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Antioxidant and Antimicrobial Activities of *Melissa officinalis L.* (Lemon Balm) Extracts

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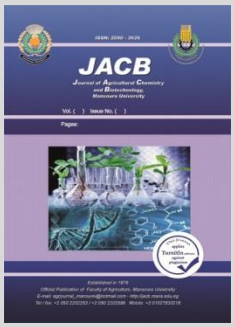

ABSTRACT

Family *lamiaceae* is an important plant family, its includes 236 genera and 250 spicies in which *Melissa officinalis* (M. *officinalis*) is the most common one Plants of this genus contain many chemical compounds like essential oils, terepenes, flavonoids, glucasinolates, anthocyanins and alkaloids. Also it exhibited different biological activities as antioxidant and antibacterial. Methanolic extract of plant was prepared where total phenolic content (TPC) and total flavonoid content (TFC) were estimated by colorimetrically. Antioxidant activity was estimated by DPPH radical scavenging activity. Also, six microbial species were used to estimate the antimicrobial activity of *M. officinalis* .Total phenolic and flavonoids of *M. officinalis* ethyl acetate extract are 143.50 mg GAE/g (dw) and 124.96 mg QE/g dw, respectively. These results confirm the antioxidant effect of the plant sample under study.The effect of *M. officinalis* methanolic extract on *Staphylococcus aureus* ranged between 9.0 and 14 mm with concentration varied between 30 and 100 µl, respectively, while, *E. Coli* inhibition zone was fluctuated between 10.0 and 11.0 mm for the same concentration. This plant extract was considered sensitive agent against *Staphylococcus aureus* and *E. Coli* . Moreover, the stronger effect of *M. officinalis* extracts was detected on *Saccharomyces cerevisiae* with a diameter of 16.0 mm when a concentration of 100 µl was used

Keywords: Antimicrobial; antioxidant; DPPH; Lamiaceae; total polyphenols, Flavonoids .

INTRODUCTION

Lemon balm (*Melissa officinalis*) is one of the most used as medicinal plant in Asia, Europe . The common name of this plant comes from southern Europe, Asia . The Mediterranean region. This plant is well known as a herbal tea for its aromatic, digestive and antispasmodic and sedative properties. The leaves issue a special aromatic lemon odour bruised (Encalada *et al*., 2011).

Many uses of the plant such as food additives, in cosmetic industries as ornamental plant, in medicine and pharmacology, phyto-pathology and for food preservation as an antimicrobial and antioxidant compound, antibacterial and stimulator agent for the immune system as reported (Sadraei *et al*. 2003). Moreover, it is commonly used for its antioxidant, antimicrobial, anticancer, anti-Herpes and anti-viral, anti- Alzheimer , anti-diabetic and anti-inflammatory Chung *et al*. (2010).

It was also reported that *M. officinalis* contains substances inhibiting protein biosynthesis in cancer cells (Beloued, 2009).

These biological activities have been attributed to the essential oil flavonoids and phenolic acids such as rosmarinic and caffeic acids, phenylpropanoid heteroside and Triterpene (Mencherini *et al*., 2007).

Various medicinal properties may be due to major constituents. Rosemaric (a derivative of coffee acid) is the most bountiful compound of the *M. officinalis* leaves extract, which is known to have antiviral and antioxidant activity (Koch Heitzmann and Schultze, 1984), while the essential oil has antibacterial, antifungal and antihistaminic activities (Burt, 2004).

Essential oils are becoming common as natural antimicrobial factors to be used for food keeping (Pazos *et al*., 2008).

M. officinalis is an aromatic herb and has amount of natural antioxidants. Antioxidant compounds can disrupt and scavenge the free radicals. Further studies suggested that methanolic extract at great concentration caused inhibition of lipid peroxidation phenolic compounds of *M. officinalis* proved antioxidant activity. Investigators indicated that extracts of *M. officinalis* have antioxidant activity due to the high portion of phenolic acids (Zandi and Ahmadi 2000).

The aim of the present study was to determine poly phenols and flavonoids of *M. officinalis* four extracts as well for investigate the antioxidant and antibacterial activities of *the plant* methanolic extract.

MATERIAL AND METHODS

1-Plant material :

Fresh leaves of *M. officinalis* were collected 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 experimental use,

2- Plant extracts :

The dried powder leaves were soaked in appropriate volume of pure methanol and kept at room temperature (25°C) over night.

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The extracts were then filtered and the residues were re-extracted twice by soaking in methanol. The combined extracts were evaporated under vacuum pressure using rotary evaporator. The extracts were kept in sterilized glass under refrigerated conditions till further use.

3- Fractionation of M. officinalis methanolic extract :

Methanol extract of plant leaves under study was fractionated by successive extraction using 4 solvents have increasing polarity i.e pet ether, CH_2Cl_2, $C_2H_5OCOCH_3$ and C_4H_9OH. The extraction process was three times for each solvent, then the solvents were removed by evaporation using rotary evaporator. Obtained fractions were kept in refrigerator tell use.

4- Polyphenols:

Polyphenols of plant extracts were estimated as indicated by **Singleton *et al 1999***. Gallic acid was chosen as a reference with a concentration (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 indicator and 1 mL (7% wt /v) Na_2CO_3. Mixture was incubated for 90 mins. at Labe condition, absorbance was measured at 765 nm and polyphenols were expressed as mg gallic / g dw extract.

5. Total flavonoids content :

Total flavonoids content of *M. officinalis* extracts was estimated by $ALCL_3$ colorimetric method as mentioned by *Lin and Tang (2007)*. 1.o ml of each extract was added to 0.1 mL of (10% wt /v) $ALCL_3$, 0.1 mL of (1 M KCH_2COOH) and 2.8 mL of dist. H_2O. the mixture was incubated for 40 mins. at Labe condition, the absorbance of samples color were measured at 415 nm. Quercetin (QE) was used as a reference between 0.005 to 0.1 mg/mL and the flavonoids were expressed as mg (QE) / g dw extract.

6- Antioxidant Activity Assay.

1 DPPH free-radical scavenging activity :

The free-radical scavenging activity was measured by the 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 min at room temperature, the absorbance was measured at 517 nm using UV–Vis spectrophotometer. High absorbance of the reaction mixture indicated low free radical scavenging activity. Butylated hydroxyl toluene (BHT) was used as positive control. Inhibition of free radical by DPPH was calculated as follows:

Antiradical activity (%) = (A control – A sample)/A control×100.

Where : A control = Absorbance of control
A sample = Absorbance of sample

The IC50 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 triplicate and the results are recorded as average values.

7- 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 0.2µm, 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 were: *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 (0.09% NaCl) was added to each slants. The antimicrobial activity was determined by 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.officinalis* 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 polyphenol Content (TPC) :

Total polyphenols are considered a secondary plant metabolites and represent an important part of human and animal diets.Flavonoids also are a large group of phenolic compounds consists of anthocyanin, flavonols and flavanols.

Table (1) showed total polyphenols content (as mg GAE/ g) of M. officinalis leaves extracts . These results demonstrate the presence of natural antioxidant phenolic compounds in all extracts.

A high content of total phenolic for ethyl acetate extract (143.50 mg GAE/gdw) was observed in comparison with other extracts. While, pet. ether extract showed to have the lowest value of total polyphenols (32.44 mg GAE/g dw).

The present data in Table (1) showed that methanol extract gave 71.02 mg GAE/ g dw for total phenolics. . These results were lower than those of other workers. For instance, Moradi *et al* 2016 gave average value of 227.6 mg GAE/ g dw of *M. officinalis* methanol extract. Some literature studies indicated that the plants belonging to the *Lamiaceae* family have TPC ranged between 61-137 mg GAE/g dw (Velickovic si colab., 2011), our results lies in this range. On the opposite trend , these results agreed with those of Tusevski *et al* (2014), who gave average value of 70.86 mg/g dw for TPC of *M. officinalis* leaves methanolic extract.

It seems that total phenolic content (TPC) depended on the cultivation region i. e methanol extract obtained from the aerial parts growing in Romania had a TPC of 22 mg GAE/g extract . But methanolic extract of the same herb from Bulgaria a TPC of 48.86 mg GAE/100g dw was detected (Atanassova *et al*, 2011).

Also the amount of polyphenols may be affected by some factors, such as type of solvent, method of drying, plant species and ripening stage . (Negro *et al*., 2003)

Table 1. Total polyphenols in *M. officinalis* extracts

Extracts	Polyphenols (mg GAE/g dw extract)
Methanol	71.02
Methylene chloride	41.6
Ethyl acetate	143.50
Butanol	93.88
Pet. ether	32.44

2. Total flavonoid Content (TFC) :

Total flavonoids content as shown in Table (2) are ranged from 124.96 to 45.44 mg QE/g dw of ethyl acetate and pet.ether extracts, respectively. However, Butanol ,methanol and methylene chloride extracts have medium values of 84.96, 72.38 and 59.76 mg QE/g for total flavonoids, respectively.previous studies of *M. officinalis* alcoholic extract have indicated that the TFC were 12.5±2.11 mg/g (milligram of rutin equivalents of dw) (Moradi *et al*., 2016). However, Tusevski *et al* (2014) gave average value of 45.71 mg CE/g dw for TFC extracted from *M. officinalis* leaves using methanol. These value is lower than those of the present results (72.38 mgQE/ g dw). Flavonoids has a positive effects on human health, which are manifested through its anticarcinogenic, antibacterial, immune-stimulating, anti-virus and the anti-inflammatory properties (Havsteen, 2002.). The benefit of fruits and vegetables consumption is largely attributable to the positive effects of flavonoids (Howard *et al*., 1997).

Table 2. Total flavonoids in *Melissa officinalis* extracts

Extracts	Flavonoid (mg QUE/g dw extract)
Methanol	72.38
methylene chloride	59.76
Ethyl acetate	124.96
Butanol	84.96
Pet. ether	45.44

3-DPPH radical-scavenging activity of the *M. officinalis* extract :

The free radical scavenging activities of *M.officinalis* were determined and the results are summarized in Table (3).

The antioxidant capacity is described quantitatively by the concentration of antioxidant required to scavenge 50% of DPPH•, which is referred as IC50.

In our study, DPPH IC_{50} value was obtained at 125.72 µg/ml for methanol extract.

Our results are higher than these of Esfahlan *et al*. (2015) who reported that the DPPH IC50 of ethanolic extract of *M. officinalis* was 2.9 µg/ml.

In another study, Moradi *et al*. (2016), found IC50 values for *M. officinalis* ethanol extract was 16.8± 1.41 µg/ml , this data are disagreed with our results (125.72 µg/ml).

Other coworkers evaluated the antioxidant activity of ethanolic extract of *M. officinalis* using DPPH method. They mentioned average value of 202.7 µg/ml for IC50 (Koksal *et al*, 2011). These results is higher than those found in the present study for IC50 methanol extract of the *M. officinalis* plant leaves under investigation (125.72 µg/ml).

Table 3 . Free radical scavenging activity of *M. officinalis* represented by IC50 (µg/ml) of five extracts as follows:

Extracts	IC50 (µg/ml)
Methanol	125.72
methylene chloride	146.67
Ethyl acetate	83.95
Butanol	94.58
Pet. ether	905.79

1 - inhibition %of antioxidant activity (AOA) :

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

The inhibition percent was calculated from the following equation:

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

The obtained data showed that using of the concentration of 200 µg/ml of different extraction of *M. officinalis* during the determination of AOA% was more effective than the others. Ethyl acetate extract had the higher value of inhibition percent followed by the extracts of butanol , methanol, methylene chloride and finally with pet. ether extract, with the values of 89.91,88.72, 83.45, 62.01 and 41.24 %, respectively.

These results were higher than those found by Albayrak *et al* (2013) who mentioned AOA 40 % for *M. officinalis* methanol extract at 250 µg/ml .

Table 4. percent (%)inhibition of antioxidant activity (AOA) induced by *M. officinalis* leaves extracts.

Conc.	Methanol extract	methylene chloride extract	Ethyl acetate extract	Butanol extract	Pet. Ether extract
50	22.85	25.66	89.02	44.43	11.79
100	30.53	42.13	89.31	77.74	22.62
200	83.45	62.01	89.91	88.72	41.24

4- antimicrobial activity

The antimicrobial activities of the methanolic extracts of *M. officinalis* are shown in Table (5).

Methanolic extract of *M. officinalis* did not exhibit any antibacterial activity against *Bacillus cereus, Pseudomonas aeruginosa and Aspergillus flavus* with any concentrations, while, the same extract in the same concentrations gave antibacterial activity against *Staphylococcus aureus and Escherichia coli.*

The effect of *M. officinalis* methanol extract on *Staphylococcus aureus* (Table 5) was ranged between 9.0 to 14 mm, while, the inhibition zone was fluctuated between 10.0 to 11.0 mm in the case of *E. Coli*. This plant extract was considered sensitive agent against *Staphylococcus aureus* and *E. Coli* .

The highest effect of *M. officinalis* extracts on *Saccharomyces cerevisiae* was 16.0 mm with a concentration 100 µl .

Table 5. Antimicrobial activities of the methanolic extracts of *M. officinalis* (diameter of the inhibition zone measured in mm)

Microorganism	Diameter of the inhibition zone(mm)		
	30 µl	60 µl	100 µl
Staphylococcus aureus	09.0	12.0	14.0
Bacillus cereus	0.00	0.00	0.00
Escherichia coli	10.0	10.0	11.0
Pseudomonas aeruginosa	0.00	0.00	0.00
Saccharomyces cerevisiae	0.00	0.00	16.0
Aspergillus flavus	0.00	0.00	0.00

The strongest activity was recorded for *Saccharomyces cerevisiae* with 16 mm zone of inhibition at 100 µl concentration . while moderate activity was recorded for *Staphylococcus aureus* (12 and 14 mm zone of inhibition) at 60 and 100 µl concentration, respectively. The lowest antimicrobial activity was observed in *Staphylococcus aureus* (9 mm) in 30 µl and *E. Coli* (10, 10 and 11 mm) in 30,60 and 100 µl, respectively.

In another study, Albayrak1 et al 2013, evaluated that antibacterial effects of M. officinalis methanol extract against Bacillus cereus and Pseudomonas aeruginosa with 12 and 8 mm zone of inhibition at 50 mg/mL concentration, respectively. this results disagreed with our results.

Similar to our results, Korcan *et al* 2018 showed that the methanol extract of *M. officinalis* exhibited antimicrobial activity against *Escherichia coli* with 10 mm zone inhibition at 50 µI concentration. On other hand, they study effect of the same extract at 100 µI on *Bacillus cereus* and *Escherichia coli* with 19 and 14 mm zone inhibition, respectively.

CONCLUSION

The extracts of M. *officinalis* contain a considerable amount of phenolic compounds and showed strong total antioxidant activities and DPPH radical scavenging activities when compared to standards such as BHT.

The results of this study show that the methanolic extracts of *M. officinalis* can be used as natural source in food and pharmaceutical industry for its strong activities as antimicrobial and antioxidant agents.

Also, it can be used in stabilizing food against oxidative deterioration. However, in vivo studies are needed to confirm the health-promoting potential of these plants.

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الانشطة المضادة للميكروبات والاكسدة فى مستخلصات تبات المليسة المخزنية (بلسم الليمون)

رمضان احمد حسن ، سامى طلعت ابو طالب ، حسان بركات حامد ومصطفى شرف الدين

قسم الكيمياء - كلية الزراعة – جامعة المنصورة

فى هذا البحث تم استخلاص المركبات الفعالة فى الاوراق الجافة لنبات المليسة المخزنية عن طريق الاستخلاص المتتابع بمذيبات الميثانول والاثير البترولى وكلوريد الميثيلين وخلات الايثايل والبيوتانول. تم تقدير المحتوى من الفينولات الكلية والفلافونيدات الكلية فى جميع المستخلصات وكان اعلى محتوى من الفينولات الكلية والفلافونيدات الكلية فى مستخلص خلات الايثايل بقيم ٥ر١٤٣ملج GAE/ جم و ٩٦و١٢٤ QUE/جم على التوالى . كما تم دراسة تاثير تثبيط المستخلص الميثانولى على نشاط الميكروبات (بكتريا – فطر – خميره) وكانت نسبة تثبيط المستخلص الميثانولى عند تركيزات من ٣٠ -١٠٠ ميكرولتر على بكتريا *Staphylococcus aureus* تتراوح بين ٩ – ١٤ مم بينما كانت نسبة التثبيط بالنسبة ل *E. Coli*. تتراوح بين ١٠ -١١ مم عند نفس التركيزات . فى حين ان اقوى تاثير للمستخلص على الخميرة ممثلة فى *Saccharomyces cerevisiae* بمقدار ١٦ مم عند تركيز ١٠٠ميكرولتر بينما لم يظهر المستخلص اى تاثير على فطر *Aspergillus flavus*.مماسبق يتضح ان لهذا النبات تحت الدراسة اهمية خاصة كمكملات اغذية وفى صناعة مواد التجميل وفى صناعة الادوية وفى حفظ الاغذية كمضاد للميكروبات ومضاد للاكسدة.