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Chemical Constituents and Biocidal Activity of the Essential Oil of *Mentha Spicata* L. Grown in Zagazig Region, Egypt

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Abstract: GC and GC/MS analysis of the essential oil prepared by hydrodistillation from aerial parts of spearmint, *Mentha spicata* L. showed the presence of sixteen compounds accounting 99.45% of the total oil composition. Fifteen components representing (98.69%) of the oil were identified. The major components were menthone (32.43%), 1.8- cineol (18.79%), cis- iso pulegone (16.65%), pulegone (10.01%), β- pinene (7.12%), a-cadinol (5.30%) and α- pinene (5.03%). The anti microbial of oil was determined by micro dilution method. The obtained result declared that the oil exhibited potential antimicrobial activity and a great acaricidal effect against spider mite, *Tetranychus urticae* Koch (Acari:Tetranychidae). After 24 hrs post treatment, the LC_{50} of *Mentha* oil were 2.82, 2.29 and 6.65% for larvae, protonymphs and adult females of *T. urticae*, respectively. Whereas, the LC_{50} values were 1.69, 1.49 and 2.31%, respectively after 48 hrs post treatment at concentrations 0.5, 2.0 and 4.0% of the oil. The essential oil showed a high repellency effect to adult female *T. utricae.* The oil shortened the oviposition period, longevity, life span, total number of eggs/female, no. of Eggs/ ♀/day of *T. urticae* as well as prolonged pronouncedly the incubation period as compared with control.

Key words: *Mentha spicata* L., *Lamiaceae* , Essential oil composition, GC/MS, Antimicrobial (MIC), *Tetranychus urticae*, Acaricidal activity.

INTRODUCTION

Since the discovery of pyrethroids, Phytochemicals biological activity have demonstrated a great utility as pharmaceuticals and pest-management agents since decades ago. Essential oils with potential and or their constituents extracted from herbs have been used as flavoring and fragrances in food, beverage and cosmetic industries. Also they have been recognized to repel insects for at least as long, and in recent years have been demonstrated to have both contact and fumigant toxicity to insect pests[1-3]. There is also growing evidence that certain essential oils are effective antibacterial and antifungal agents[4].

Mentha is a well-known genus belonging to family *Lamiaceae* shows a reputed medicinal and aromatic values. That genus includes about 30 species that grow in the temperate regions of Eurasia, Australia and South Africa[5]. *Mentha spicata* L.*(spearmint; Syn. Mentha Viridis)* is a herbaceous perennial rhizomatous. It produces pink or white flowers[6,7]. With high essential oil content. The oil possesses an antibacterial, antifungal and anticonvulsive action[8]. Previous studies on the volatile oil prepared from *M. spicata* revealed that the oil was rich in monoterpenes[9-15]. Yet ,no recent reports were published concerning the volatile oil of *Mentha spicata L.* growing in Zagazig region of Egypt as well as its biological potential.

The two-spotted spider mite, *Tetranychus urticae* Koch, is one of the most important pests of fruits, vegetable and ornamental plants and worldwide[16]. The mite has been reported to attack about 1200 species of plants[17] of which more than 150 are economically important[18]. The economic threat posed by these mites is constantly increasing because of the development of pesticide resistance and the resurgence of mite populations following the use of non-selective synthetic pesticides that eliminate natural enemies such as predaceous mites and spiders[19]. Spider mites have evolved resistance to more than 80 acaricides to date, and resistance has been reported from more than 60 countries[20]. Essential oils from aromatic plants provide potential alternative in the place of currently used insect pest control agents because they constitute a rich source of bioactive chemicals[21] and act in many way on various types of pest complex[22]. They are also selective to pests, have no or little harmful effects on non target organisms such as pollinators, natural enemies and rapid disappearance from the environment. Adding to this the fact, many of these substances are an exemption from pesticide registration in the USA[23-25]. Using this bio-rational approach has not

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demonstrated arthropod resistance and has not been as successful at controlling pest numbers but has been an important factor in effecting behavior such as oviposition[26]. Because of that, essential oils from aromatic plants have been recently researched as potential miticides where *T. urticae* become a worldwide and a significant greenhouse and field pest[27-32].

Only few reports were published concerning the volatile oil prepared from the leaves of *Mentha spicata* L. grown in Giza, Egypt[10]. Thus, the present investigation is considered the first report on the physical characters, chemical composition, acaricidal and antimicrobial activity of the essential oil prepared from the whole aerial parts of *M. spicata* L. grown in Zagazig region of Egypt.

MATERIALS AND METHODS

Plant Material: The fresh whole aerial parts of *Mentha spicata* L. Var. *viridis,* family lamiaceae were collected in July, 2008, from the plants cultivated in the experimental station of Faculty of Pharmacy, Zagazig University, Zagazig, Egypt. A voucher specimen has been deposited in the herbarium of Pharmacognosy Dept., Faculty of Pharmacy, Zagazig University, Egypt.

Preparation and Analysis of Essential Oil: The oil was obtained from the fresh minced herbs by hydrodistillation for 6 hours in a clevenger type apparatus[33]The oil was dried over anhydrous sodium sulfate (to give 0.69%v/w) and was kept in a freezer until further analysis. Qualitative and quantitative analysis of the oil were done on 2 ml sample oil solutions "approximately 1% oil in ether" using GC and GC/MS. The GC analysis of the oil was done on Shimadzu GC/ MS- QP5050A equipped with a split-less injector, attached to an DB1 fused silica column (30 m x 0.53 mm; film thickness 1.5 mm), fitted with FID, under the following conditions; helium as carrier gas at 1 ml/min; injector temperature was 280°C; detector temperature 300°C; column temperature programme: 40°C for 1 min., ramp 7.5°C/1`min. to 150°C (5min) at 10°C/ min to 250 (2 min) at 5°C/ min to 280 (2 min) at 3.5°C/ min. The total run time for GC was 47 min. The relative amount (%) of individual compounds of the oil is expressed as percent peak area relative to total peak area from the GC/ FID analyses of the whole extracts .For GC-MS analysis; GC conditions as mentioned above, and the capillary column was directly coupled with a Shimadzu GC/MS-QP5050A.EI-MS were recorded at 70 ev, Full scan type, Mass Rang 40-400, scan time: 5 sec`. Identification of the components was performed by aid of the computer library search (NIST-Mass lab software package, fissions), comparison of mass spectra with literature data and by comparison of their retention times and mass fragmentation patterns with those of the library database [Wiley (Wiley Int. USA)] [34-35].

Screening of Antimicrobial Activity:

Escherichia coli, Staphylococcus epidermidis, Staphylococcus aureus, Micrococcus luteus, Corynebacterium jeikeium and *Candida albicans* were subjected *in vitro* to susceptibility testing on Mueller-Hinton agar medium by agar dilution method[36]. Each of these strains was tested against *M. spicata* L. essential oil at concentrations of 100, 50, 25, 12.50, 6.25, 3.12, 1.56 µl/ml. For each strain, minimum inhibitory concentrations **(**MIC) of the essential oil was determined by the standard agar dilution assay according to guidelines of the National Committee for Clinical Laboratory Standards (NCCLS 2003) with Mueller-Hinton agar (Difco Laboratories, Detroit, Mich., USA). *Mentha spicata* L. essential oil-containing agar plates were incubated with 5 µl of an inoculum corresponding to about 10^4 CFU per spot and were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration that prevented visible growth of bacteria.

Mite Culture: For establishing colonies of *Tetranychus urticae* in the laboratory, individuals of adult females of the mite were collected from castor been, leaves at Zagazig region, Egyppt. Adult females of the mite were reared on sweet potato, *Iponoea batatas* cuttings holding about 4 leaves each. Those cuttings were placed in water in 250 ml. bottles, after being thoroughly washed under running water and then wipped out with a piece of cotton wool to remove dirt and possible eggs. Two sweet potato cuttings were used for each colony. Sweet potato cuttings were changed twice a week before each colony was transferred. Mite colonies were kept isolated in separate cabinets (160 x 150 x 100 cm) under constant temperature (28±2°C) and relative humidity (65±5%). The side and the top of the cabinet being made of muslin cloth held in position by wooden frame work. Fluorocent tubes (40 watt) were used to maintain continuous illumination inside the cabinet.

Acaricidal activity: Leaf spray method was used to determine acaricidal activity of *M. spicata* L essential oil against *T. urtica*e with a potter spray tower. Five adult females of the two-spotted spider mite *T. urtica*e were allowed to oviposit on sweet potato (*Ipomea potato*) leaf discs (1 inch in diameter) resting on wet cotton wool in a Petri-dish. They were removed after 24 hours, where 30-40 eggs per leaf disc

were laid. There were three leaf discs per Petri- dish and a minimum of four Petri -dishes per concentration. The leaf discs were sprayed with six concentrations (0.25, 0.5,1.0,2.0,4.0 and 8.0%) of *M. spicata* L. oil as emulsifying solution and the control mites were held using the potter tower. The Petri-dishes were stored at 28 ˚C ± 2˚C and approximately 70±5 % R.H. *T. urticae* larvae and protonymphs were reared from eggs laid on existed sweet potato leaves resting on wet cotton wool. Four sweet potato leaf discs (1-inch in diameter) were placed on wet cotton wool in a Petri-dish to each concentration. Each disc was considered as a replicate contained 20 larvae or protonymphs. The leaf-discs with larvae or protonymphs were sprayed with above concentration then placed under laboratory condition (28˚C±2˚C) and approximately 70±5 % R.H. This experiment was repeated using adult females of *T. urticae*. The number of live and dead mites of immature stages and adult female were assessed daily for 24 and 48 hrs, while eggs for 7 days. Mites were considered dead if their appendages did not move when prodded with a fine paintbrush. Mortality counts for eggs, immature stages and adult females were corrected using Probit analysis to determine the lethal concentration (LC_{50}) value, using the EPA, probit analysis program version 1.3.with their lower and upper confidence limits(CL,95%).

Repellency Effect: The repellency of *M. spicata* oil at 0.5, 2.0 and 4.0% against *T. urticae* was assessed. Leaf discs of sweet potato were placed in Petri-dishes lined with moist cotton wool. Half of each disc was painted with the proper concentration, while the other left untreated. Twenty females of *T. urticae* were placed of the midrib. Orientation of *T. urticae* females on treated or control half was recorded after 1,3,20 and 24 hours from the beginning of the experiment, also the number of eggs laid on the control versus the treated half was counted after 24 hours. The percentage of repellency value was calculated using the equation: D = (1-T/C) x 100 [37] where T and C represent the mean number of eggs oviposited per female of the treated and control test, respectively.

Biological Aspect: Adult females of the same age of *T. urticae* were placed individually on sweet-potato leaf-discs resting on wet cotton wool in a Petri-dish. The Petri-dishes were sprayed with the respective *M. spicata* at the LC_{50} levels by potter tower and twenty replicates were made. The different effects on the biological aspects were estimated. Analysis of variance (ANOVA) was carried out for the obtained data according to the method of [38].

RESULTS AND DISCUSSION

Chromatographic Analysis of Essential Oil of *Mentha Spicata* L.: The essential oil was prepared by hydro distillation [33]of fresh whole aerial parts to yield (0.69% v/w). Results of analysis of the essential oil of *Mentha spicata* L. is summarized and presented in (Fig. 1) and Table (1). The different chemical classes of the identified compounds are present in Table (2).

Valuable increase was noticed in the yield of volatile oil prepared from the plant under investigation is attributed to the different ecological conditions where the plant grows. The oil was light- green in color, lighter than water and exhibited characteristic aromatic, minty odor and its refractive index is (1.495).

GC/MS analysis of the oil led to identification of the majority of the components. Which where listed in Table (1), along with their quantitative data and their retention time. The identification of the compound was based on comparison of their mass spectra with those described in literature [10,34].From these results it could be concluded that fifteen compounds were identified representing (98.69%) of the total oil sample. The major components were found to be menthone (32.43%), 1.8- cineol (18.79%), cis- iso pulegone (16.65%), pulegone (10.01%), β- pinene (7.12%), α-cadinol (5.30%) and α- pinene (5.03%).

Grouping the constituents, the results indicated that, oxygenated compounds constituted (83.88%) Table (2), mainly attributed to monoterpenes (78.18%) and sesquiterpenes (5.7%). menthone (32.43%) constituted the highest percentage of the oil and as oxygenated monoterpene, while α- cadinol (5.3%) was the major oxygenated sesquiterpene. Furthermore, the oil contains (13.88%) terpenoids hydrocarbons, β- pinene and α-pinene (7.12% and 5.03%, respectively) representing the major monoterpenes hydrocarbons, while trans-caryophyllene (0.81%) is the major sesquiterpene of the oil. Essential oil of *Mentha spicata L.* grown in Zagazig, Egypt showing a characteristic aromatic and minty odor due to the presence of oxygenated compounds (83.88%), as well as menthone (32.43%). Qualitative, quantitative and composition variations noticed between the constituents of essential oil of *Mentha spicata L.* under investigation and those previously reported by [10], are attributed to climate, soil composition, plant organ, age and vegetative cycle stage [39,40]. So, in order to obtain essential oils of constant composition, they have to be extracted under the same conditions from the same organ of the plant which has been growing on the same soil, under the same climate and has been harvested in the same season.

Antimicrobial Activity: The antimicrobial results showed that *M. spicata* L. essential oil possessed antibacterial effect against all tested bacterial isolates with MIC values in the range of 3.12 µl/ml to 12.5 µl/ml (Table.3). *M. spicata* L. essential oil exhibited very strong antibacterial activity, in particularly against *Escherichia coli* strain. In addition, the tested oil showed significant antifungal activity, against *Candida albicans* (MIC, 6.25 µl/ml). Therefore, it is excellent for treatment of infections caused by these organisms, e.g. diarrhea and skin infections. The activity is confirmed with its high concentration of oxygenated terpenoids (83.88%). The oil is rich in oxygenated terpenoids, thus it was reported to show antimicrobial activity [41]. These results concluded that essential oil prepared from the plant under investigation had great potential of antimicrobial activity, in contrast with previously reported data [10].

Acaricidal Activity: 3.1.Toxicity to eggs, immature stages and adult females The toxicity results in Table 4. showed that immature stages of *T. urticae* were more susceptible to the action of essential oil of *M. spicata* L. than egg stage and adult female after 24 and 48 hrs of treatment. After 24 hrs post treatment, the LC_{50} values of *M. spicata* were found to be 2.29 %(95% confidence interval (CI) = 1.2-5.4), 2.82 %(95%CI = 1.6-6.4), and 6.65 %(95% CI = 3.3-7.9) for protonymphs, larvae and adult females of *T. urticae*, respectively. Whereas, the LC_{50} values were 1.49% (95% CI = 0.7-2.5), 1.69 % (95% CI = 0.8-2.8) and 2.31% (95% CI = 1.32-4.5), respectively after 48 hrs of treatment. After 7 day of inoculation period, the LC50 value of *M. spicata* L. for egg stage of *T. urticae* was 4.34% (95% CI =2.10-62.39). The immature stages are less likely to spin-down or aerially disperse than adult which may increase the exposure period. Therefore, immature stages of *T. urticae* were more susceptible to action of oil than adult stage [42]. Essential oil of *Mentha* are effective against some arthropod pests [43-45,1,2,3,46].The mode of action of essential oils on arthropods, is largely unknown, due to the complexity of the chemical constituents .There are fifteen major constituents in *M. spicata* L. essential oil as described in Tables (1,2), those oils may operate through more than one mode of action due to the diversity of terpenes and terpenoids in each plant extraction [47]. Pulegon which may be associated with their insecticidal activity may be acting as an acetyl cholinesterase inhibitor [48]. The mode of action for botanical oils and products containing essential oils including peppermint oil (Ecotrol®) cause convulsions in insects following ingestion or topical administration.They have recently been discovered to act as octopamine agonists in the American cockroach, *Periplaneta americana* (Linnaeus), which may explain their toxicity to insects but not to mammals [49]. Midgut microsomal monooxygenase activity of variegated cutworm, *Peridroma saucia* (Hubner) larvae was induced up to 45-fold by feeding on peppermint leaves compared with that of larvae fed an artificial diet [50]. The induction was apparently due to high concentration of certain monoterpenes such as menthone, α-pinene, and β- pinene in the peppermint leaves. These allelochemicals, when fed to cutworm larvae, all increased midgut aldrin epoxidase activity up to 24-fold and the cytochrome P450 content, 6-fold induction by peppermint leaves in midgut and other unspecified tissues was also observed in larvae of the alfalfa looper, *Autographa californica* (Peyer), and the cabbage looper[51,52].

The Repellency Tests: Results in Table (5) showed that adult females of *T. urticae* preferred the untreated section of the leaves to feed and deposit eggs. At the three concentrations of *M. spicata* L. essential oil (0.5, 2.0 and 4.0%) and after 24 hour of exposure, small percent of two mites were recorded on the treated section. The above concentrations were found to be highly repellent to adult females of *T. utricae*. Females of the mite showed an ovipostion preference for residue-free substrated where the mean number of eggs laid on the water treated control halves of the 50% residue leaf discs were higher than for the compounds treated halves (Table 5). Spearmint essential oil was found to be the second best repellent of the oils screened after cinnamon bark oil, with an exposure concentration of 45–180 ppm. [53]. *Mentha* oil inhibited the settling caused by their repellency and probing inhibitory, sucking inhibitory, and locomotion stimulatory activities against green peach aphid *Myzus persicae* [54]. *M. spicata* could be used to both repel and curb insect damage on stored kidney beans [55].Also, results showed that many plant essential oils proved to be a high repellent to *T. urticae* females [56-59].

Biological Effects: Data in Table 6 revealed the changes in some biological aspects of the two mite *T. urticae* after exposure to *M. spicata* L. essential oil at the level of LC_{50}. The essential oil of *M. spicata* highly significantly shortened the oviposition period, longevity, Life span, number of eggs/female, no. of Eggs/ ♀/day of *T. urticae* .While prolonged pronouncedly the incubation period as compared with control.. Common mint oils, including *M. spicata* oil, prevented egg hatching and provoked pupal malformation with the fly *Drosophila aurea* [60].Several authors showed that the biological aspects of *T. urticae* Koch were more affected by plant essential oils [61-63].

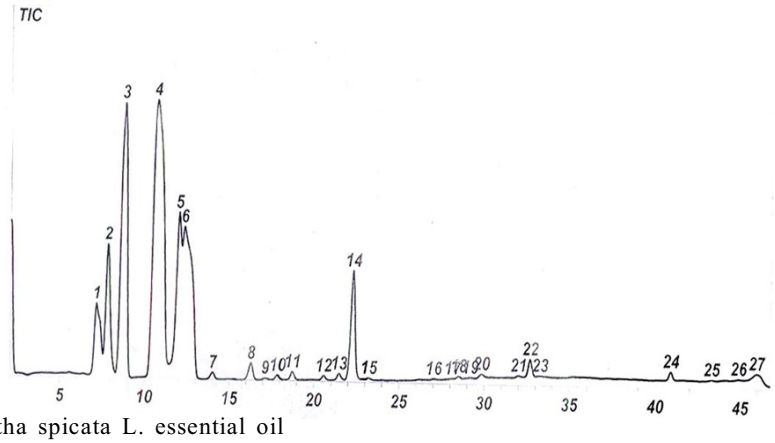

Fig .1: GC/ MS of Mentha spicata L. essential oil

Table 1: The GC/ MS analysis of the essential oil of Mentha spicata L.

	Compound	R_t	M^+	B.P	Major peaks	Area %
1	α- pinene	7.17	136	93	77, 39, 105, 67, 53	5.03
2	β- pinene	7.83	136	93	41, 69, 79, 53, 107	7.12
3	1.8- cineol	8.82	154	43	81, 108, 71, 139, 125	18.79
4	Menthone	10.7	154	41	112, 55, 139, 69, 97, 83	32.43
5	Pulegone	12.0	152	81	41, 152, 67, 95, 109, 137	10.01
6	Cis-iso pulegone	12.33	152	41	67, 108, 152, 109, 137, 53	16.65
7	Piperitenone	14.03	150	39	107, 150, 91, 135, 67	0.30
8	Trans-caryophyllene	16.3	204	41	69, 93, 133, 107, 161, 204	0.81
9	α- Humulene	17.15	204	41	93, 67, 79, 121, 161, 189	0.07
10	γ- cadinene	17.86	204	161	41, 105, 91, 119, 133, 204	0.17
11	δ- cadinene	18.7	204	161	41, 204, 91, 105, 119, 134	0.33
12	Caryophyllene oxide	20.6	220	41	79, 93, 177, 121	0.15
13	Cadinol	21.5	222	161	204,179,41, 119, 105, 135	0.25
14	α- cadinol	22.3	222	161	204, 43, 105, 134, 119, 77	5.30
15	Unknown	32.7	360	191	253,41,95,109,81,215,242	0.76
16	Lanost-8- ene	45.97	412	43	397, 275, 351, 255 , 231	0.93

R_t, Retention time; M^+, molecular ion peak; B.P, base peak.

Table 2: Essential oil composition of Mentha spicata L. with constituent categories

Constituent category	Relative area percentage
Monoterpenes hydrocarbons	12.5
Oxygen containing monoterpenes	78.18
Sesquiterpenes hydrocarbons	1.38
Oxygen containing sesquiterpenes	5.7
Others	1.69

Table 3: Antimicrobial activity (MICs) of Mentha spicata L. essential oil.

Microorganisms	MIC (µl/ml)
E. coli	3.12
Staphylococcus epidermidis	6.25
S. aureus	12.5
Micrococcus luteus	6.25
Corynebacterium jeikeium	6.25
Candida albicans	6.25

Table 4: Toxicity of Mentha spicata L essential oil to adult females of T. urticae individuals.

Treatments	LC_{50}	LC_{90}	Slope	Toxicity index	LC_{50}	LC_{90}	Relative potency LC_{50}	LC_{90}
				`After 24 hrs				
Adult female	6.65	59.02	1.35	34.90	26.04		1.0	1.0
Larval stage	2.82	15.37	1.73	81.20	95.76		2.36	3.84
Protonymphal stage	2.29	16.05	1.51	100	100		2.90	3.67
After 48 hrs								
Adult female	2.31	12.2	1.77	64.50	64.91		1.0	1.0
Larval stage	1.61	8.61	1.76	92.55	91.75		1.41	1.42
Protonymphal stage	1.49	7.90	1.76	100	100		1.54	1.54
Egg stage after 7 days of application	4.37	72.2	1.05					

Table 5: Distribution and repellency of adult females T. urticae on treated plant discs with essential oil of Mentha spicata L.

Concentrations (%)	*Tetranychus urticae*						
	Average% distribution of mites on treated leaf part(±SD) after				Average number of eggs/ after 24hrs		
	1h	3h	20h	24h	TC	Significantly	% Repellency
4.0	0 ± 0c	0.0±0.0d	6.7±0.0d	0.0±0.0c	0.0 ± 0.0b	3.21±0.08 **a	95.3
2.0	0 ± 0c	17.8±3.8c	15.6±3.8c	15.6±3.8b	0.24± .03b	4.23 ±0.2 **a	94.3
0.5	8.9±3.8b	29.6±5.1b	22.3±3.8b	15.6±3.8b	0.29±0.1b	4.32±0.34**a	93.3
Control Significantly	82.6±3a **	51.4±3.9a**	51.4±3.8a**	42.4±3.8a**	2.3 ±0.3b	3.22 ±0.33*a	29

Table 6: Effect of treatments with Mentha spicata L. on the biological aspects of T. urticae.

Periods in days			
	Mentha spcicata L. essential oil	Control	Significantly
Incubation period	6.28 ± 0.61 a	5.33 ± 0.47b	0
Active larva	3.17 ± 0.6 a	2.66 ± 0.23a	NS
Quiescent larva	1.31 ±0.46 a	1.16 ± 0.23a	NS
A. protonymph	2.28 ± 0.67 a	2.17 ± 0.23a	NS
Q. protonymph	0.75 ± 0.32 a	1.00 ± 0.0a	NS
Pre oviposition	1.10± 0.44 a	0.67 ± 0.23a	NS
Oviposition	2.75 ± 1.59 b	18.67 ± 1.69a	**
Post oviposition	1.15 ± 0.49 a	1.00 ± 0.8a	NS

Table 6: Continue

Longevity	5.00 ± 2.07 b	25.1 ± 4.32a	**
Life cycle	17.65 ± 1.85 a	15.8 ± 0.62a	NS
Life span	22.66 ± 3.45 b	41.0 ± 3.47a	**
No. of Eggs/ ♀	3.27 ± 2.56 b	45.3 ± 4.19a	**
No. of Eggs/ ♀/day	0.28 ± 0.61 b	5.33 ± 0.47a	**

N.S. = Not significant and ** = P ≤ 0.01 and * = P ≤ 0.05

a,b,c, . = Means within each column having similar superscripts are not significantly differ (P ≤ 0.05).

Synthetic acaricides usually contain a single active compound; however, like other essential oils, *M. spicata* L. oil is a complex mixture of compounds (Table 1). These constituents exert a wide range of behavioral and physiological effects on mite. Therefore, it is difficult for mites to develop resistance easily against these compounds. Based on the results, we suggest that the acaricidal effects of *M. spicata* essential oil were because these components. It has been reported that green peach aphids (*Myzus persicae* Sulzer) developed resistance to pure azadirachtin (the major ingredient of neem insecticide), but not to a refined neem seed extract containing the same absolute amount of azadirachtin but with many other constituents present [64]. Finally the results obtained have provided a potential use of *M. spicata* L. as a carcidial against the two-spotted spider mite and antimicrobial.

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