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Chemical Composition and Insecticidal Activities of *Mentha Longifolia* and *Mentha Mozaffarianii* Essential Oils against *Callosobruchus Maculatus*

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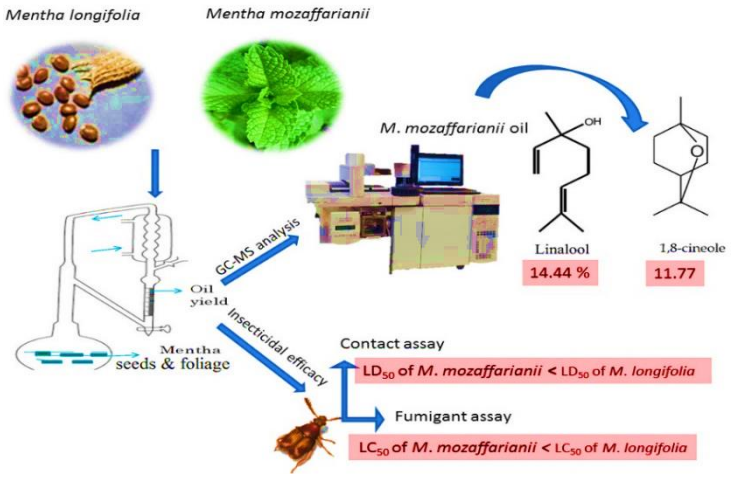
ABSTRACT

Essential oils from two species of *Mentha* genus, (Mentha *longifolia* L. and *Mentha mozaffarianii* Jamzad), from the mint family Lamiaceae that grow in Iran were isolated and analyzed by GC and GC-MS and their insecticidal and repellent activities were investigated against adult cowpea beetles. Mortality rates increased with increasing of the time of exposure and concentration, where *M. mozaffarianii* oil showed more potent toxicity. Using the topical application, LD_{50s} values of *M. mozaffarianii* and *M. longifolia* oils at 96 h after exposure against adults were 22.7 and 39.4 µg/adult, respectively. In the fumigant test, LC_{50s} at 96 h after exposure was between 38.5 and 55.4 µL/L air against adults, respectively. The results demonstrated that no concentration-dependent effect was observed in the test of the repellent effect and *M. longifolia* oil was most repellent to *C. maculatus* adults at 10 µl/l air (29.00%) while *M. mozaffarianii* oil's repellency was 9% at a lower concentration of 5 µl/l air. The main chemical components of the *M. longifolia* oil were Pulegone (48.67%), Menthone (14.31%), 1, 8- Cineole (9.37%) and Camphor (6.60%) while the major constituents of oil of the *M. mozaffarianii* were Piperitone (23.59%), Linalool (14.44%), 1, 8- Cineole (11.77%) and Piperitenone oxide (9.39%).

GRAPHICAL ABSTRACT



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Introduction

Food legumes play an important role in human nutrient contents such as essential amino acids in third world countries including Iran [1-2]. Insect pests of grain legumes annually lose large quantities of stored products [3]. In Iran, pulse beetles such as the cowpea beetle, *Callosobruchus maculatus* (Coleoptera: Chrysomelidae: Bruchinae), are one of the most economically important pests causing significant damage to beans and different type of legumes [4]. So far, numerous chemical and non-chemical methods have been used to manage this storage pest.

Plant secondary natural products include terpenoids (polymeric isoprene derivatives and biosynthesized from acetate via the mevalonic acid pathway), Phenolics (biosynthesized from shikimate pathways, containing one or more hydroxylated aromatic rings) and non-protein nitrogen-containing such as alkaloids, cyanogenic glucoside, glucosinate (biosynthesized from amino acids such as tyrosine, with a long history in medication) are mostly small organic molecules, that are not essential for plant growth, development and reproduction, but have a special role in plant–environment interactions, adaptation to environmental stresses, and defense against biotic threats, thus they are indispensable for plant survival. Moreover, many secondary metabolites have been reported to have antimicrobial, antioxidant and phytochemical properties, among which the biochemical defense against insects and other organisms is one of the most important ones and can be used as excellent alternatives to synthetic or chemical pesticides [5-9].

Terpenoids are an important part of secondary metabolites in plant essential oils (EIs) and seem to be a good alternative to chemical pesticides and act as eco-friendly agents for sustainable agricultural pest management. Plant EOs have diverse biological and insecticidal properties including repellent, antifeedant and oviposition deterrent activity against various stored product insects [10, 11]. Species of the genus *Mentha* have been reported to contain a range of components, including cinnamic acids [12], aglycon, glycoside or acylated flavonoids [13], and steroidal glycosides [14]. However, the main active component of *Mentha* is OI, which is reported to govern its various properties.

The genus *Mentha* (Lamiaceae) comprises 25-30 species that grow worldwide mainly in Africa, Australia, North America and temperate areas of Eurasia [15]. Six species and several subspecies of the genus *Mentha* such as *M. piperata*, *M. suaveolens*, *M. spicata*, *M. longifolia*, *M. arvensis*, *M. aquatica* and *M. mazaffarianii* are found in Iran, among which just *M. mozaffarianii* is Iranian native and is known locally as Pooneh-koohi. [16]. *Mentha longifolia* L., from Labiatae (Lamiaceae) is widely growing in Eurasia, Australia and South and North Africa [17]. This species is found in many habitats and is one of the most common medicinal and aromatic plants in Iran. Also, its insecticidal properties have been widely demonstrated on different insect pests [18].

Literature survey revealed that no study has been previously reported on the insecticidal activates of the oils of *M. mozaffarianii* against *C. maculatus*. Also, no experiment has been conducted to evaluate the oil of *M. longifolia* seeds against *C. maculatus*. Only insecticidal activity of the foliage oil of *M. longifolia* has been reported against *C. maculatus* [19-21]. In the previous studies, insecticidal activity of the oil of *M. longifolia* has been reported against other insect and acari species, such as *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae) [22], *Tetranychus urticae* Koch (Acari: Tetranychidae) [23-25], *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) [18-25], *Sitophilus oryzae* (Coleoptera: Curculionidae) [26], *Varroa destructor* (Acari: Varroidae) [27], and *Oryzaephilus Surinamensis* (L.)(Coleoptera: Silvanidae) [28]. Also, numerous studies carried out on insecticidal properties of other species of *Mentha* genus

Although *M. mozaffarianii* L. has been frequently studied for its chemical constituents, none of the studies has approached its insecticidal activity. In this study, we investigated the repellent and insecticidal properties and chemical composition of *M. mozaffarianii* aerial parts and seed oil of *M. longifolia* obtained from plants cultivated in Iran

against *C. macullatus* adults, as one of the most popular pests of stored grain.

Material and methods

Insects

Parent adults of bean beetles, *C. maculatus* were used in bioassays derived from stock cultures in laboratory and kept at the Department of Plant Protection, University of Zabol and then reared on the pinto bean in plastic boxes (20 × 25 × 15 cm) closed by covers with openings for the exit of air under laboratory conditions (32±2 °C and 60±5% RH) in darkness. Every week, beetles were killed and transferred to another box with new foods. Every week, the adult insects were collected and transferred to another box containing the new seeds. In this case, the adults in the next generations could be the same from each box. (7 ± 2 days old). These adults were used in bioassays.

EOs

Fresh seeds of *Mentha longifolia* and *Mentha mozaffarianii* foliage were collected in February 2021 from field located in Institute of Agricultural Research at the University of Zabol, Zabol City, Iran. The identity of both plants was confirmed by the Biology Department of the University of Zabol. Plant samples were dried and powdered and hydrodistilled for 3 hours. The isolated oils dried with anhydrous Na_2SO_4, transferred and then stored at 4 °C to be used for following bioassays.

GC and GC-MS analysis

Major constituents of two *Mentha* species oils were determined on an Aligent 6890N gas chromatograph equipped with a HP-5975 capillary column (30m × 0.25mm × 0.5µm). Helium (99.99% pure) was the carrier gas with 1 mL/min of flow and 2 µL of the sample was injected at 250°C. The oven temperature was kept at 50 °C for 2 min and programmed to reach 100°C at a rate of 25 °C/min and was held for 2 min, and finally raised to 290°C for 5 °C/min. The GC-MS analysis operated in an electronic ionization system and was kept at 70eV ionization energy [29]. The compounds were identified by comparison of their retention Kovats index with those reported in the literature, or by comparison of their relative retention index to a homologous series of C_8-C_{22} *n*-alkanes and by comparison of their mass spectra of standard compounds with publication data [30-31].

Contact toxicity

The contact toxicity of the *Mentha* oils were measured by topical application method against *C. maculatus* adults as reported by Liu and Ho [32]; preliminary tests were carried out to calculate a graded series of six concentrations of each oil. The EOs were diluted 1:10 in acetone; then, a dose of 0.5 µl from each tested oil (5 µg/adult) was applied dorsally to the thorax of adult beetles by using the proper applicator. Controls were determined using 0.5 µL of acetone per adult. Ten adult insects from each treatment and control were transferred in 9 cm Petri dishes containing culture media and covered with fine nylon mesh and kept under suitable conditions for rearing. Every treatment and control had four replicates. Mortality percentages were recorded after 48 and 96 h of treatment until no longer mortality increased with time. Data adjusted for mortality in the control by Abbott's formula [33]. Probit analyses (IBM SPSS Statistics 24 software) were carried out to calculate the LD_{50} values [34].

Fumigant toxicity

This assay was performed based on the khani *et al*. [35]. The dose-finding tests were conducted to calculate the appropriate testing concentrations. The obtained concentration from the preliminary tests (3, 10, 15, 20, 25 and 30 µL of oils) was impregnated with a filter paper (2 cm diameter) without the use of solvent. In order to prepare the concentrations used in the final tests, the filter paper was air dried for two minutes and placed in a 60 ml glass vial with a lid on the glass to generate concentrations of 50, 166.66, 250, 333.33, 416.66 and 500 µl/l airs. The studied adult insects were transferred to glass vials in groups of ten and completely covered by parafilm tape. This experiment was performed in a completely randomized design with four replications for each concentration and ten

individuals per replicate. Insects were exposed to essential oil for 24 hours, and then the treated insects were transferred to vials with culture media and kept in the same rearing conditions. Insect mortality was recorded after 48 and 96 hours. The observed mortality data were corrected for control mortality using Abbott's formula. The achieved data were subjected to probit analysis to calculateLC_{50} and LC_{95} values (IBM SPSS Statistics 24 software).

Repellency tests

The repellent activity was performed using arenas test similar to that used by Negahban *et al.* with minor modifications [36]. In this experiment, two plastic containers with a volume of 65 ml were used, which were connected to a central plastic chamber through plastic tubes (2 cm long and 3 mm in diameter). Three concentrations of plant oils used (5, 10, and 15 µl) were selected and dissolved in 1 ml of solvent and 1 ml from each concentration was applied on 20 g of cowpea grains. Also, the control container (without the oil) was treated similarly as described. To evaporate the solvent, the treated and control beans were kept for 20 minutes and then placed in the center of the chambers. Fifty-one-day-old female beetles were released into the central box. Then, the center box was covered with plastic wrap, but the treated and control containers were covered with lids, and the whole designed device was placed in darkness. This experiment was conducted in a completely randomized design with three replications for each concentration. The number of beetles in each box was counted after 24 hours and the percentage of the repellency was determined using the following formula: $\%PR = \left(\frac{C-E}{T}\right) \times 100$, where C = the number of insects responding to the control, E = the number of insects responding to the oil treated chamber and T = total number of insects released in the chamber [37].

Statistical analysis

In toxicity and repellency tests, when the observed mortality ranged from 5 to 20%, the data were corrected using the Abbott's formula [33]. Probit analysis [38] was performed to calculate L_{C50} and LC_{95} values and their fiducial limits. An analysis of variance (ANOVA) was carried out to determine the significance between treatments and control. Also, the mean differences were compared using Tukey's test (IBM SPSS Statistics 24 software).

Result and Dissection

Data in Tables 1 and 2, show that both the test oils and the time of exposure, exhibited variant degrees of toxicity and were activity determinant factors against *C. maculatus*. The toxicity of EOs increased with rising concentration and longer exposure times. The two EOs tested were topically effective to *C. maculatus* (Table 1). In all treatments, *M. mozaffarianii* oil was the more toxic than *M. longifolia.* The LD_{50s} of the test oils after 48 h exposure of adults were 28.4 and 54.1 µg/adult in case of *M. mozaffarianii and M. longifolia*. These values decreased to 22.7 and 39.4 µg/adult after 96 h of exposure. On the basis of the LD_{50} and LD_{95} values, *C. maculatus* adults were more susceptible to the seed EOs of *M. mozaffarianii* than *M. longifolia* (Table 1).

Table 1: Toxicity of *Callosobruchus maculatus* exposed to the plant oils using the topical application bioassay

Plant species	Period (h)	LD_{50} (95% CL)	LD_{95} (95% CL)	Slope ± SE	χ2 (df)
M. mozaffarianii	48	28.4* (21.4-34.4)	72.8 (62.1-72.3)	1.8 ± 0.20	0.35 (5)
	96	22.7 (16.1-25.9)	60.2 (56.4-67.1)	2.1 ± 0.19	0.68 (3)
M. longifolia	48	54.1 (48.6-67.17)	140.6 (123.6-147.11)	3.0 ± 0.31	3.88 (4)
	96	39.4 (33.8-48.8)	132.7 (120.4-145-5)	2.0 ± 0.25	2.34 (3)

***** Ten individuals per replicate, four replicates per concentration, six concentrations per assay, total number of tested insects were 240 adults for every plant; LD: lethal dose µg/adult; CL: confidence limits.

In the fumigant bioassay, based on lower and upper confidence interval 95%, the EO of *M. mozaffarianii* also possessed strong fumigant activity against *C. maculatus* with a LC_{50} and LC_{95} values of 38.5 and 96.8 µL/L air after 96 h of exposure. Also, the LC_{50} after 48 h of exposure of adults belonged to *M. mozaffarianii and M. longifolia,* were 52.5 and 73.7 µL/L, air respectively (Table 2).

Table 2: Toxicity of *Callosobruchus maculatus* exposed to the plant oils using the fumigation bioassay

Plant species	Period (h)	LC_{50} (95% CL)	LC_{95} (95% CL)	Slope ± SE	χ2 (df)
M. mozaffarianii	48	52.5* (49.7-58.4)	115.3 (103.4-123.6)	1.8 ± 0.20	0.76 (5)
	96	38.5 (34.7-31.8)	96.8 (85.6-106.2)	2.0 ± 0.19	0.88(2)
M. longifolia	48	73.7 (67.1-80.2)	149.3 (137.7-150.0)	2.1 ± 0.22	1.22 (3)
	96	55.4 (50.4-62.9)	134.3 (123.4-155.5)	1.8 ± 0.21	1.20 (6)

***** Ten individuals per replicate, four replicates per concentration, six concentrations per assay, total number of tested insects were 240 adults for every plant, LC: lethal concentration µL/L air ,CL: confidence limits

The EO of *M. longifolia* repelled the cowpea beetle in all concentration except 5 µl/l air acetone. Therefore, repellency of *M. longifolia* was significantly higher than that of *M. mozaffarianii* to the *C. maculatus* adults with overall repellency. The data showed that high fumigant toxicity did not necessarily lead to increased repellency (Table 3).

Table 3: Percent repellency (mean ± SE) of the essential oils from *M. mozaffarianii and M. longifolia* and on *Callosobruchus maculatus* adults

Plants	Concentration of essential oil (µl /food)		
	5	10	15
M. mozaffarianii	9.00 ± 0.58a (B)	11.50 ± 0.50b (B)	16.50 ± 1.26b (B)
M. longifolia	7.50 ± 0.50a (B)	29.00 ± 3.00a (A)	24.00 ± 0.82a (A)

Means followed by the same letter in a column (same letter) and within a row (capital letter) are not significantly different using Tukey's test at $\rho < 0.05$.

The main compounds of the EOs from *M. longifolia* and *M. mozaffarianii* was determined and quantified by GC/MS (Table 4). A total of 19 and 23 components of the essential oil of *M. longifolia* and *M. mozaffarianii* were identified, accounting for 97.95 % and 97.42 % of the total oil, respectively. The major components of wild mint oil was Pulegone (48.67%) followed by Menthone (14.31%), 1,8- Cineole (9.37%), Camphor (6.60%), Germacrene D (3.38%), Ppiperitenone 2.67%), β -Caryophyllene (2.54%), α-Terpineol (1.94%), Piperitenone oxide (1.80%), Limonene (1.14%), Piperitone (1.05%), and Linalool (1.03%). Twenty three compounds were identified in Pooneh-Koohi oil. Piperitone (23.59%) was the most abundant compound, followed by Linalool (14.44%), 1,8- Cineole (11.77%), Piperitenone oxide (9.39%), Piperitenone (6.69%), Menthone (5.98%), Pulegone (4.65%), Menthol (4.56%), trans-Jasmone 2.99%), Thymol (2.98%), β- Pinene (1.98%), Menthyl acetate (1.46%), β -Caryophyllene (1.22%) and Bornyl acetate (1.08%).

Herein we, for the first time, reported insecticidal efficiency of the EO of *M. mozaffarianii* cultivated in Iran to *C. maculatus*. Various experiments were carried out to evaluate the biological activity of EO of *M. longifolia* against different insect and arthropod pests [20, 27, 39, 40, 41, 42]. In our study, *M. mozaffarianii* oils showed more fumigant toxicity against *C. maculatus* adults. It has already been proven that one of the most valuable properties of plant oils is their fumigant toxicity against storage pests, since it makes it possible to use them successfully during storage

without the need for direct use on pest insects. Generally, insects absorb EOs by inhalation, ingestion or penetration via spiracles pores [43].

Table 4: Chemical composition of the essential oils *M. longifolia* and *M. mozaffarianii* under investigation

Compounds	Kovats index	Composition (%)	
		M. longifolia	***M. mozaffarianii***
α- Pinene	934	0.55	0.68
Camphen	949	0.16	-
Sabinene	973	0.75	-
β- Pinene	977	0.34	1.98
Myrcene	990	-	0.34
p-cymene	1016	-	0.62
Limonene	1030	1.14	-
1,8- Cineole	1034	9.37	11.77
γ- Terpinene	1055	0.56	0.41
Linalool	1108	1.03	14.44
Menthone	1155	14.31	5.98
Camphor	1164	6.60	-
α-Terpineol	1171	1.94	0.23
3-thujanol	1173	-	0.31
Menthol	1180	0.86	4.56
Pulegone	1243	48.67	4.65
Piperitone	1251	1.05	23.59
Bornyl acetate	1286	-	1.08
Carvone	1293	-	2.98
Menthyl acetate	1304	-	1.46
Ppiperitenone	1339	2.67	6.69
Piperitenone oxide	1372	1.80	9.39
trans-Jasmone	1390	-	2.99
β -Caryophyllene	1419	2.54	1.22
Germacrene D	1489	3.38	-
Bicyclogermacrene	1496	0.23	0.81
Spathulenol	1580	-	0.77
Caryophyllene oxide	1585	-	0.47
Total		97.95	97.42

This mechanism of action is becoming more imperative to managing stored product insect pest when accumulation of harmful chemical residues entering the food is very undesirable [44]. The two oils were highly toxic against test insects via topical application method. Toxicity of plant oils against stored product pests, except for phytochemical diversity patterns, also includes multiple different factors. Insects such as *Callosobruchus maculatus* have been shown to be sensitive to contact or topical bioassays and their active ingredients [45]. Our results revealed that the oil of *M. mozaffarianii* showed significant contact toxicity. This might be because of high level concentration of Linalool (23.59%). Ling Chang *et al*. [46], Vicenço *et al*. [47] and Wang *et al*. [48] reached the same conclusions on the toxicity of Linalool as the major constituent of the oil of *Oscimum basilicum*, *Cinnamomum camphora* and *Zanthoxylum schinifolium*, respectively. Other major monoterpenoid constituents in the oil of *M. mozaffarianii* such as 1,8-Cineole, Pulegone, Menthol and Carvone has been well documented to have insecticidal toxicity against stored product pests [49-53], which have been isolated from other plant species.

EOs of two *Mentha* species exhibited contact and fumigant in a dose-dependent manner against *C. maculatus* adults. Our results showed that repellency is not necessarily correlated with the toxicity. Our results on repellent effect of *M. mozaffarianii* were in agreement with those of Talukder and Howse [54], who showed that *Aphanamixis polystachya* seeds extract had highly toxicity against pulse beetle but low repellency values were observed for this insect. Also, Abdel-Rahman *et al*. [55] found that among twenty botanical extracts and products, guava and black

seed exhibited significant toxicity against the almond mouth, *Cadra cautella* eggs but no repellent activities was observed on *Cadra cautella* larvae. Contrariwise, a study on the psocid, *Liposcelis bostrychophila*, demonstrated that repellent activity of *Citrus aurantium* oil was higher than that of other five test plant oils in which they had a low level of fumigant toxicity [48]; therefore, authors concluded that the active ingredients involved in toxicity and repellency might be chemically different.

The major constituents of the tested oils from two *Mentha* species were similar to previous reports on the chemical analysis of *Mentha* plants cultivated in Iran or other countries [20-59]; but the number of major constituents of different oils varied significantly or partially. Various species of *Mentha* show significant differences in the content of EOs and chemical components; these variations in oil components are due to ecological and various environmental conditions such as temperature, humidity, and photoperiod [60]. Similarly, agronomic conditions and plant-genotype-specific features, such as plant age at harvest and plant density affect the chemical variability of major components of the plant oils.

The plant oils activity is related to the activity of low molecular weight of the main components [61]. Various components of plant oils are involved in cell activities such as the uptake of substances into cells, the uptake and stabilization of hydrophobic or lipophilic molecules on membranes and cell walls, and the distribution of components into cells. The oil compartmentation in the cell defines production of various types of radical reactions, so, the distribution of different components into cells is very important. Therefore, it is more useful to study the whole plant essential oils rather than some of its constituents, because synergistic effects of plant oil constituents seem to be more effective. However, plant EOs and their insecticidal activities correlated to number of monoterpenoids [62-64]. The instant action of plant oils or their components indicate their mode of action in the nervous system of pests [65], which might be due to acetyl-cholinesterase inhibitory activity [66]. Insecticidal activity of plant oils and/or their constituents could also be attributed to the blocking ectopamine receptors resulting in poisoning of insects [65-67]. Undoubtedly, the advantage of using EOs and their constituents in insect-pest management is their low mammalian toxicity as protectants of stored products.

Conclusion

For several decades, the efficacy of pesticides based on plant EOs has been well demonstrated against a broad range of stored product insect. According to the obvious effects on *C.maculatus*, the tested *M. mozaffarianii* oil appears to be promising alternative for controlling the major pest of stored legumes. Due to the fact that the amount of essential oils in aromatic plants is very low, efforts should be made to remove existing barriers to commercialization and mass production of these compounds on a large and commercial scale. Further studies may dwell on the mode of action of *M. mozaffarianii* oil in other storage pests and its adverse or indirect effects on non-target organisms. Finally, it is necessary to identify and introduce compounds that can reduce the possibility of insect resistance by creating synergism.

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Authors' contributions

All authors contributed toward data analysis, drafting and revising the paper and agreed to be responsible for all the aspects of this work.

Conflict of Interest

We have no conflicts of interest to disclose.

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