Original Research Article

Evaluation of the antifungal activity of Cinnamon, Clove, Thymes, Zataria multiflora, Cumin and Caraway essential oils against ochratoxigenic *Aspergillus ochraceus*

Running head: essential oils

Abstract

Introduction: Mycotoxin producing fungi are major contributors to food corruption and many epidemics in humans and animals. The adverse effects of the use of chemical preservatives have led to a special focus on the use of natural compounds, especially plant derivatives. The aim of this study was to investigate the antifungal properties of herbal essential oils of Cinnamon, Clove, Thymes, and Zataria multiflora, Cumin, and Caraway on the growth of *Aspergillus ochraceus*.

Materials and Methods: Briefly, the presence of mycotoxin producing gene was investigated using PCR. Furthermore, production of mycotoxin in a medium with high performance liquid chromatography with fluorescence detection (FLD) was evaluated. Ingredients of essential oils were determined using GC/MS. The amount of antifungal activity of essential oils was assessed by disc diffusion and well diffusion method. Additionally, the minimum inhibitory concentration (MIC) was determined by macrodilution method.

Results: Gene presence and mycotoxin production were confirmed by PCR and HPLC-FL. Among all studied essential oils, Cinnamon with MIC and MFC of 0.078 µl / ml exhibited the greatest effect on *A. ochraceus* as compared to other essential oils.

Conclusion: This study indicated that essential oils have an effective role on controlling *A. ochraceus* growth and have shown promising to be a good bioactive natural preservative in food industry.

Keywords: *Aspergillus ochraceus*, mycotoxin, essential oils, MIC, antifungal activity .

Introduction

Aspergillus species are opportunistic fungi that are abundantly isolated from contaminated food (1). These fungi are able to infect crops before and after harvest, during the process and handling. Loss of nutritional value and quality loss such as loss of sensory characteristic - appearance, loss of color and taste, and Food Rotting are considered as consequences of fungal damage (2). Some Aspergillus species are also able to produce mycotoxins in addition to undesirable appearance of the food (3) .Some of these toxins include ochratoxin, aflatoxin, patulin, strigmatoxin, penicillic acid and cyclopiazonic acid (4). Ochratoxin (OTA) is known as a nephrotoxin for mammals, and OTA was also categorized in Group 2B of the International Agency for Cancer Research (IARC) in 2004 (5). This mycotoxin has carcinogenic, teratogenic and immunotoxic effects for rats and probably human (3). Regarding to the adverse effects of fungicides on health and the environment, and on the resistance of strains to drugs and synthetic chemical compounds, researchers have focused on more natural and safe chemical compounds (6-8). Studies have revealed that secondary metabolites of many plants are capable of preventing the growth of fungi. Due to their less toxic effects, they can be favorable alternatives to synthetic compounds and toxins (9 -12)

Essential oils as secondary metabolites of plants are a complex combination of fugitive molecules that can be obtained by distillation from different parts of the plant such as stems, roots, leaves (13, 14). Antimicrobial properties of essential oils are revealed to be related to their chemical compounds, including phenolic, alcoholic and oxygenated terpenoids (7, 9, 10) and can be a good alternative to synthetic compounds and toxins (9, 10). Essential oils, secondary metabolites of plants (15), are a complex of volatile organic compounds that can be obtained by distillation from different parts of the plant such as stems, roots, leaves (13, 14).

Antimicrobial properties of essential oils can be related to their chemical compounds, including phenolic, alcoholic compounds and oxygenated terpenoids (7, 9, and 10). The antimicrobial properties of these compounds are affected by the geographical and climatic conditions of the site of planting, harvesting season, plant body, concentration of active compound and microorganism target (16-18) .In Iran, many studies have been carried out for evaluating antimicrobial effects of various essential oils such as Thymus vulgaris, Clove (19), Satureja (20) Eucalyptus, Mentha (21), Caraway (22), Echinophora (23) Nigella sativa (24) and Salvia officinalis (25), on *Aspergillus niger*, *Aspergillus flavus* , *etc.* and aflatoxin production by them in a variety of culture and food environments.

The study by Shokri et al. demonstrated the antifungal activity of Zataria multiflora and Geranium pelargonium essential oils against toxigenic species of *Aspergillus. Flavus*, *A. parasiticus*, *A. ochraceus* and *Fusarium. verticillioides* (26).

A study by Hadi Fakoor et al., reported that the extent of aflatoxin production is dependent on the concentration of essential oil of *Cuminum cyminum* L from Alborz Mountain, Iran. They showed that the increase in the concentration of the essential oil was associated with the decrease in aflatoxin production (21). The aim of this study was to investigate the antifungal properties of essential oil of Cinnamon, Clove, Thymes, Zataria multiflora, Cumin, and Caraway on growth of *Aspergillus ochraceus* K.Wilh by disk diffusion method and well diffusion method.

Material and methods

The strain used in this study, freeze-dried culture of *A. ochraceus* CBS 263.67, was provided by the Westerdijk Fungal Biodiversity Institute. The essential oils used in this study were obtained from Magnolia (Saveh, Iran), Barij Essence (Kashan, Iran) and golghatrehtoos (Mashhad, Iran) Companies with a purity of over 90%. Furthermore, sabrouraud dextrose agar and potato dextrose agar (PDA) were purchased from Merck Company. Moreover, the liquid medium of RPMI 1640 (Glutamine, Phenol Red, without bicarbonate) was prepared from Solarbio. Additionally, agar solution was prepared (Difco, Bitek;

Becton, Dickinson and Company, Sparks, MD, USA). Extraction solvents with HPLC grade and sodium phosphate buffer were also applied (Merck, Germany).

Preparation of fungal suspension

A.Ochraceus was cultured on potato dextrose agar medium for 7-14 days. For fungal suspension, normal saline containing 0.01% Tween 80 was used (27). Concentration of 10^7 was prepared by diluting a lower concentrated solution (10^5) using neubauer and counting spores.

Identification of presence of mycotoxin gene by PCR method

In this study, two primers listed in Table 1 were used to confirm the presence of mycotoxin gene in Aspergillus (28).

Table 1: Primers for confirming the presence of the of mycotoxin gene

Primer Name	Sequence 5'-3'	Reference
AoLC35-12L	GCCAGACCATCGACACTGCATGCTC	Dao et al (2005)
AoLC35-12R	CGACTGGCGTTCCAGTACCATGAGCC	Dao et al (2005)
AoOTA-L	CATCCTGCCGCAACGCTCTATCTTTC	Dao et al (2005)
AoOTA-R	CAATCACCCGAGGTCCAAGAGCCTCG	Dao et al (2005)

DNA extraction and PCR reaction

DNA extraction was performed according to the standard method of Cenis (1992) with a few changes (29). The PCR reaction was conducted with two primers including AoLC35-12L / AoLC35-12R and AoOTA-L / AoOTA-R according to the method of Dao et al (2005) with a slight change (MWG PCR machine; Biotech, Ebersberg, Germany), (28). Furthermore, the PCR reaction was carried out in a mixture of 25 µl containing: 18.25 µl dd H2O, 2.5 µl PCR buffer, 1 µl MgCl2 50 mM, 0.75 µl dNTP, 0.5 µl Taq polymerase and 0.5 µl of the primers and 1 µl of genomic DNA. The reaction conditions included 94 ° C for 4 minutes per cycle, 30 cycles of 94 ° C for 30 seconds, 54 ° C for 30 seconds, 72 ° C for 40 seconds, and 72 ° for 5 minutes. At the end, agarose gel electrophoresis (1.5% agarose gel) was used for separating *DNA* (1.5% agarose gel) (30).

92

93 **Determination of mycotoxin by HPLC**

94 To this end, a HPLC Waters e 2695 equipped (United States) with a fluorescence detector 2475 and
95 Chromolith® columns (4.6 mm column diameter, 20 cm column length) were applied for this purpose.
96 The column temperature was 50 °C with a reversible phase of acetonitrile/methanol, a 150 µL injection
97 volume and a total run-time of 9.5 minutes.

98 The limit of detection (LOD) and limit of quantification (LOQ) were 0.3 and 1 ng/g, respectively.

99 LOD and LOQ of the method were reported by spiking sample at 1 ng/g. The recovery of Ochratoxin was
100 102%. Calibration curves were obtained using OTA standards from 8 different concentration at the range
101 of 0.25-15 ng/mL for samples, with r^2>0.997. Typical chromatograms obtained for OTA is shown in
102 Figure 10. The retention time of OTA was about 7.3 min.

103

104 **Extraction of OTA from the medium**

105 Extraction was carried out according to standard 9238 of Iran. Mycotoxin A was extracted by high-
106 performance liquid chromatography and followed by purification with immunoaffinity chromatography
107 (IAC), (31). The fungi cultivated on Malt Extract Agar at 27 ± 2 ° C were removed from the incubator
108 after ten days to determine the production of toxins. Afterward, the medium was mixed with ethanol. The
109 extract was then diluted with a specific volume of the PBS buffer (24). The extract was passed through an
110 immunoaffinity column (France) followed by column washing with methanol. Determination of the
111 amount was made using the inverse phase method. The fluorescence detector was adjusted with an
112 excitation wavelength of 333 nm and an emission of 477 nm. The area under standard peaks compared
113 with the specimens, taking into account the dilution factor.

114 **Determination of compounds of essential oils with GC**

Sample analysis was performed according to the procedures described by Satyal et al., with minor modifications (32). Briefly, each essential oil was diluted with acetone (1 % v/v), then 0.1 µL of the sample was injected to GC/MS.

GC-MS analysis was performed using a 7890A GC system from Agilent Technologies (Palo Alto, CA, USA) equipped with a 5975C inert MSD network mass selective detector and split / splitless injector. Separation of compounds was performed using an HP-5 MS capillary column (ID 30 m × 250 µm, 0.25 µm film thickness) under a helium flow rate of 0.8 mLmin-1 and in a split mode (1:50 ratio). The oven temperature was programmed at 50 ° C (kept for 5 minutes) to 280 ° C at a rate of 5 ° $Cmin^{-1}$ (run time 46 min). Total ion monitoring (TIs) was used to measure the main component. A hot-plate magnetic stirrer (Heidolph, Germany) with a temperature-controlling probe was used for stirring and heating the sample solution.

Determination of antifungal activity by disc diffusion method

Disc diffusion method was used to evaluate the antifungal activity of six essential oils according to the Carins method (2003-2004) with a slight change (33). Briefly, 100 µl of the fungal suspension prepared on the Malt Extract Agar medium was cultured. After thirty minutes, drying the plates, the paper discs (6 mm, HiMedia Company) were impregnated with pure essential oil (ten microliters) and then placed on the center of the plate. Finally, the plates were closed with parafilm and incubated at 25 ± 2 ° C for 7 days.

Determine antifungal activity by well diffusion method

The determination of antifungal property by well diffusion method was done according to the Rana method (34). One hundred µl of suspension was cultured on Malt Extract Agar medium. After drying the environment, an 8 mm diameter pit was made using a micropipette tip and well was filled with 50 µL of pure essential oil.

Minimum inhibitory concentration (MIC)

In order to determine the MIC of fungi, the macrodilution technique was used with a few changes (35). The RPMI1640 liquid medium was then prepared at pH 7 in a test tube of 1 ml volume. Essences were prepared at a concentration of 1% to 0.00048 % (v / v) in the RPMI medium containing Agar 1% by serial dilution method. Finally, 100 µl of fungal suspension was added to it and the MIC of the essential oils was examined on days 3 and 7 of incubation at 25 ° C.

Minimum fungicidal concentration (MFC)

MFC is the minimum concentration that no growth is observed on the subculture. MFC is the least concentration that no growth is observed on the subculture. Fifty microliters were removed from experimental tubes in which no fungal growth was observed and cultured on a PDA medium, followed by incubation at 27 ± 2 ° C for three days. Plate, which did not show any growth after this period, was considered as MFC (36).

Results

In order to investigate the antifungal activity of essential oils on *A. ochraceus*, impregnated discs were used in two replicates. All essential oils showed a 72-hour growth delay, indicating that there was evidence of growth beginning just after the 48 hours at the margin of the plate compared to a control plate that had good growth after 48 hours.

According to the results, Cinnamon showed a higher inhibitory effect with a mean diameter of 37.82 for zone of inhibition and a standard deviation of 0.68, followed by Cinnamon, Clove, and Zataria multiflora, Thymes, Cumin and Caraway (Table 3). Over time, the essential oils were reduced, this value was higher for essential oils of Cumin and Caraway. After 144 hours, the diameter of the growth halo was less than other essential oils. After 6 days, the diameter of the inhibition zone was not visible (results not shown after day 6), (Table 2).

Table 2: Inhibition zone (mm) of different essential oils with Disk diffusion test against *A. ochraceus* (n=3)

Essential oil type	Incubation time				
	48 h	72 h	96 h	120 h	144 h
Cinnamon	Growth at the margin of the plate	42.4 41 41.5	37.65 37 38.3	36.9 36 37.5	35.4 34.55 35.7
Clove	Growth at the margin of the plate	36.35 36.85 40.25	33.8 35 39.6	32.6 34.3 37.7	31.9 33 34.8
Cumin	Growth at the margin of the plate	24.25 22.95 23.5	18.8 17.5 20.35	17 18 19	15.5 16.35 16
Caraway	Growth at the margin of the plate	30 30.65 30.35	22.2 24.6 25.2	17.4 17.3 17	15.3 16.25 15.75
Thymes	-	32.6 32.75 33.35	31.6 32 32.3	30.65 31.2 31.25	30 30.8 31
Zataria multiflora	-	34.5 36.45 33.35	33.5 34.75 31.35	31.75 33.2 30.2	29.4 31.4 29

Table 3: Mean ± SD inhibition zone for of *A. ochraceus* by disk diffusion method

Incubation time	Cinnamon Mean ± SD	Clove Mean ± SD	Thymes Mean ± SD	Zataria Mean ± SD	Cumin Mean ± SD	Caraway Mean ± SD
T1	41.63±.71	37.82±2.12	32.95±.48	34.77± 1.57	23.57± .65	30.33±.33
T2	37.65± .65	36.13± 3.06	31.97± .35	33.20± 1.72	18.88± 1.43	24.00± 1.59
T3	36.80± .75	34.87± 2.60	31.03± .33	31.72± 1.50	18.00± 1.00	17.23± .21
T4	35.22± .60	33.23± 1.46	30.60± .53	29.93± 1.29	15.95± .43	15.77± .48

The antifungal activity of the essential oils was investigated by well diffusion method. The result of the absence of growth was investigated every 48 hours, the results are shown in Table 4. With a very small

difference, Cinnamon had a more inhibitory effect, followed by Zataria multiflora, Thymes, Clove and, as well as Cumin and Caraway. Over time, the effect of essential oils was found to be reduced and the growth of the *A. ochraceus* has increased. After 7 days, no significant change was observed in the diameter of the inhibition zone (Table 4).

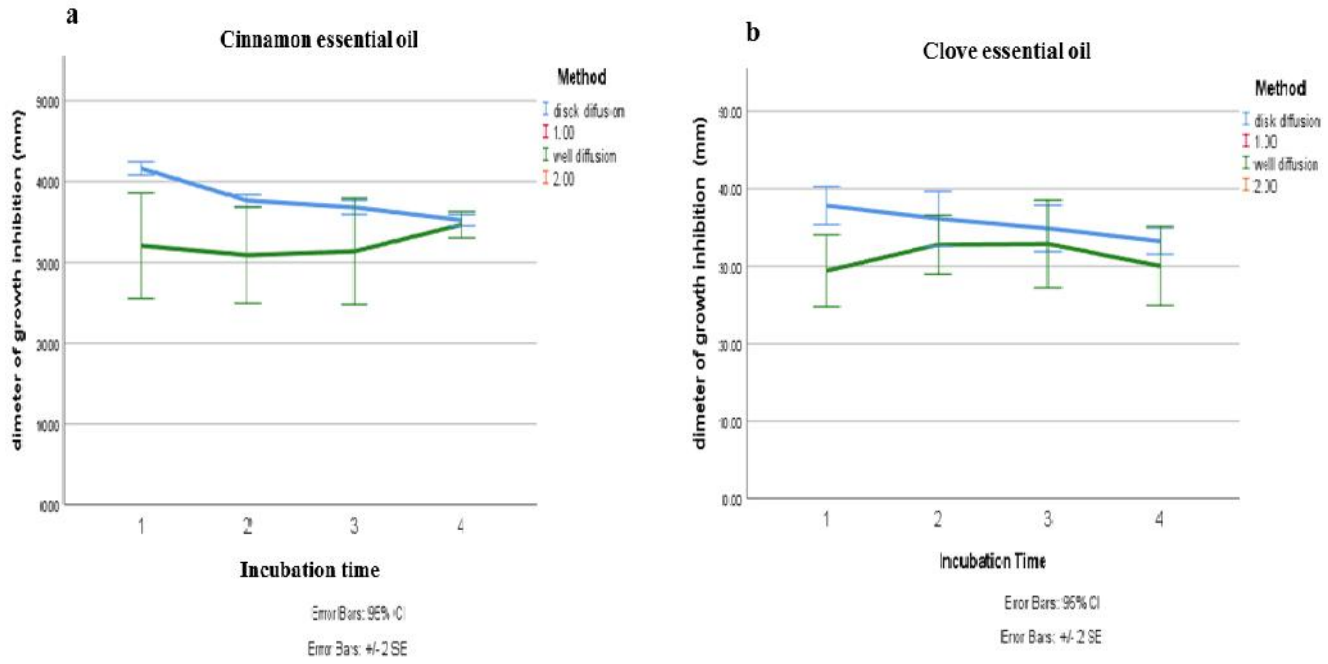
Table 4: Inhibition zone (mm) of different essential oils observed with well diffusion test against *A. ochraceus* (n=3)

	Incubation time			
Essential oils	**48h**	**96h**	**144h**	**192h**
Cinnamon	38.8 39.15 42	30.15 32.17 33.62	29.5 31.27 32.17	29 27.75 28.55
Clove	38.8 37.52 36.35	38.15 37.05 35.5	37.35 36.2 35	36.25 35.1 34
Thymes	42.4 40.9 39.8	40 38.9 37.5	40 38.9 37.5	36 35 34.6
Zataria multiflora	38.8 39.35 42.1	37.75 36.4 40.5	35.5 34.9 38.25	34.8 34.3 36.5
Cumin	36.85 36.6 35.7	35.85 36 35.1	35 35.1 34.2	33.05 34 33.2
Caraway	29.85 31.75 30.6	28.4 29.25 28.7	26.5 27.25 26.55	25 25.7 24.8

Table 5: Mean ± SD inhibition zone for of *A. ochraceus* by well diffusion method

Incubation time	Cinnamon Mean± SD	Clove Mean± SD	Thymes Mean± SD	Zataria Mean± SD	Cumin Mean± SD	Caraway Mean± SD
T1	32.07 ±5.65	29.42± 4.02	36.23± 1.24	35.25± 5.66	32.51± 2.84	39.50± 1.21
T2	30.88± 5.15	32.77± 3.29	35.23± 2.92	34.02± 4.68	34.30± 3.66	41.25± 1.66
T3	31.37± 5.70	32.87± 4.90	34.84± 3.49	34.57± 5.48	35.48± 3.32	39.20± 2.88
T4	34.67± 1.40	30.03± 4.42	33.68± 3.65	37.62± 2.45	33.69± 3.13	38.71± 1.14

By applying well diffusion method, the inhibition zone was appeared after 72 hours, and the diameter of the inhibition zone formed in this method was revealed to be greater than well diffusion test; however, after 48 hours, inhibition zone appeared in well diffusion test which indicates the greater sensitivity of this method, but the inhibition zone in this method was found to be less than disk diffusion method. The effects of essential oils on growth of *A. ochraceus* using two methods of disc diffusion and well diffusion are shown in Fig1.



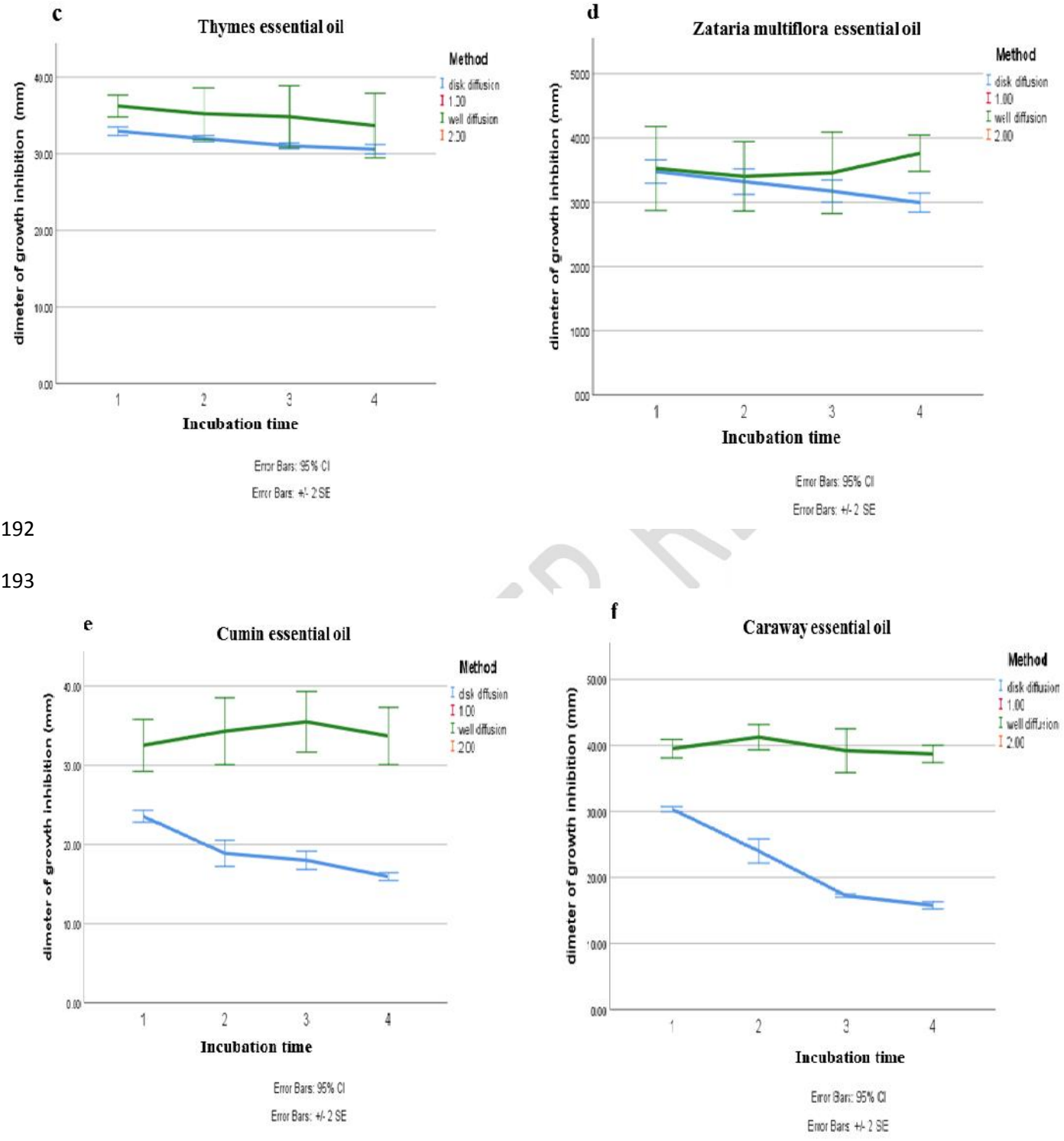


Figure 1: The effect of essential oils on the growth of *A. ochraceus* using disc diffusion and well diffusion methods. a) Essential oil of Cinnamon, b) Essential Oil of Clove, c) Essential oil of Thymes, d) Essential oil of Zataria multiflora, e) Essential oil of Cumin, f) Essential oil of Caraway.

MIC and MFC

MIC of six essential oils was determined on days 3 and 7 of incubation. In addition, MFC obtained after the three-day incubation at 28 ° C in µl per milliliter is shown in Table 6.

Table 6: MIC (µl/ml) and MFC

essential oil	MIC on the third day	MIC on the seventh day	MFC
Cinnamon	0.039	0.078	0.078
Clove	0/156	0/312	1.25
Thymus	0/078	0/312	0/312
Zataria multiflora	0/156	0.625	1.25
Cumin	1.25	2.5	2.5
Caraway	0/156	0.625	1.25

ANOVA test demonstrated a significant difference between the two methods used (p value = 0.000). Among the essential oils studied, Cinnamon had the highest rate of inhibition. The important chemical compositions of essential oils obtained from GC were indicated in Table 7. Figures from 2 to 7 depict the peaks of essential oils.

Table 7: Chemical compositions of essential oils

	Type of essential oil					
Composition	**Thymus**	**Zataria multiflora**	**Cinnamon**	**Clove**	**Cumin**	**Caraway**
E-Cinnamaldehyde	-	-	95.45	0.19	-	-
Carvacrol	0.08	26/62	-	-	-	7.43
Thymol	41.71	39.14	-	-	0.92	1.02
Eugenol	0.02	-	0.53	93.56	0.06	0.01
α –Pinene	1.17	3/38	-	-	2.35	1.16
β - Pinene	1.57				25.22	
p-Cymen	28.97	9/44	-	-	37.78	34.5

γ-Terpinene	13.15	6/59	-	-	10.79	0.22
Caryophyllene	0.24	2/22	-	-	0.1	0.25
Caryophyllene oxide	-	-	-	-	-	0.5
Camphor	0.05	0.06	-	-	3.78	-
Carene	-	1.44	-	-	0.05	27.54
Limonene	2.69	0.45	-	-	2.91	2.55
Terpineol	0.02	0.7	-	-	0.1	-
Borneol	0.18	-	-	-	-	-
Spthulenol	-	0.52	-	-	0.05	-
α –phellandrene	-	0.09	-	-	0.46	1.83
β –phellandrene	-	-	-	-	-	-
Camphene	0.02	0.15	-	-	0.5	0.13
β–myrcene	0/43	0.63	-	-	0.43	0.51
Linalool oxide	0.01	-	-	-	-	0.01
Thujone	-	0.02	-	-	0.51	0.02
Nerodiol	-	-	-	-	0.02	-
Pulegone	-	0.01	-	-	0.04	-
Cuminal	1.38	-	-	-	11.65	-
Estragol	-	-	-	-	-	5.86

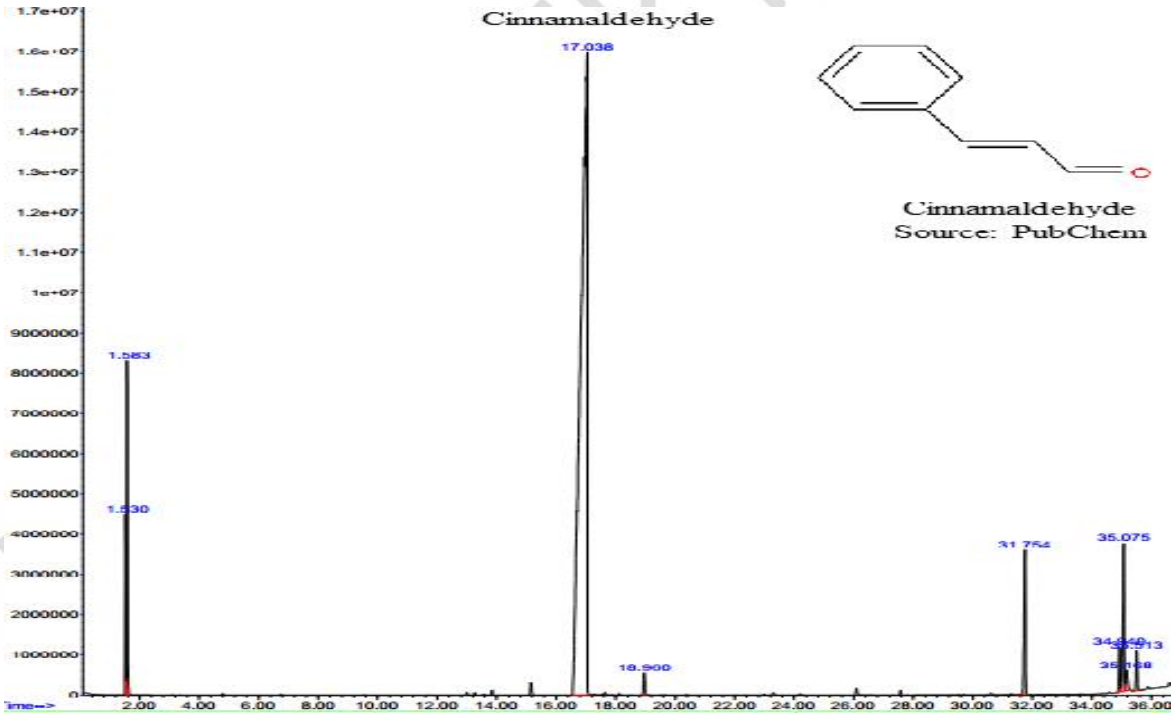


Figure 2: The GC/MS chromatogram of Cinnamon essential oils

Cinnamaldehyde (C6H5CH=CHCHO) is the main compound obtained from the analysis of Cinnamon essential oils, which is most frequently found in Cinnamon essential oils (37). Studies have indicated that cinnamaldehyde is more potent than other antifungals containing an aldehyde group. The chemical structure of this material (outer CH chain and conjugated double bonds) plays a key role in demonstrating these properties (38).

The studied Clove contains about 93% of the eugenol.

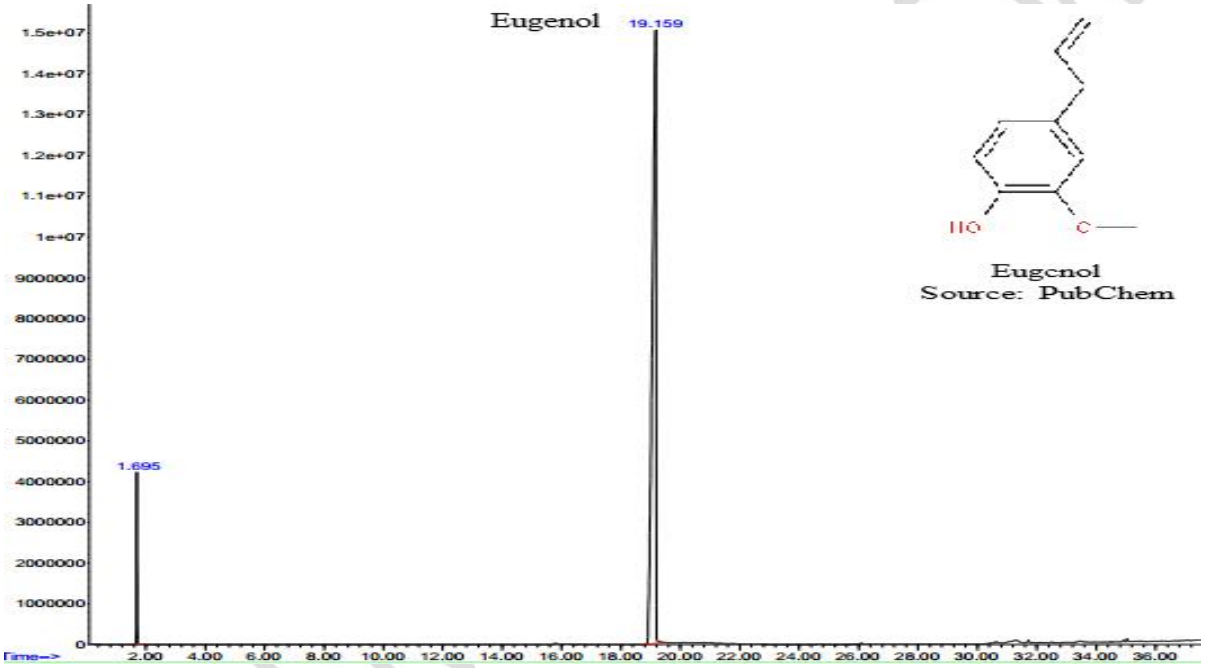


Figure 3: The GC/MS chromatogram of Clove essential oils

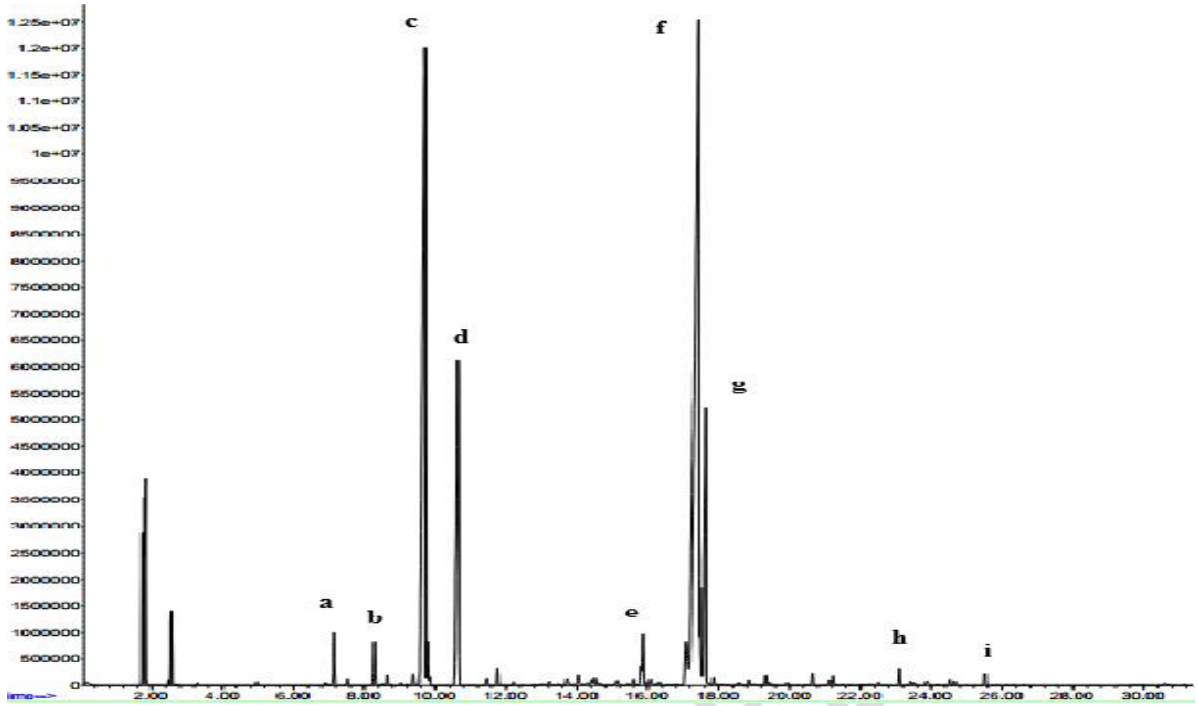


Figure 4: The GC/MS chromatogram of Thymus essential oils (Camphene:a, b-Pinene:b, Benzene:c, T- Linalool Oxide:d, Pulegone:e, Thymol:f, Carvacrol:g, α-Terpineol:h, Apiol:i)

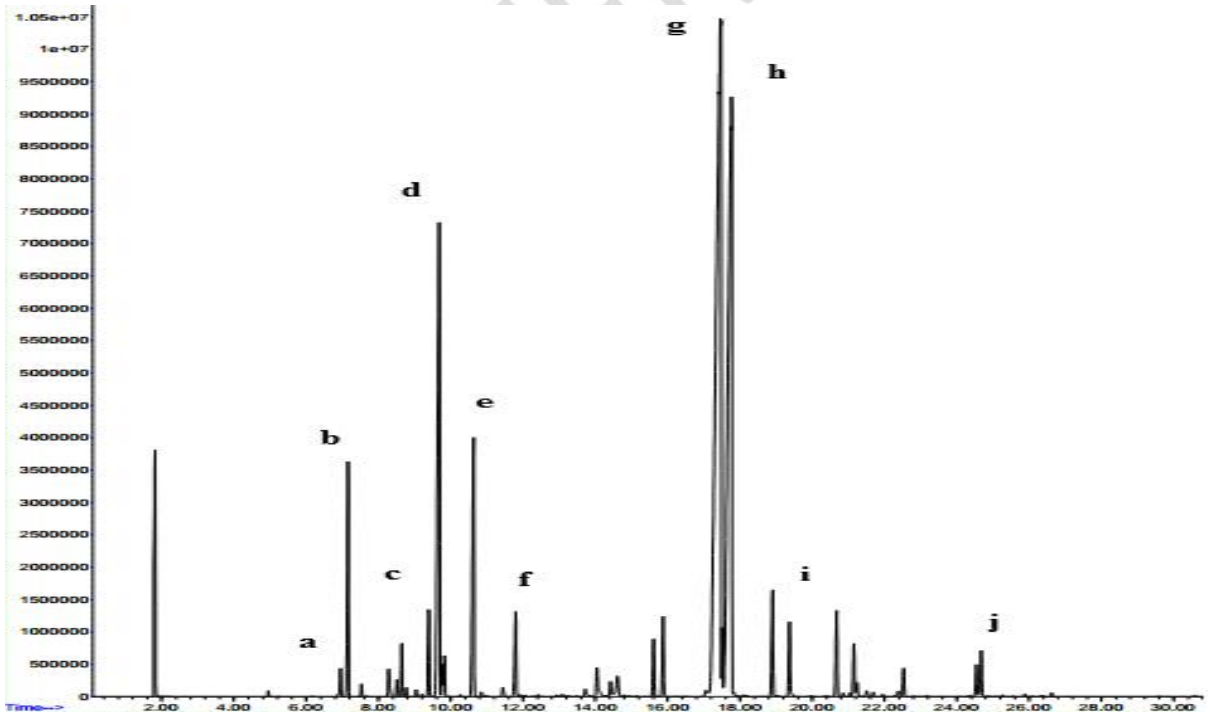


Figure 5: The GC/MS chromatogram of Zataria multiflora essential oils (α-Pinene:a, b-Myrcene:b, Limonene:c, Eucalyptol:d, Terpineol:e, Thujone:f, Thymol:g, Thiophene:h, caryophyllene:i, , Caryophyllene oxide:j)

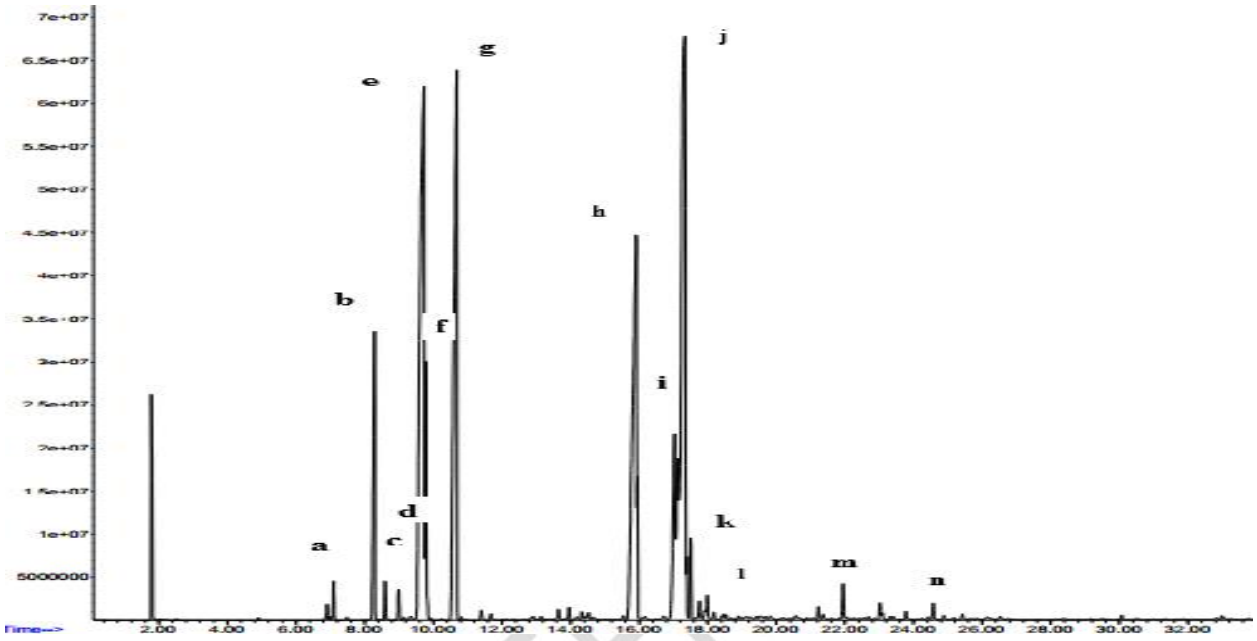


Figure 6: The GC/MS chromatogram of Cumin essential oils (Camphene:a, b-Pinene:b, b-myrcene,:c, α-phellandrene:d, p-Cymen:e,D-Limonene:f, Carene:g,Propanol-2methyl:h, hedroxy-2methylacetophene:i, Vinylguaiacol:j, Thymol:k, Thiophenealdehyde:l, α-Amorphene:m, Carotol:n)

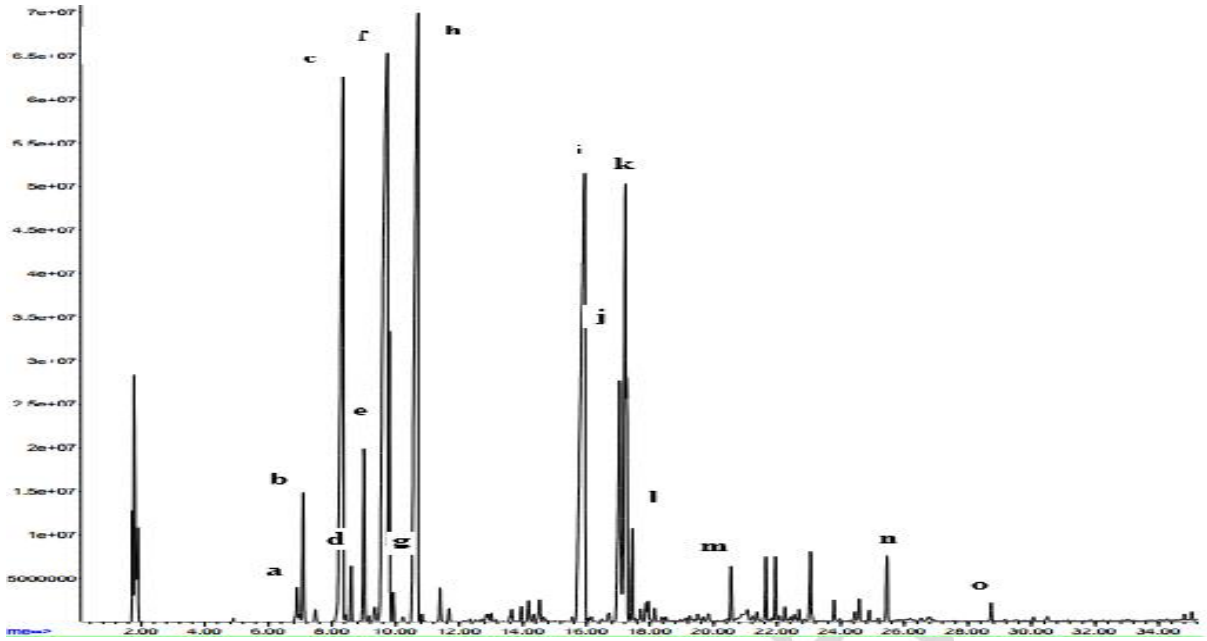


Figure 7: The GC/MS chromatogram of Caraway essential oils (Xylene:a, Bicyclohexan4-methyl-1:b, 3-Carene:c, b-Myrcene:d, 4-Carene:e, p-Cymen:f, D-Limonene:g, Carene:h, P-Cymen:i, Estragol:j, Crvacrol:k, Thymol:l, Benzoic acid:m, Apiol:n, 7-Hydroxy2-methyisoflavone:o)

The findings of the analysis exhibited that the main components of the Cumin and Caraway essential oil are p-Cymene, Carvacrol, Carene and Limonene, respectively.

The gene producing mycotoxin

The PCR reaction indicates the presence of the mycotoxin producing gene in *A. ochraceus* (Fig. 8, 9).

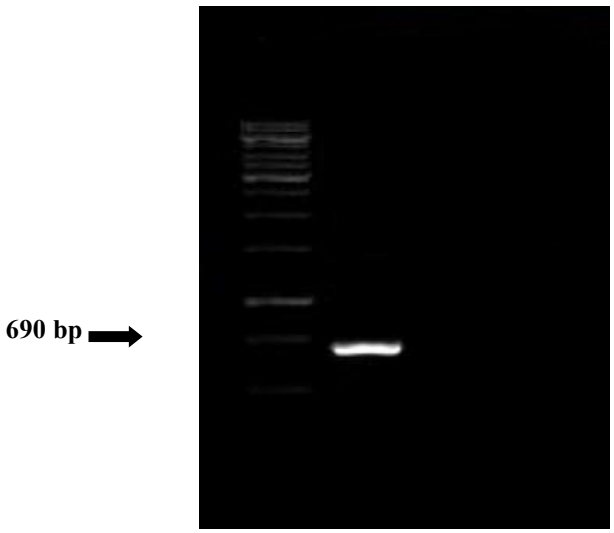


Figure 8: Results of the PCR reaction using AoOTA primer. Lane 1: Ladder, lane 2: PCR product of mycotoxin gene (690 bp)

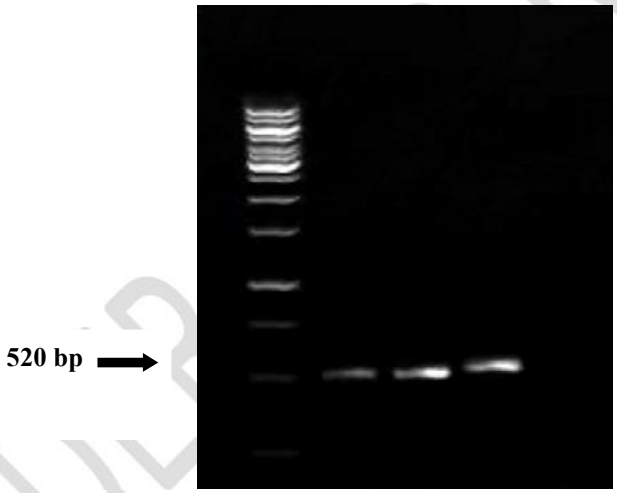
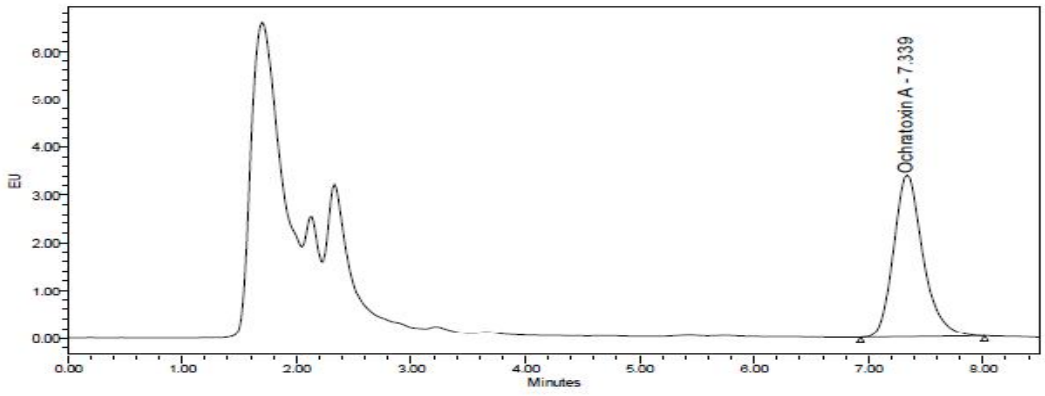


Figure 9: Results of the PCR reaction using primer AOL35-12L Primer. Lane 1: Ladder, lane 2: PCR product of mycotoxin gene (520 bp)

Given the confirmation of the presence of AoOTA and AOL35-12, the findings revealed that Aspergillus is a mycotoxin-producer.

Toxin production

HPLC results of *A. ochraceus* CBS 263.67 demonstrated the production of toxins in the culture medium (Fig. 10). The peak level in minutes between 7 and 8 represents the amount of mycotoxin produced by the fungus.



	Peak Name	RT	Area	% Area	Height
1	Zearalenon	6.300			
2	Ochratoxin A	7.339	605530	100.00	33938

Figure 10: The result of HPLC confirmed the presence of the mycotoxin producing gene and the production of toxin by the *A. ochraceus*.

Discussions

The presence of secondary compounds in medicinal plants has attracted particular attention in recent years. The researchers have shown that the essential oils of aromatic plants contain *v*olatile aroma*,* the main constituents of which are hydrocarbons, aldehydes, ketones, alcohols, phenols, ethers, esters with phenolic and terpenoids origin (39, 40). Recognizing medicinal plants and extracting their pure active ingredient will pave the way for mass production of effective natural pesticides with the least adverse effects (41, 42). The sensitivity of the fungal species varies depending on the essential oil and concentrations used. It is worth noting that the difference in the antifungal activity of essential oils depends on their composition (43) .A compound may be antifungal activity alone or in combination with other compounds (38).

Shakarami et al. evaluated inhibition effects of essential oils of some plant species (Myrtus communis, Vitex agnuscastus, Thymus daenensis, Mentha aquatica, and Artemisia aucheri) on the mycelial growth of plant pathogenic fungi including *Fusarium oxysporum,* and *Rhizoctonia solani*. They showed that essential oil of M. aquatica and T. daenensis herbs inhibited the mycelial growth of these fungi (93.70 and 92.74%) (44). another study indicated that *Acacia catechu*, Dedonia viscosaare, and Lawsonia alba are capable of showing a strong inhibitory against mycelium growth of *Fusarium solani* (45).

In 2015, Patel and his colleagues assessed antifungal effect of Eucalyptus essential oil on Aspergillus species. The results of these experiments indicated that Eucalyptus extract has a strong inhibitory effect on the growth of Aspergillus species (46).

In the present study, the inhibitory and lethal effects of oily essential oils of Cinnamon, Zataria multiflora, Thymes, Clove and, as well as Cumin and Caraway on the mycotoxin generative species of *A. ochraceus*. The essential oil of Cinnamon exhibited a more inhibitory effect than other essential oils, especially caraway.

The major components of Clove in Iran are Eugenol, β-caryophyllene, Eugenol acetate, α-humelene and Caryophyllene oxide (47), respectively. This essential oil is a strong antimicrobial agent due to the presence of Eugenol. This issue has been confirmed by disc and well diffusion methods due to the diameter of inhibition zone. Major compounds of thyme are carvacrol (45.54%), α-Terpineol (22.96%) and Endo-borneol (14.29%) (48).

The most important compounds of Zataria multiflora are indicated to be Thymol and carvacrol (both of them: 71%), as well as p-Cymen 7.78%), alpha-Terpinene (3.88%) and Beta-caryophyllene (2.06%).

The acidic nature of the hydroxyl group in the carvacrol and thymol compounds and its hydrogen bonds are capable of enhancing the antimicrobial activity of these essential oils. In Zataria multiflora, other compounds such as para-cymene, terpinene-α, 1,8-cineole, terpinen-4-ol and spathulenol along with carvacrol and thymol, improve the antimicrobial activity of this essential oil (49).

297 Cinnamon is a spice belonging to the several tree species of genus Cinnamomum, which is mainly native
298 to Sri Lanka, India and China. The oily essential oil of this plant has a strong antimicrobial effect and is
299 used as an aromatic condiment and flavoring additive. The components of these essential oils have been
300 studied frequently and it has been shown that cinnamaldehyde in Cinnamon has antibacterial activity.
301 Cinnamaldehyde, by attaching a carbonyl group to the protein of microbial cells and preventing the
302 decarboxylation of amino acids, exerts its own antimicrobial properties. As shown in this study, the
303 diameter of the inhibition zone in the Cinnamon essential oil indicated the appropriate inhibitory effect of
304 cinnamaldehyde on *A. ochraceus* (37).

305 Prevention of toxin activity by plant essential oils can indirectly result from disturbances in a series of
306 factors such as transcription, translation or direct inactivation of toxins. However, herbal essential oils can
307 interrupt the production of toxin in several ways via their components such as aldehyde esters, ketones
308 and terpenes. These natural herbal essential oils are superior to many commonly used antimicrobial
309 agents that affect only one target (50).

310 There is a considerable variation in the content and composition of the Cumin essential oil; for instance,
311 essential oil from the Cumin fruits contains carvone and limonene, while carawayaldehyde is the most
312 compound in essential oil obtained from Cumin seeds.

313 By comparing the active ingredients obtained from the essential oils of two Cumin and Caraway
314 (regardless of the planting area), many common compounds can be seen. Ketones such as carvone, trans
315 and cis-Dihydrocarvone are important components of the essential oil of Cumin seeds. In Comparison
316 with Cinnamon and thyme, this essential oil has an antimicrobial effect, which is confirmed by the results
317 of disc and well diffusion methods. The antifungal effect of these plants can be due to the presence of
318 antioxidant compounds through damage to DNA, and mitochondria, as well as cell wall damage, leading
319 to death of microorganisms (51).

The results of analysis of variance reveled that there was no significant difference between the inhibition zone in two disc and well diffusion methods (95% CI, p value = 0.000).

Among the studied groups, Cinnamon with the mean of inhibition zone of 38.24 mm, had the highest effect against *A. ochraceus*. Cumin, with the mean of inhibition zone of 19.93 mm, was found to have the least effect. As shown in Table 6, Cinnamon essential oil has the highest minimum inhibitory and fungicidal effects on *A. ochraceus* compared to other essential oils. Based on the findings presented in the current study, With the exception of Cumin and Caraway, the effects of essential oils in the studied concentrations were statistically significant.

Conclusion

Our result was indicated that Cinnamon, Clove, Thyme, Zataria, Cumin and Caraway essential oils have antifungal activity against *A.ochraceus*. Cinnamon and Clove essential oil were higher antifungal activity than the others due to two component of cinnamaldehyde and eugenol. Furthermore all our studied essential oils have fungistatic effect at different concentrations, considering the toxin production by our Aspergillus this becomes more important.

Given the effectiveness of the essential oils against *A.ochraceus growth,* and the increasing interest of consumers to use food products without preservatives, it was concluded that they can be used as natural preservatives instead of artificial preservatives in medicine, food flavoring and food preservation.

Conflict of interest

No potential conflict of interest was reported by the authors.

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