

Antimicrobial activity :

The antimicrobial activities of *M. longifolia* methanolic extract are shown in Table (5).This extract did not exhibit any antimicrobial activity with *A. flavus* with any concentrations, while, the same extract with the different concentrations gave antimicrobial activity against other microbes i.e *Staph. aureus, B. cereus, E. coli, P. aeruginosa and S. cerevisiae* .

Table 5. Antimicrobial activities of the methanolic extract of *M. longifolia:*

Microorganism	Diameter of the inhibition zone (mm)		
	30 µl	60 µl	100 µl
Staph. aureus	11.0	18.0	20.0
B. cereus	18.0	19.0	21.0
E. coli	20.0	22.0	25.0
P. aeruginosa	14.0	16.0	19.0
S. cerevisiae	0.00	0.00	19.0
A. flavus	0.00	0.00	0.00

The effect of *M. longifolia* methanolic extract ranged between 11 to 20 mm and 18 to 21 mm for *Staph. aureus* and *B. cereus*, respectively. While, the inhibition zone was fluctuated between 20.0 to 25.0 mm and 14 to 19 mm in the case of *E. coli* and *P. aeruginosa*, respectively.

This plant extract was considered sensitive agent against *Staph. aureus, B. cereus, E. coli and P. aeruginosa.*

The strongest activity was recorded for *E. coli* with 22 and 25 mm inhibition zone at concentrations 60 and 100 µl. While moderate activity was observed for *B. cereus* (18,19 and 21mm) at 30, 60 and 100 µl. Both *P. aeruginosa and S. cerevisiae* gave the same inhibition zone 19 and 19 mm at 100 µl, respectively. While, *Staph. aureus* gave inhibition zone 18 and 20 mm at 60 and 100 µl, respectively.

The lowest antimicrobial activity was observed in *Staph. aureus* (11 mm) at 30 µl . Also, *P. aeruginosa* gave inhibition zone 14 and 16 mm at levels of 30 and 60 µl, respectively.

Hajlaoui *et al*., (2009) evaluated that antibacterial effects of *M. longifolia* methanol extract at 300 µg remained inactive against 10 microorganisms including *Staph. aureus, B. cereus, E. coli* and *P. aeruginosa* .

Mothana *et al*., (2010) reported that the methanolic extract of *M. longifolia* at 200 µg was not effective against

E. coli and *P. aeruginosa*, but was effective against *Staph. aureus* (11 mm).

Antimicrobial activity in plants happens due to phenolic compounds as well as changes caused in the cell membrane and permeability (Carson *et al.*, 2006).

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مضادات الاكسدة والانشطة البيولوجية لمستخلصات نبات النعناع طويل الاوراق

رمضان احمد حسن سامي طلعت ابو طالب - حسان بركات حامد و مصطفى شرف الدين

قسم الكيمياء - كلية الزراعة – جامعة المنصورة – المنصورة - مصر

تعتبر نباتات العائلة الشفوية من اهم النباتات التي تحتوى على الكثير من المركبات الفعالة مثل التربينات والمركبات الفينولية. والنعناع طويل الاوراق *Mentha* (*longifolia*) هو واحد من جنس النعناع الذي ينتمى الى هذه العائلة و يضم ٢٥ إلى ٣٠ نوعا. تم استخلاص المركبات الفعالة من الاوراق الجافة لهذا النبات بالميثانول عن طريق الاستخلاص التجزيئي لمستخلص الميثانول بمذيبات متدرجة القطبية وهى الاثير البترولي وكلوريد الميثيلين وخلات الايثايل والبيوتانول . امكن الحصول على اربعة مستخلصات تجزيئية لكل من هذه المذيبات. تم تقدير المحتوى من الفينولات الكلية (TPC) والفلافونيدات الكلية (TFC) ودراسة نشاط مضادات الأكسدة باستخدام طريقة DPPH في جميع المستخلصات. وكذلك تم تقدير النشاط المضاد للميكروبات فى المستخلص الميثانولي ضد اثنين من البكتيريا الموجبة لجرام ، واثنان من البكتيريا السالبة لجرام و واحد من كل من الخميرة والفطريات. أظهرت النتائج أن أعلى تركيز لـ TPC كان فى المستخلص التجزيئي لخلات الايثايل بقيمة ٢٢٨ر٣٦ ملجم GAE / g وزن جاف من المستخلص التجزيئي بينما لوحظ أن أقل تركيز كان فى الاثير البترولى ١٥٤ر٥٦ مجم GAE / جم وزن جاف. فى حين تراوحت قيم ال TFC بين ٢٩٣ر٨٦ و ١٥٣ر٣٩ ملجم QE / g وزن جاف فى المستخلص التجزيئي لكل من الاثير البترولي والبيوتانول على التوالي. من ناحية أخرى تم العثور على أعلى نشاط مضاد للأكسدة في المستخلص التجزيئي لخلات الايثايل حيث كانت اقل قيمة لل IC_{50}= ١٠٧ر٠١ ميكروجرام / مل.