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Antifungal and Antiaflatoxigenic Effects of Mentha Longifolia Essential Oil Against Aspergillus Flavus

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***Abstract*— Mycotoxin contamination in foods and feeds poses serious health hazard to humans and animals. Aflatoxins, which are mutagenic, carcinogenic, teratogenic, hepatotoxic and immunosuppressive, could be produced by certain strains of Aspergillus parasiticus, A. nominus and A. flavus. An aim of this study was to examine the antifungal activity of various essential oils(EOs) obtained fromM. longifolia, Nepeta asterotricha, Ziziphora clinopodioides, Ferula assa- foetida and Heracleum persicum against A. flavus. Comparing the effect of EOs obtained from five plant species mentioned above showed that at 2000 ppm concentration all EOs tested were capable of inhibiting mycelia growth of A. flavus ranging from 100% to 18%. The highest and lowest levels of antifungal effect were obtained using M. longifolia and Heracleum persicum, respectively.Gas chromatography (GC) and GC-mass spectrometry analyses were applied to determine the constituents of M. Longifolia oil, as the most effective EO in suppressing A. flavus growth. Obtained data indicated that the main compounds of this EO were limonene (1.7%), 1,8-cineole (2.2%), 1-borneol (1%), isopiperitenone (1.3%), piperitenone (18.7%) and piperitenone oxide (70%). Spore germination of A. flavus was completely inhibited by IC50 and MIC concentrations of M. longifolia EO. Whereas, these concentrations of the EO had no inhibitory effect on sporulation of the fungus. Morphological changes of A. flavus via application of M. longifolia EO at IC50 concentration were compared with control and considerable alterations in the hyphae and conidiophores of treated samples were observed. Aflatoxin production by A. flavus significantly decreased at MIC concentration of M. longifolia EO compared to control. Only aflatoxin B1 was detected at low concentration using the MIC level of this EO. So, the EO obtained from M. longifolia might be used as a biological agent to decrease mycelial growth and aflatoxin production of A. flavus for protecting crops from this toxigenic fungus.**

***Index Terms* — Antifungal, Anti aflatoxigenic, Mentha longifolia, Aspergillus flavus**

I. INTRODUCTION

Essential oils (EOs) are aromatic substances which are obtained from various plant parts by steam distillation; many of which exhibit antifungal, antibacterial, and antiviral activity(1,2,3).The Lamiaceae family has great diversity and distribution in Iran with 46 genera, 410 species and subspecies from which 124 are endemic (4,5). Among this rich array of plants yielding EOs, the genus Mentha, distributed worldwide, is very important, including 20 species with considerable economic value. Mentha longifolia(L.) Hudson is a wild mint species native to Asia, Europe, and Africa. It is a perennial herb widely used in pharmaceutical, perfumery, food, confectionary and cosmetic industries. Many biological activities such as antifungal (6,7) and antioxidant (8) effects have also been reported from different Mentha species.

The EO of M. longifolia has interesting antimicrobial activity against A. flavus, Botrytis cinerea, Fusarium oxysporum, Pseudomonas aeruginosa, Aspergillus niger(9), Trichophyton longifusus, Microsporm canis(10)and Mucor ramamnianus(11).

Mycotoxins are toxic contaminants produced by fungi via their secondary metabolism (12).Aflatoxins (AFs) are a group of mycotoxins produced by various species of the anamorphic fungal genus Aspergillus, particularly A. flavus (13). They are most commonly known for causing acute or chronic liver disease but they are also considered immunosuppressive, hepatotoxic, mutagenic, teratogenic, and carcinogenic. Various agricultural products might be contaminated with aflatoxin producing fungi or aflatoxins (14). Aflatoxins have been detected in cereal grains, oil seeds, fermented beverages made from grains, milk, cheese, meat, nut products, fruit juice and numerous other agricultural commodities (15). The four major aflatoxins are B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2), which are each distinguished by the color of their fluorescence under ultraviolet light (B: blue; G: green)(16).Among 4 different types of aflatoxins, AFB1 has the highest level of toxicity on consumers (17,18).

While previous studies have identified reduction in the growth of A. flavus by application of various EOs, there is no any detailed report on their effects on production of aflatoxins so far. Therefore, the objectives of this study were (i) to screen and investigate the effect of EOs obtained from 5 plant species on mycelial growth of A. flavus, and (ii) to determine the capability of different concentrations of the most effective EO in reducing production of various aflatoxins by the fungus.

II. MATERIALS AND METHODS

A. *Plant materials*

Plant materials comprising of flowers, leaves and stems of 5 plant species were collected from Yazd in Iran (Table 1). The collected samples were dried in darkness at room temperature. Plant species were identified and a dried voucher sample was kept in the laboratory of botany in Ferdowsi University of Mashhad, Iran.

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B. Preparation of the plant EOs

Fifty gram of air dried and ground flowers, leaves and stems of several plants (Table 1) were submitted to hydro distillation for about 3 h using a Clevenger apparatus(19). The obtained EO was dried over anhydrous sodium sulfate, preserved in sealed glass bottle and stored in the dark at 4°C until used.

C. Fungal culture conditions

The isolate of A. flavus (CNR ITEM code: 16499) was obtained from the soil of pistachio orchards in Kerman province, Iran. This isolate was maintained on potato dextrose agar (PDA) medium at 4°C and subcultured at monthly intervals.

D. Antifungal assay

Inhibition of mycelial growth was assayed using the food poison method described by Tatsadjieuet et al., 2009(20). The PDA plates were amended with various concentrations of plant EOs (0 to 2000 ppm). For enhancing the EO solubility, 0.5% Tween-20(v/v) was added. Each plate was inoculated with a mycelial plug (10 mm diameter) of A. flavus. All plates were incubated in triplicate for each concentration at 28°C for 7 days. Plates with Tween-20 but without any EO were used as control. Observation of fungal growth was done at a time interval of 12 h up to 7 days after incubation. The mycelial growth inhibition percentage was calculated according to the following formula: MIC (%) = ((Dc-Dt)/Dc) × 100; Where MIC is minimum inhibitory concentration, Dc is the mean diameter of colony in the control (mm), and Dt is the mean diameter of colony in the treatment (mm). Three replicate plates were used per treatment and the experiment was repeated three times. The MIC values were determined as the lowest concentration of EO that completely prevented visible fungal growth.

To determine minimum fungicidal concentration (MFC), the mycelial plugs were obtained from each Petri dish treated with the oil concentrations where no growth was observed transferred into a new plate without the EO and incubated at 28°C for 7 days. MFC was defined as the lowest concentration at which no colony growth was observed after sub culturing into fresh PDA medium.

IC50 (concentration that produces 50% inhibitory effect) values were graphically calculated from the dose–response curves based on measurement at different concentrations as described by Chang et al.(2008)(21).

Three replicates were used per treatment, and the experiment was repeated three times. The EO with highest level of inhibiting A. flavus mycelial growth was selected for the rest of experiments. Efficacy of the selected EO was also compared with common fungicides, such as carbendazim and copper oxychloride by the agar medium assay described before.

E. Effect of the wild mint EO on sporulation of A. flavus

Effect of various concentrations of EO obtained from M. longifolia on the ability of A. flavus for sporulation was evaluated on PDA medium. Different concentrations of M. longifolia EO (0 andIC50) were mixed into each of three replicate plates of PDA, just before it solidified. Then, the plates were inoculated with a mycelial plug (1 cm diameter) of A. flavus. They were incubated at 28°C. After 7 days of incubation, 1 cm diameter mycelial plugs from a plate of each of the wild mint EO concentrations were randomly immersed in 5 ml distilled water in test tube, which was shaken to dislodge the spores. The number of spores from the replicates of every concentration were counted using hemocytometer. The percentage of sporulation inhibition was determined using the following formula:

Inhibition of sporulation (%)= ((Nc- Nt)/Nc) × 100

Where, Nc is the number of fungal spores in control and Nt is the number of fungal spores in treatment (22, 23).

F. Effect of the EO on conidial germination of A. flavus

Conidia of A. flavus cultured on PDA plates were taken and conidial suspensions (105spore/ml) were prepared. Different concentrations of M. longifolia EO (0, IC50 and MIC) were mixed into PDA plates. One ml of conidial suspension was spread in each PDA plate containing the EO and they were incubated at 28°C for 7 days. Germination of spores was investigated with stereomicroscope and inhibition of spore germination was determined using the following formula: Inhibition of spore germination (%)=((Nc- Nt)/Nc) × 100, Where Nc is the number of fungal colonies in control and Nt is the number of fungal colonies in treatment(16).

G. Morphological studies

For evaluating morphological alterations caused by the EO in A. flavus a sample of mycelium was taken from the periphery of a 7daysold fungal colony grown on PDA at 28ºC containing IC50 concentration of the EO. The samples were observed under microscope to examine morphological abnormalities. Control assay without the EO was tested in the same way(24, 25).

H. Chemical analysis of the M. longifolia oil

Qualitative and quantitative analyses of the oils were performed using GC and GC-MS, respectively, as mentioned below.

- Gas Chromatography (GC)

The qualitative analyses of the EO constituents were performed in a gas chromatography equipped with a Flame Ionization Detector (FID). Ten µl of the EO were dissolved in 100 µl pentane and 2 µl of the solution was injected into a Thermo Quest Finnigan Trace GC. The column used was SUPELCOWAX fused silica (film thickness: 30 m * 0.25 µm i.d., 0.25 mm). Column temperature was programmed at 60 to 250 °C with a rate of 4 °C/min. Injector and detector temperatures used were 220 and 280 °C, respectively. Carrier gas was nitrogen. Quantification was carried out by the peak area calculations (GC/FID using a non-polar column). Identifications were carried out by comparing the retention time of each compound with that of known compounds.

- Gas Chromatography- Mass Spectrometry (GC-MS)

The chemical composition of M. longifolia EO was investigated using gas chromatography mass spectrometry (GC-MS). Analysis of the EO was performed using a Thermo Quest Finnigan Trace MS (E.I Quadrapole) equipped with a

SGE-BPX5 MS capillary column (60 m * 0.25 µm i.d., 0.25 mm). For GC-MS detection an electron ionization system with ionization energy of 70 eV was used whereas mass ranges were between 40 to 460 amu. Helium was the carrier gas, at a flow rate of 1.1 ml/min. Injector temperature was 250 °C, interface heating was 280°C and ion source temperature was 200°C. The components were identified based on the comparison of their relative retention time and mass spectra with those of standards, Wiley7N, TRLIB library data of the GC-MS system and literature data (26).

I. Aflatoxin assay by high performance liquid chromatography (HPLC)

The isolate of A. flavus was cultured on PDA amended with different concentrations of M. longifolia EO (0, IC50 and MIC) for 15 days at 28°C in the dark. Agar of PDA was extracted with a solution of methanol: water (80:20, v/v) on a shaker for 30 min, using a volume five times higher than the weight of each sample. The HPLC analysis was carried out on a Waters Alliance 2695 separations module coupled to a Waters 474 scanning fluorescence detector (Waters Corp, Milford, Massachusetts, USA)that was set at 365 nm excitation and 440 nm emission. To perform aflatoxin derivatisation, 700 µL of trifluoroacetic-glacial acetic acid–water (2:1:7 v/v/v) was added to extracts and the mixture heated at 65 °C for 10 min. The extracts were re-suspended in 200 µL of benzene and cleaned up with a C18 Spherisorb5 µm (250 * 4.6 mm, Merck, Germany) column maintained at 50 °C. The mobile phase was water–acetonitrile–methanol (78:12:10 v/v/v) with a flow rate of 2.5 mL/min. Aflatoxin production was measured in µg/kg of culture medium. A standard comprising a mix of the AFB1, AFB2, AFG1 and AFG2 was used to construct a five-point calibration curve of peak areas versus concentration. The injection volume was 50 µL for both the standard solution and sample extracts (12).

J. Statistical analysis

All experiments were repeated at least three times. The data were analysed by the analyses of variance (ANOVA) and Duncan's multiple range test (DMRT) at the 5% level. Statistical analyses were carried out using with SPSS (Statistics for Windows, Version 21.0. Armonk, NY, USA) software.

III. Results

A. Antifungal assay

A preliminary screening showed that various concentrations of the Eos obtained from different plant species (Table 1) exhibited various degrees of antifungal activity against A. flavus (Figure 1).Comparing antifungal effect of the EOs obtained from five plant species showed that at 2000 ppm concentration all EOs tested were capable of inhibiting mycelia growth of A. flavus ranging from 100% to 18% (Figure1). The highest and lowest levels of mycelial growth inhibition were obtained using the EOs of M. longifolia and Heracleum persicum, respectively.

The EO of wild mint (M. longifolia), which showed the highest growth inhibitory effect on flavus, was selected for using in the rest of experiments. The IC50 of 300 ppm was obtained using M. longifolia EO. Investigating fungistatic and/or fungicidal activities showed that the EO of M. longifolia had fungistatic or growth inhibitory capability on the fungus at 650 ppm concentration, which is MIC of the tested EO. Whereas, this EO revealed fungicidal effect on A. flavus at 1900 ppm. The effects of copper oxychloride and carbendazim on growth inhibition of A. flavus at 8000 ppm concentration were 30% and 90%, respectively.

B. Sporulation of A. flavus

The results of counted spores in IC50 concentration of M. longifolia EO compared to control revealed that the EO was not significantly effective on sporulation of A. flavus at the used concentration (Table 2).

C. Conidial germination of A. flavus

Germination of A. flavus spores was completely inhibited by both concentrations of M. longifolia EO(including MIC and IC50) compared to control in which spore generation occurred about 24 h after inoculation (Table2).

D. Morphological studies

Morphological changes of A. flavus cultured on PDA plates treated with the M. longifolia EO atIC50 were compared with control (Figure 2). Microscopic investigations showed considerable alterations in the fungal hyphae such as fragmentation and cytoplasmic coagulation (Figure 2A). Also, cytoplasmic coagulation was observed in conidiophores of the fungus (Figure 2B). These alterations in the morphology of vegetative growth structures were not observed in controls (Figurs 2C and 2D).

E. Gas chromatography- Mass spectrometry analysis

The major constituents of M. longifolia EO were shown in Table 4 and Figure 3 Chemical analysis of the M. longifolia EO components indicated that the main constituents of this oil were limonene (1.7 %), 1,8-cineole (2.2), 1-borneol (1 %), isopiperitenone (1.3), piperitenone (18.7%) and piperitenone oxide (70%), presented in Table 4 and Figure 3. These constituents were compared with the components of EOs obtained from other samples of M. longifolia reported in the literature (Table 5).

F. Aflatoxin assay by HPLC

Investigating aflatoxin B1 and B2 production by A. flavus in PDA media containing at IC50 concentration of M. Longifolia EO didn't show any significant decrease compared to control samples. Whereas, production of both aflatoxin types was significantly reduced by application of the M. longifolia EO at MIC level (Table 5).

IV. Discussion

Since the identification of aflatoxins, extensive efforts have been made for more than 50 years to clarify biochemical and molecular mechanisms involved in biosynthesis of these harmful fungal secondary metabolites. Especially, considerable progress has been made in understanding biosynthetic pathways, pathway intermediates, genes, corresponding enzymes, and regulatory mechanisms. To date, more than 30 genes are known to be involved in aflatoxin

biosynthesis, which are clustered within a 75-kb region of the fungal genome on chromosome III (27,28).Even with the broad spectrum of current knowledge on aflatoxins biosynthesis and their harmful effects on various ecosystems, applicable and effective strategies to decrease or suppress aflatoxin contamination of food and feed products are lacking so far.

Several agricultural practices, plant breeding strategies and biocontrol methods were used to eliminate production of the carcinogenic, mutagenic and immunosuppressive aflatoxins pre- and post-harvest of various crops (reviewed by Bhatnagar-Mathur et al., 2015)(29). In the case of biocontrol, several microorganisms and different plant species with inhibitory effect on the fungal growth and aflatoxin production, could be used(29,30). But, most of the researches were focused on application of various bacterial and fungal species against A. flavus and aflatoxin contamination of agricultural crops till now. So, our knowledge on the possibility of using plant extracts or essential oils to prevent aflatoxin production is in its infancy stage. Among natural antifungal and anti aflatoxigenic constituents characterized in plants, none of them cause a considerable risk to public health at the levels of intake when used as additives(31). So, the plants could be considered as natural sources of useful and safe antimicrobial compounds against harmful effects of various microorganisms, including A. flavus.

In this study antifungal capability of the EOs obtained from several plants against A. flavus was investigated. Our data showed that all of used EOs in this study had inhibitory effect on mycelial growth of A. flavus. The M. longifolia EO had complete inhibition and N. asterotricha and Z. clinopodioides had high effect on A. flavus growth. The F. assa- foetida and H. persicum EOs had moderate and low effect on vegetative growth of A. flavus, respectively. The EO of M. longifolia revealed the highest level of inhibitory effect on mycelial growth of A. flavus among all EOs tested. So, it was selected to be used in the rest of experiments on analysis of its antifungal and anti aflatoxigenic activities against A. flavus. Previous studies revealed interesting antimicrobial activity of M. longifolia EO against plant and human pathogens(11,32,33).Our results about fungistatic and fungicidal activity of M. longifolia EO against A. flavus growth were consistent with previous findings(34,35).

In previous reports, the EO obtained from M. longifolia was found to have high level of inhibitory effect(between 80 to 100 %) on spore germination of several phytopathogenic fungi belonging to Ascomycota(36).Similarly, our findings showed that spore germination of A. flavus was completely inhibited by the MIC and IC50 concentrations of M. longifolia EO compared to control. Therefore, the EO of this plant species is capable of controlling a wide range of fungi including plant parasites and saprophytes.

To our knowledge, there is not any report on the effect of M. longifolia EO on fungal structures such as hyphae and conidiophores so far. In this study, microscopic observations of A. flavus treated with M. longifolia EO showed clear changes in morphology of hyphae and conidiophores. Appearance of shriveled aggregates was observed in the conidiophores exposed to the EO, compared to normal conidiophores growth in control. Fragmentation, cytoplasmic coagulation and shriveled aggregates were observed in the fungal hyphae and conidiophores exposed to M. longifolia oil, compared to normal vegetative growth of controls. The results of our microscopic analyses are in agreement with other reports in which EOs of aromatic plants caused morphological changes in the fungal structures (1,2).

The effects of copper oxychloride and carbendazim on growth inhibition of A. flavus at 8000 ppm concentration were 30% and 90%, respectively. So, it can be concluded that the EO had higher antifungal effect against A. flavus compared to both fungicides tested. This is a novel finding, suggesting that the EO of M. longifolia might be used as a powerful biological or natural agent instead of synthetic fungicides for reducing or suppressing A. flavus growth and aflatoxin production in various crops and agricultural products.

The EO of M. longifolia has been a subject for several investigations and different chemotypes of this plant species have been reported worldwide, which are compared with the major chemical components of our sample in Table 5. The major compounds identified in our sample were limonene (1.7 %), 1,8-cineole (2.2%), 1-borneol (1 %), Isopiperitenone (1.3%), piperitenone (18.7%) and piperitenone oxide (70%). Among these constituents, the percentage of piperitenone oxide was higher compared to other reports on the percentage of this chemical compound of M. longifolia EO sampled from Iran(37, 38).As observed in our study, piperitenone oxide was reported to be one of the main constituents in M. longifolia EO with high percentage in the oil in several plant samples obtained from various Asian countries as Iran (37, 38, 39) and Pakistan (34, 40). In accordance with these findings, several researchers reported the presence of piperitenone oxide as a major component in the oil of this plant species sampled from Southern Africa (41)and several European countries such as Croatia (42), Turkey (43), Greece (44), and Italy (45).

This is the first report on presence of piperitenone at high percentage in the EO of M. longifolia sampled from Iran. However, this chemical compound has also been reported to be a main constituent of the oil of this plant species sampled from Pakistan (40), which is in agreement with our data (Table 5).

The other main component of M. longifolia oil investigated in this research was 1,8-cineole, which was also reported to be one of the major constituents in the oil of this Mentha species sampled from Iran (37, 39, 46), Tunisia (47, 48), and Italy (45) with variable percentages in the oil from 1.6% to 15.58% (Table 5).

Another main constituent of our oil, Limonene, was also reported to be a main component of M. longifolia EO in Iran (49) and Croatia (42). But in the EO obtained from this plant species sampled from African countries, limonene was not reported.

Presence of isopiperitenone as a main component of M. longifolia EO was observed in our study, which is in accordance with another report from Iran (39). So far, this compound is only found in the EO of M. longifolia sampled from Iran, indicating unique chemotypes in the flora of this wide country which needs more investigations in the future.

The other main constituent of M. longifolia EO in our research was 1-borneol, which was also reported to be a major compound in the oil of this plant species sampled from Pakistan (34, 40), Tunisia (48). Whereas, there is not any report on the presence of 1-borneolas a main component of this EO in Europe till now (Table 5). Variation in plant chemotypes might be due to the differences in geopgraphic region of collection, altitude, climatic conditions of each season, interaction of various microorganisms with plants, and soil composition.

Our data revealed that M. longifolia essential oil not only had high level of capability in growth inhibition of A. flavus and making microscopic alterations in fungal hyphae and conidiophores, but also was able to significantly decrease aflatoxin production by the fungus. Anti aflatoxigenic capability of M. longifolia EO increased with increasing its concentration. This case is in agreement with a previous report on increasing anti aflatoxigenic activity of the EOs of Thymus vulgaris, Citrus aurantifolia and Carum carvi by increasing the oil concentration (31).

Previous findings showed that extracts and EOs of some plant species inhibited aflatoxin production together with inhibitory effect on mycelial growth of A. flavus(11, 14, 15, 16, 50, 51, 52).However, this is the first report on the inhibitory effect of M. longifolia EO on AFB1 and AFB2 production by A. flavus, which were observed in oil concentration dependent manner. Similarly, Gibriel et al. (2011) observed antifungal and anti aflatoxigenic capability of M. viridis EO on A. flavus, which was reported to be higher with using high doses of the oil(11). In a previous study, no antifungal effect against A. parasiticus and no anti aflatoxigenic activity of M. longifolia EO on AFB1 and AFG1 produced by this fungal species were reported (31). Differences between the data obtained by the present study and the experiments of Razzaghi-Abyaneh and associates (2009) could be due to different Aspergillus species and plant samples used in these researches(31).

Findings of the present research demonstrated the high level of antifungal and anti aflatoxigenic capability of M. longifolia EO against A. flavus. Therefore, this oil might be used as a natural preservative instead of synthetic chemical fungicides in food and agricultural products against biodegradation and storage contaminations caused by A. flavus.

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FIGURE CAPTIONS:

Table 1: List of plant species and family used in the preliminary screening for antifungal properties.

No.	Plant species	Family
1	*Mentha longifolia*	Lamiaceae
2	*Nepeta asterotricha*	Lamiaceae
3	*Ziziphora clinopodioides*	Lamiaceae
4	*Ferula assa- foetida*	Apiaceae
5	*Heracleum persicum*	Apiaceae

Table 2. Effect of different concentration of *Mentha longifolia* essential oil on inhibition of sporulation and spore germination of *Aspergillus flavus.*

Concentration of EO	Inhibition of sporulation (%)	Inhibition of spore germination (%)
Control	0	0
IC50[1]	0	100
MIC [2]	0	100

[1]: IC50 = Inhibitory concentration of the essential oil with 50% inhibitory effect on *A. flavus* hyphal growth (300 ppm).

[2]: MIC = Minimum inhibitory concentration of *Mentha longifolia* essential oil (650 ppm).

Table 3: Effect of *Mentha longifolia* essential oil on aflatoxin production by *Aspergillus flavus*.

Treatment	Aflatoxin B_1(µg/Kg)	Aflatoxin B_2(µg/Kg)	Total (µg/Kg)
Control	36 a*	3.3 a	39.3 a
IC50[1]	34.3 a	0.6 a	34.9 a
MIC [2]	0.36 b	ND b	0.36 b

ND: Not Detectable

*: Different letters in each column indicate significant difference according to according to Duncan's multiplerange tests at $P \leq 0.05$.

[1]: IC50 = Inhibitory concentration of the essential oil with 50% inhibitory effect on *A. flavus* hyphal growth (300 ppm).

[2]: MIC = Minimum inhibitory concentration of *Mentha longifolia* essential oil (650 ppm).

Table 4: The chemical composition of the essential oil obtained from *Mentha longifolia***.**

Retention index (RI)	Common Name	Area %
933	α-Pinene	0.3
972	Sabinene	0.3
978	β-Pinene	0.5
988	β-Myrcene	0.5
993	3-Octanol	0.5
1029	**Limonene**	**1.7**
1032	**1,8-Cineole**	**2.2**
1171	**1-Borneol**	**1.0**
1278	**Isopiperitenone**	**1.3**
1354	**Piperitenone**	**18.7**
1388	**Piperitenone oxide**	**70.0**
1393	β-Bourbonene	0.2
1406	cis-Jasmone	0.6
1426	trans-Caryophyllene	0.7
1453	Geranylacetone	0.3
1457	Farnesene<(E)-beta->	0.1
1485	Germacrene D	0.3
1500	Bicyclogermacrene	0.1
1584	spathuleno	0.1
1589	Caryophyllene oxide	0.2
1693	n-heptadecane	0.2
1963	Palmitic acid	tr
2149	(E)-9-Octadecenoic acid	tr
2299	n-Tricosane	0.1
Total		99.9

Tr: Trace

Table5. Comparison of major compounds in *Mentha longifolia* essential oil in previous studies and the present research.

Origin Country-Continent	References	Main components
Iran-Asia	Piperitenone oxide (70%), Piperitenone (18.7%),1,8-Cineole (2.2%), Limonene (1.7 %), Isopiperitenone (1.3%) and 1-Borneol (1 %)	This study
	Carvon (1.1- 26.6%); 1,8-cineol (3.4-11.2%); cis-piperitone oxide (1.59-34.94%), Pulegone (14.96-31.25%); Menthone (2.8-15.05%) and iso-Menthone (0.96- 43.79%)	(46)
	Cis-piperitoneoxide (36.4 and 40.5%), piperitenone oxide (22.5 and 37.3%) and caryophyllene oxide (13.65 and 7.43%)	(38)
	1,8-Cineole (15.58%), Piperitenone oxide (15.05%), Pulegone (9.58%), Sabinen (9.52%), p-Mentha-3,8-diene (10.531%), 2,6-Dimethyl-2,4,6-octatriene (10.132%), Sabinene (6.98%), β-Caryophyllene (6.971%), Piperitone oxide (6.77%) and Pulegone (6.60%)	(37)
	Piperitone (43.9%),l imonene (13.7%)and*trans*-piperitol(12.9%)	(49)
Pakistan-Asia	Sample1; Piperitenone oxide (33.91%), Isopiperitenone (11.98%) and Piperitone (8.40%) Sample2:Isopiperitenone (57.96%), Piperitone oxide (19.99%) and 1,8-cineol (5.49%) Sample3:Piperitone (43.96%), 1,8-cineol (13.73%), *trans*-piperitol (12.92%) and *cis*-piperitol (9.34%)	(39) (34) (40)
	Piperitenone oxide (40.1 and 64.6%), Piperitenone (16.4 and 1.97%) and Borneol(13.3 and 4.36%)	
	piperitenone oxide (28.3%), piperitenone(24.9%), Germacrene D (8.16%), Borneol (5.96%), and	

	β-caryophyllene (5.94%)	
Ethiopia-Africa	Epizonarene (29.7%), Caryophyllene (11.3%), Dimethyl malonate (7.5%), β-eudesmol (10.3%), β-cubebene (6.1%), α-cadinol (6.9%),	(53)
Tunisia-Africa	β-bourbonene (4.9%) and -α-guaiene (3.8%)	(48)
	Pulegone (54.41%), Isomenthone (12.02%), 1,8-cineole (7.41%), Borneol (6.85%),and Piperitenone oxide (3.19%)	(9)
		(47)
Southern Africa	pulegone (47.15%), 1,8 cineole (11.54%), menthone (10.7%), α-pinene (3.57%), α-terpineol (3.17%) and d-cadinene (3.53%) and Isomenthone (2.4%)	(41)
	menthol (19.4-32.5%), menthone (20.7-28.8%), 1,8-cineole (5.6-10.8%), terpineol-4 (3.1-4.9%), pulegone (7.8-17.8%) and piperitone (2.2-3.3%)	
	Menthofuran (51-62%), Piperitenone oxide (15-66%) and *cis*-piperitone oxide (15-36%)	
Croatia- Europe	*trans*-dihydrocarvone (23.64%), piperitone (17.33%) and *cis*-dihydrocarvone (15.68%).	(8)
		(42)
Greece- Europe	Carvone (33.48%), piperitenone oxide (28.95%), limonene (10.29%) and *β*-caryophyllene (5.84%)	(44)
Turkey- Europe	Piperitoneoxide (50-66.4%)	(43)
Serbia- Europe	*cis*-piperitone epoxide (18.4%), pulegone (15.5%) and piperitenone oxide(14.7%)	(54)
Italy-Europe	Piperitone (8.8%), Menthone(11.2%) , Pulegone(4.88%), Neo -menthol(3.55%) and Isomenthone(3.13%)	(45)
	Piperitenone oxide (77.43%),Germacrene D (3.7%) and 1,8-cineole (1.61%)	

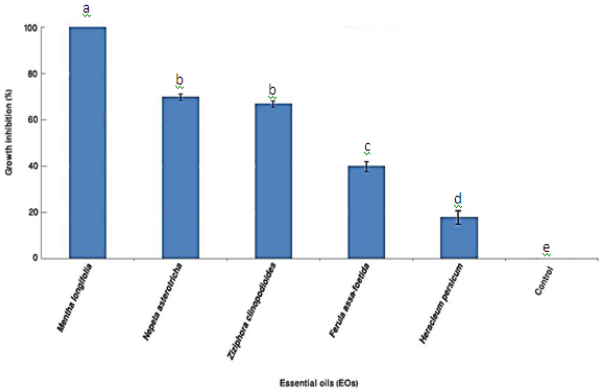

Figure 1: Effect of essential oils obtained from five plant species at 2000 ppm concentration on mycelial growth of *Aspergillus flavus.*Different letters indicate significant differences according to Duncan's multiple range tests at $P \leq 0.05$. The bars indicate standard errors.

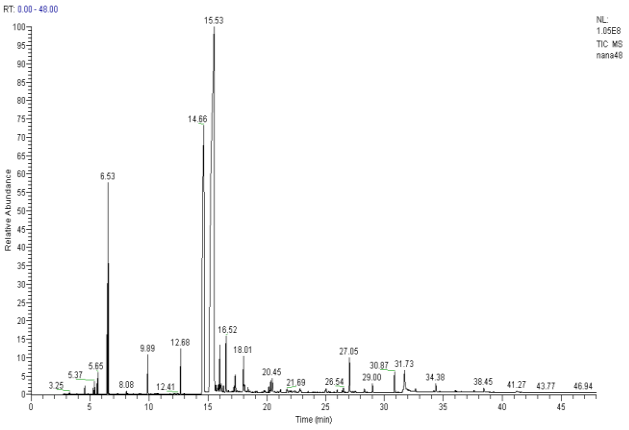

Figure 3: Chromatogram of the essential oil obtained from *Mentha longifolia*.

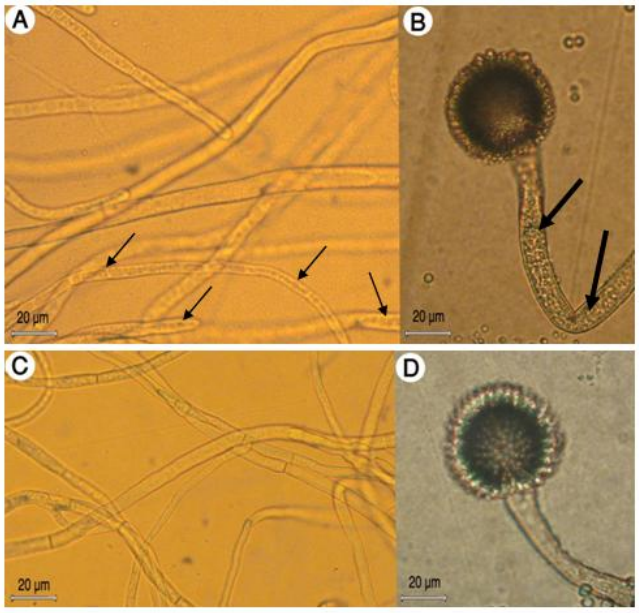

Figure 2: Hyphal morphology of *Aspergillus flavus* grown in potato dextrose agar medium amended with the IC50 concentration of *Mentha longifolia* essential oil (A and B), and in control medium without the oil (C and D). Small arrows marked fragmentation and cytoplasmic coagulation in vegetative hyphae. Large arrows indicated cytoplasmic coagulation in a conidiophore.