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Chemical and Biological Evaluation of the Essential Oil of Egyptian Moldavian Balm

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Abstract: The essential oil of *Dracocephalum moldavica* L. growing in Egypt was analyzed by TLC, GC-MS, HPLC and NMR. More than 90 components were detected. Of which 44, comprising 97.18% of the total oil were identified. The principal constituents were gerneyl acetate (24.93%), general (23.67%), geraniol (14.96%), nerol (11.0%), neryl acetate (5%), neral (3.3%) and linalool (1.38%). The antimicrobial potential of the oil against six bacterial and four fungal strains were investigated. *D. moldavica* essential oil exhibited a significant high antibacterial and antifungal activity in comparison to positive reference standards (chloramphinecol). The minimum inhibitory concentration (MIC) values were about 0.07 mg/mL and 0.080 mg/mL for tested bacterial and fungal strains, respectively. There was responsible agreement between MICs and clear zones of inhibition against tested bacterial strains. Inhibition zones of bacterial growth in the Bioautograms had R*f*s = 0.1, 0.30, 0.42, 0.54 and 0.73. These active components were identified by GC-MS after separated with preparative TLC as geraniol and nerol; gernyl acetate; general; neral; neryl actetate and methyl nerolate, respectively. Antioxidant activity was also tested, the oil showing a moderate antioxidant than that of α-tecopherol, BHT and BHA. Use of *D. moldavica* essential oil can constitute a power tool in control of pathogen microbial in food and pharmaceutical industrial.

Key words: *Dracocephalum moldavica* · Moldavian balm · Antimicrobial activity · Essential oil · Bioautography method · GC-MS

INTRODUCTION

Moldavian balm (*Dracocephalum moldavica L.,* syn. Moldavin dragonhead) is an annual herbaceous aromatic plant belonging to the family Lamiacece (Labiatae). It is native to central Asia and in eastern and central Europe [1]. Plants of the genus *Dracocephalum* is reported to use as a food ingredients, as a tea and as a herbal drug in folk medicine as a general tonic and for treatment of stomach, kidney and liver disorders, headache and congestion [2, 3]. The essential oils of *D. moldavica* have been investigated previously in Hungarian, Iran, Magnolia and Finland [4, 5]. The oil was characterized by a high containing oxygenated acyclic monoterpenes, having a ketone, aldehyade, and alcoholic function groups, which in dependent on plant origin. Recently, *D. moldavica* has introduced to Egypt, so fewer investigation were conducted on their plants [6, 7].

The antimicrobial properties of essential oils were stated long ago (since antiquity), and in past decade intensive studies were carried out indicting the possibility of their use in the control microbial pathogens such as *Escherichia* and *Salmonella* species contributing reduction in the employment of antibiotics [8-11]. Acquisition of antimicrobial resistance by food pathogens will compromise human drug treatments [12]. In addition, the use of essential oils is important not only in preservation of food but also in control of human and plant diseases of microbial origin [13]. However, essential oil and their constituents have been used extensively as flavor ingredients in a wide variety of food, beverage and confectionery products. Many such compounds are classified as generally recognized as safe [14].

Although essential oil of *D. moldavica* has been investigated for their chemical composition, a little is known about the possible the antioxidant and notably antibacterial and antifungal efficacy in their essential oil against diverse microorganisms. Therefore, the present study was conducted to evaluate the antioxidant, antibacterial and antifungal properties of essential oil Maldivian palm and identify the chemical compounds responsible for antimicrobial activity in their plant, which has not been done previously.

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MATERIALS AND METHODS

Plant Material and Preparation of the Essential Oil: Air dried herbs of *D. moldavica* L (100 g) subjected to hydrodistillated in a Clevenger apparatus for 2 h. The yield of the producing pale yellow oil was found to be 0.21%.

Gas Chromatography/Mass Spectrometry (GC/MS) Analysis: The hydrodistillated essential oil was analyzed on GC/MS system containing: HP5890 Series II Gas Chromatograph, HP 5972 Mass Detector and Agilent 6890 Series Auto-sampler (Agilent Technologies, USA). A Supelco MDN-5S capillary column (30 m x 0.25 mm i.d., film thickness 0.5 μm) was used with helium as the carrier gas at a flow rate of 1.0 ml/min. GC oven temperature was programmed at initial temperature of 40°C for 5 min, then heated up to 140°C at 5°C /min and held at 140°C for 5 min, then heated to 280°C at 9°C/min and held for 5 additional minutes. Injector and detector temperature were set at 250°C. Mass spectrometry was run in the electron impact mode (EI) at 70 eV. The identification of the chemical constituents of the oil was determined by their GC retention times, interpretation of their mass spectra and confirmed by mass spectral library search using the National Institute of Standards and Technology (NIST) database.

Antioxidant activity: The ability of essential oil to scavenge DPPH• free radical was assessed as described by Tagashira and Ohtake [15]. A 50 μL of different concentrations of oil (ranged 100-1000 μg/mL) was mixed with 25 ml of 100 mmol L^{-1} DPPH radical. The mixture were incubated in dark at 30° ± 1°C, the absorbance was recorded at 517 nm for 90 min, at 15 min intervals, against blank (pure methanol). The BHT, BHA and α-tocopherol (200 ppm) were used as reference standard. The radical scavenging activity of oil was calculated from a calibration curve. All tests were run in triplicate and averaged. The oil concentration providing 50% inhibition (IC_{50}) was calculated from graph plotted of inhibition percentage against oil concentration.

Bioassay for Antibacterial Activity

Preparation of bacterial cultures: Six different strains of bacteria were purchased from the Fisher-Co (Texas, USA). Four species of gram positive bacteria (*Bacillus subtilis, Bacillus cereus*, *Staphylococcus aureus*, *Micrococcus luteus)* and two gram negative bacteria *(Klebsiella pneumoniae, Serratia Marcescencs)* were used for the antimicrobial assay. Bacteria were sub-cultured on nutrient agar at 37°C prior to being grown in nutrient broth overnight. All overnight cultures were standardized by matching to the McFarland 0.5 turbidity standard using sterile saline to produce approximately 1.5 x 10^8 colony forming units (cfu) per mL.

Disc diffusion bioassay: The antibacterial activity of the essential oil was carried out by the disk diffusion assay as described by Gulluce *et al.* [16]. Muller Hinton agar (MHA) plates were swabbed with the respective broth cultures of the organisms (diluted to 0.5 McFarland Standard with saline). Sterile 6 millimeters diameter filter paper discs were impregnated with the appropriate equivalent amount of the essential oil dissolved in sterile dimethylsulfoxide (DMSO) at concentration 5 mg/ml. Negative controls were prepared using the same solvents employed to dissolve the essential oil. Chloramphenicol (Sigma Co) was used as positive reference standard to determine the sensitivity of one strain in each bacterial species tested. Plates were incubated overnight at 30°C. The antimicrobial activity was evaluated by measuring the inhibition zones expressed as millimeters of inhibition against the tested organism. The assays were performed in triplicate.

Minimal inhibitory concentration: The minimal inhibitory concentration (MIC) tests were determined according to the European Pharmacopoeia methods [17]. The MIC was defined as the lowest concentration of tested samples showing no visible bacterial growth after incubation time for 24 h at 37°C.

Thin layer chromatography (TLC) bioautographic methods: A TLC bioautographic method was used to detect active component in Maldivian palm oil. A series of three TLC plates (5 x 20 cm, silica gel G, Merck, Germany) were used, one plate for each bacterial strains in each experiment, a 5 μL each of the neat oil were applied on each plate. The plates were then developed with toluene-ethyl acetate (95: 5, v/v) and dried for complete removal of solvents. The dried TLC plates were then cut with a diamond knife into three strips each one representing one applied spot. One of the strips was sprayed with sulfuric acid /vanillin mixture for spot visualization and the second was used for the bioautography, while the third strip was used for elution of the active constituent. TLC bioautography was carried out using the previously

identified four bacteria strains (*B. subtilis, S.aureus and K. pneumoniae)* which showed the highest effectiveness in the diffusion disk assay. Suspensions of the bacteria in Difco-nutrient broth containing 0.5% agar and 0.1% iodonitrotetrazolum chloride media were individually distributed over the TLC plates (second strip); the plates were then incubated at 37°C for 48 h. Inhibition zones were shown as clear areas against a pink colored background. The third strip of the TLC plates were used for eluting the region of the plate that showed the inhibition zones for identifying the chemical composition of the active compound(s), TLC zones that showed inhibition activities were scraped from the plates, and eluted with dichloromethane and filtrated followed by nitrogen stream concentrated to a final volume of 100 μL and analyzed by GCMS as mentioned before.

Antifungal assay: Antifungal activity was carried out according to the Daw *et al.* [18], based on the measured of the inhibitions of linear growth mycelia of different mold strains (*Aspergillus niger, Penicillium notatum, Mucora heimalis* and *Fusarium oxysporum)* in potatoes dextrose broth medium.

RESULTS AND DISCUSSION

Forty-four compounds accounting for 97.18% of the *D. moldavica* essential oil (DMEO) were identified (Table 1). Of which 30 constituents representing 90% of the essential oil were characterized as oxygenated monoterpenes. Quantitatively (>1.0%), the most important were geranyl acetate (24.93%, total of E and Z form), neryl acetate (5%), genarial (23.67%, total of E and Z form), geraniol (14.96%), neral (11.99%), nerol (3.16%) and linalool (1.38%). Some interesting minor compounds (< 1.0->0.10%) were identified: eugenol (0.24%), 3-octanol 0.11%, 3-octanone (0.13%), 4-terpenol (0.51%) and nerolidol (0.23%). In additional, fifty two more compounds consisting mainly of thymol, isopulegol, germacrene and docosane have been identified as traces constituents (< 0.10%) of oil. In general, these results are in accordance with the results in other reports on essential oil of *D. moldavica* [4,7, 19]. These results emphasized that the oil of *D moldavica* consists mainly of oxygenated acyclic monoterpenes, e.g. geraniol, geranyl acetate, neral, genarial, linalool, and neryl acetate. However, as can be seen from Table 1 more compounds have been identified in our oil than those reported by some authors on essential oil of *D. moldavica* cultivated in Egypt [6, 7].

Table 1: Constituents of the essential oil of *Dracocephalum moldavica L.*

Compounds	Area %
5-methyl-3-Heptanone,	0.13
1-Octen-3-ol	0.50
6-methyl hept-5-ene-2-one	0.74
3 N-octanol	0.11
Benzeneacetaldhyde	0.17
Linalool	1.38
2,6-Dimethyl--heptan1,6-diene	0.17
Cyclohexane, 1-methyl-4-(1-methyle)	0.29
Cyclohexane, 1,3-dimethyl-2-(1-methyle)	0.44
Cyclohexane,1-ethyl	0.06
Spiro[2.5] octane	0.95
1- Pentadecyne	0.45
4-Terpineol	0.51
Nerol	3.16
Neral	11.99
Geraniol E	11.87
Geraniol Z	3.09
Methyl nerolate	4.86
Geranal Z	17.97
Geranal E	5.70
Geranate	0.63
Isopulegol	0.14
Undecanol-3	0.14
Neryl actetate Z	5.00
Cyclobuta [1,2:3,4] dicyclopentene	0.21
Gernyl acetate E	18.07
Gernyl acetate Z	6.86
Eugenol	0.24
Caryophyllene	0.58
1,8- Cineol	0.34
α-Caryophyllene	0.12
Elemene	0.28
α- Farnesene	0.15
Epizonarene	0.12
Spathulenol	0.28
Caryophyllene oixde	0.28
Carotol	0.25
Germacrene B	0.17
P-menth-1(7)-en-9-ol	0.29
Anisic acid	0.60
Cyclohexene, 1-methyl-4-(1-methyl)	0.12
Phytol	0.20
2,6,10-Dodecatrien-1-ol, 3,7,11-tri methyl acetate	0.27
3,7- dimethyl-2,6-oc Butanoic acid	0.12
Total identified compounds	97.18
Unknown	2.82

Percentage are the mean of three runs and were obtained from electronic intergration measurement using flame ionization detection (FID)
Gernyl acetate= gernyl acetate E and gernyl acetate Z = 24.96%
Geranal = Geranal E and Geranal Z= 23.67,
Geraniol = geraniol E and geraniol Z= 14.96
There are many compounds trace (<0.01%)

Antioxidant activity: Free radicals are involved in the process of lipid peroxidation, play a cardinal role in numerous chronic diseases such as cancer, coronary heart disease, atherosclerosis and ageing [20, 21]. Thus, the ability to scavenge free radicals is an important antioxidant property in order to minimize oxidative damage to living cells. Numerous synthetic free radicals are available for use in the assessment of this activity such as BHT and BHA [22]. BHT and BHA need to replace with

Table 2: Scavenging activity of *Dracocephalum moldavica L.* oils on DPPH●

Samples	Inhibition %[a]	IC_{50}[b] (µg/mlL)
D. moldavic	60.33±1.12	410.0
TBA	93.00±1.68	13.8
BHT	95.58±1.87	18.3
α-Tocopherol	91.54±1.95	16.0
LSD at level (P<0.01)	2.11	1.83

a: Percentage of antioxidant inhibition was calculated from following equation: $\%a = (A_{blank} - A_{sample} / A_{blank}) X 100$
Where A_{blank} = absorbance of methanolic DPPH
A_{sample} = absorbance of DPPH radical + samples
IC50[b]: Concentration (µg/ml) for a 50% inhibition was calculated from the plot of inhibition (%) against *Dracocephalum moldavica L. oils* concentration Tests were carried out in triplicate

natural antioxidant, however they were found to be toxic, responsible for liver damage, promoters of carcinogenesis and general consumer rejection of synthetic food additives [23]. Thus, it was considered important to screen the *D moldavica* essential oil as natural scavenger against free radical species.

The potency of *D moldavica* essential oil (DMEO) on the DPPH radicals is represented in Table 2. The oil exhibited a slight scavenging activity (IC_{50} 400 µg/ml) with comparable to that of positive controls: α-tocopherol, BHT and BHA (IC_{50} 16.0, 18.3 and 13.8 µg/ml, respectively), but not able active. Based upon the estimated IC_{50} values could be notes that the Moldavian palm essential oil was not as potent an electron donating agents as the positive controls. These mean that DPPH radical scavenging was not greatly induced by DHEO. This phenomenon is considered related to high contents of oxygenated acyclic monoterpenes in the oil, which had a generally lower antioxidant activity than phenolic monoterpenes [13, 24]. However, it seems that the DHEO contained several compounds are able to reduce the DPPH radical such as nerol, geraniol, terpinen-4-ol, eugenol and thymol. These components are known to possess antioxidant property due to ability to reduce free radical stability via electron or hydrogen -donating mechanisms [24-26].

Antibacterial activity: The antibacterial effect of the *D. moldavica* essential oil (DMEO) and chloramphinicol (CA) as a reference standard was assayed against four species of Gram-positive bacteria and two species of Gram-negative bacteria. The activity considered in this study was qualitatively and quantitatively assessed evaluating the presence on inhibition zone, zone diameter and MIC values (Table 3 and Fig. 1). The DMEO had a great potential of antibacterial activities against all bacterial strains tested when compared with that of CA. The maximal inhibition zones and MIC values for bacterial strains were in the ranged of 26-32 mm and 30-38 at 0.4 and 0.8 µg/dick, and 0.070 - 0.080 mg/mL of DMEO, respectively. Generally, the both Gram-positive and -negative bacteria seemed to be having the quite similar sensitive to *D. moldavica* essential oil. The inhibitory activity of the oil was found in concentration depended.

Table 3:Inhibition zone and minimum inhibition concentration of *Dracocephalum moldavica L* essential oil against the tested bacteria.

	Bacteria Strains					
Applied Doses mg/disk	*B. cereus*	*B. subtilis*	*K. pneumoniae*	*S. aureus*	*M. luteus*	*S.marcescencs*
0.2	22.00	23.00	24.00	25.00	29.00	28.00
0.4	26.00	27.00	27.00	31.00	32.00	32.00
0.6	27.00	30.00	28.00	27.00	33.00	35.00
0.8	28.00	32.00	33.00	38.00	34.00	38.00
MIC for the oil mg/mL	0.08	0.07	0.07	0.07	0.07	0.07
Chloroamphenicol 10 µg/disk	10.00	10.00	10.00	10.00	10.00	10.00
MIC for Chloroamphenicol mg/mL	0.02	0.02	0.02	0.02	0.02	0.02

Values represent the mean of three replicates and rebated three times
MIC: Minimum inhibition concentration, values given as mg/ml for samples and for chloramphenicol

Table 4: Inhibition % and minimum inhibition concentration of *Dracocephalum moldavica L.* essential oil against the tested fungal strains

	Fungus Inhibition %			
Applied doses mg/µl	*Aspergillus niger*	*Rhizopus stolonifer*	*Fusarium oxysporum*	*Mucor hemalis*
0.01	32.000	33.500	35.600	28.500
0.02	53.000	55.900	62.300	47.500
0.04	77.000	79.100	80.100	71.500
0.08	100.000	95.500	100.000	94.100
0.12	100.000	100.000	100.000	100.000
MIC for the oil mg/mL	0.075	0.085	0.078	0.080
Amphotericin B 10 µg/disk	35.000	30.000	30.000	35.000
MIC for Amphotericin B mg/mL	0.030	0.025	0.030	0.030

Values represent the mean of three replicates and rebated three times
MIC: Minimum inhibition concentration, values given as mg/ml for samples and for Amphotericin B

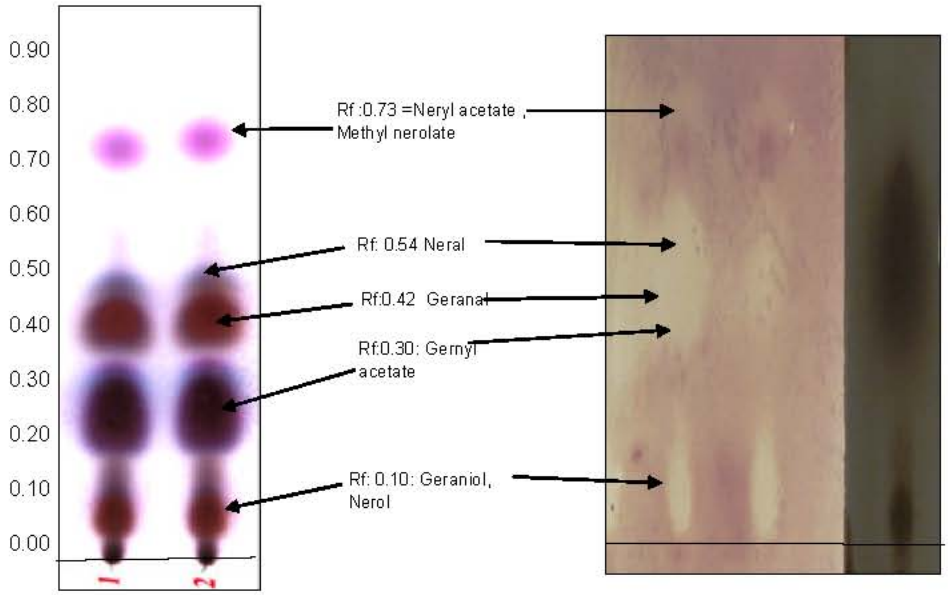

Fig. 1: Thin layer chromatogram (a) and bioautograpghy (b) of the antibacterial compounds of the of *Dracocephalum moldavica* L. essential oil

The active components of DHEO as antibacterial agents were assessed by bioautographic method. These procedure products four active inhibition zones (antibacterial components) had R_f values of 0.11, 0.30, 0.42, 0.54 and 0.73 on TLC. These active spots were characterized using GS-MS after separated with preparative TLC. These bioactive compounds towered their identification as geraniol and nerol (R_f=0.11), gernyl acetate (R_f=0.30), geranal (R_f=0.42), neral (R_f=0.54) and neryl acetate and methyl nerolate (R_f=0.73). Some of these compounds are previously reported to possess antibacterial activity [14].

Antifungal activity: With regard to antifungal activity, Table 4 shows the inhibitory effect of *D. moldavica* essential oil towered four fungal strains *A. niger, P. notatum, M. heimalis* and *F. oxysporum*. Moldavian palm oil at 0.01, 0.02, 0.04, 0.08, 0.12 mg/mL broth was able to inhibit the growth of the common spoilage fungus *A. niger* with 32, 53, 77, 100 and 100%, respectively. Also, the oil exhibited similar influence on *P. notatum, M. heimalis* and *F. oxysporu* growth, which the oil completely prevented these molds growth at concentrations of ≥ 0.08 mg/mL broth. In general, Moldavian palm oil showed a highly antifungal activity and completely inhibited the growth of all tested mold strains at concentration level were of ≥=0.08 mg/mL (expressed as MIC), and all fungus were susceptible in similar degree to the oil.

It appears that there is a relationship between the chemical constituents of Moldavian palm oil and antimicrobial activity. A large number of essential oil components have been found to have antimicrobial activity, many of these the structure identified as ketones and alcoholic and phenolics monoterpenes [11, 25, 18, 27]. Again, the oil containing a large quantity of geraniol, nerol, linalool geranial and neral as oxygenated compounds were demonstrated in bioautography assay to be a patents antimicrobial activity than that other constitutes in the oil. The presence of that component as main compounds could be explaining the higher antimicrobial activity found in their oil. That compounds are in fact responsible for the antimicrobial activity of many essential oils which contain them [14]. Citral, geraniol and nerol compounds exert antimicrobial activity by (1) impairing a variety of enzyme system, especially of those involved in the production of cellular energy and synthesis of structural components; (2) interfering with the phospholipids bilayer of the cell membranes causing increased permeability and loss of cellular constituents and/or (3) inactivation or destroying genetic material [14]. On the other hand, the oil contained some phenolics (eugenol) and alcoholic (terpinen-4-ol) terpeneoids which exhibited higher antifungal effect on mold growth and toxin production [25, 18]. Nychas [28] and Mundt *et al.* [29] reported that essential oil compounds had a high antifungal properties, which could denature the enzymes responsible for spore

germination or interfere with the amino acid involved in germination; interactions with membrane enzymes and proteins would cause an opposite flow of protons, affecting cellular activity or disturb genetic and interact with membrane proteins, causing a deformation in there structure and functionality.

This study shown that *D. moldavica* essential oils have significant antimicrobial activity against the growth of food poisoning and spoilage organisms such as *S. aureus, B. subtilis and P. aeruginosa*, organisms of fecal origin *S. fsecalis and the* spoilage fungus *A. niger*. Furthermore, the oil showed antioxidant activity. These results confirm the potential use of Moldavian palm essential oils in the food and pharmaceutical industries in the preservation of foodstuffs against bacterial and fungi. The oil could be used as antimicrobial supplement in the developing countries towards the development of new therapeutic agents. This oil is also able to incorporated into creams, lotions, drops, etc. which are applied externally on the body to treat diseases caused by molds such as *A. niger*. However further *in vivo* studies and clinical trials would be required to justify and uses Moldavian palm oil as an antimicrobial agent in topical or oral applications.

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