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Control of *Aspergilus niger in vitro* and *in vivo* by three Iranian essential oils

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Abstract

The objective of present study was to determine the antifungal activity of thyme (*Thymus vulgaris* L.), savory (*Satureja hortensis* L.) and sage (*Salvia officinalis* L.) essential oils (EOs) at different concentrations (500, 1000 and 1500 ppm) against *Apergillus niger in vitro* and *in vivo* (tomato paste) as a natural food preservative. Gas chromatography-mass spectrometry (GC-MS) was used to analyze the chemical composition of the three EOs.Thymol (30.91%), ρ-cymene (14.66%), carvacrol (11.96%) and borneol (11.39%) were the main components in thyme EO. However, carvacrol (31.32%), γ-terpinene (30.03%), thymol (20.56%); and carvacrol (46.11%), camphor (9.77%), α-thujene (9.09%) were the main components in savory and sage EOs, respectively. Furthermore, the results of disc diffusion test and minimum inhibitory concentrations (MICs) showed the highest antifungal activity for thyme, followed by savory and sage EOs. *In vivo* tests in tomato paste exhibited the greatest antifungal properties for thyme and sage EOs. Sensory evaluation results showed that all tested EOs had negative effects on the organoleptic properties of tomato paste, however application of them in a food (pizza) were mask their high odorous.

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Introduction

Molds are usually one of the main problems for food products which causes loss of quality and quantity of foods by create a bad appearance, production of mycotoxins, and loss of nutrition properties. According to the Food and Agriculture Organization (FAO), mycotoxins contaminate about 25% of foods, annually around the world (Bhat *et al.,* 2010). As a solution, addition of synthetic preservatives is a common method to inhibit fungal growth in food products. However, they show harmful effects on human health, and thus consumers are demanding for food products without any chemical preservatives.Therefore, manufactures are looking for safe alternatives to chemical additives (Brul and Coote, 1999; Baydar *et al.*, 2004).

Application of plant EOs with antimicrobial activity is a promising alternative to chemical preservatives. EOs are known as "GRAS" by FDA and are biodegradable, which remains no residual in the earth (Stevic *et al*., 2014; Prakash *et al.*, 2015). EOs, are volatile liquids and contain a mixture of different constituents with different contents. Terpenoids and phenylpropanoids include major constituents of EOs, which provide typical aroma and antimicrobial properties of EOs. The antimicrobial properties of EOs have been investigated for a long time. Having hydrophobic nature enable EOs to penetrate from the cell wall and mitochondria, interact with cell organs and destroy them. This leads to leakage of cell contents and consequently microorganism death (Burt, 2004). Several studies have reported the antimicrobial activity of spices or their EOs (Raut and Karuppayil, 2014; Noshirvani *et al.*, 2017; Fasihi *et al*., 2017). Thyme (*Thymus vulgaris* L.), savory (*Satureja hortensis)* and sage (*Salvia officinalis* L.) have been used in Iranian folk medicine from many years ago and are cultivated in many parts of Iran. Previous studies have shown the antimicrobial and antifungal properties of *Satureja hortensis* (Gulluce *et al*., 2003; Dikbas *et al*., 2008); savory and oregano (Ozcan and Chalchat, 2004); and *Thymus vulgaris* (Kohiyama *et al*., 2015). Ivanovic *et al.*(2012) evaluated the antibacterial effects of thyme, sage and rosemary EOs on some food-associated bacteria and showed the highest antibacterial activity for thyme, followed by rosemary and sage.

Tomato paste, which is usually used as a food seasoning agent, is mainly contaminated by molds when its package is opened. Mariutti and Soares. (2009) have indicated, *Alternaria, Aspergillus, Botrytis, Cladosporium, Colletotrichum, Fusarium, Geotrichum, Mucor, Penicillium, Phytophthora, Rhizophus,* and *Stemphyliumare* as the main fungal genera in tomato. In another study, Kalyoncu *et al.*

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(2005) identified the common fungi associated with tomato and tomato paste and indicated *A. niger* constituted 1/6th of all the identified species in tomato paste. Sinha and Saxena. (1987) have also reported *A.niger* is responsible for tomato spoilage. *A.niger* is the most important member of the genus *Aseprgillus*, which can grow in a wide range of food products and consequently, causes food spoilage and produces hazardous mycotoxins in foods products (Wu *et al.,* 2014).

Considering to the fact that addition of any chemical preservative to tomato paste is forbidden from Iranian National Standards Organization. In addition, because of increasing consumers demand for safe foods without any chemical agents, researchers and manufactures are looking for new methods for preservation of tomato paste from mold growth. As mentioned in previous section, application of EOs is a promising method to inhibit fungal development in food products. Numerous studies have reported the effects of EOs *in vitro* conditions, whereas there are limited researches about *in vivo* conditions. Feng and Zheng (2007) studied the effectiveness of different concentrations (100-500 ppm) of thyme, sage, nutmeg, eucaptus and cassia EOs against *Alternaria alternaria in vitro* and *in vivo* (cherry tomato). In another study, Omidbeygi *et al.* (2007) studied on the effects of thyme, savory and clove EOs against *A.flavus* in tomato paste and the results showed the strongest antifungal properties of thyme and savory among all EOs. In addition, Tian et al. (2011) reported a good potential of dill EO to control of fungi spoilage in cherry tomatoes. In another study, Kalantary *et al.* (2014), represented antifungal effects of cinnamon and oregano EOs against *A. flavus* in tomato paste. Most of the studies have evaluated the antifungal effects of EOs and to the best of our knowledge, there is no research to study the effects of EOs on the natural organisms in tomato paste. Thus, the aim of present study is to evaluation of the antifungal properties of thyme, savory and sage EOs in culture media and tomato paste against *A. niger*, as well as on the normal flora of tomato paste.

Material and Methods

Extraction of EOs

Steam distillation was used to extraction of the EOs. The plants of thyme, savory and sage were obtained from Bu-Ali Sina herbal garden of Jihad-e-Agriculture (Hamedan, Iran). The leaves were then dried under ambient conditions (30–40°C) for 3 days. Five hundred (500) g of each three dried plants, placed in a 2-L flask and 1 L of distilled water was added. A continuous steam extraction was performed using a Clevenger-type apparatus for approximately 3 h. The obtained EOs were dried over anhydrous sodium sulfate then stored at 4°C until used (Skandamis *et al*., 2000). The overall yield of EOs was calculated as described by Langa *et al.*(2009):

Yield of EOs = Yield of EOs = (mass of solute)/ (mass of dried plant) × 100

GC and GC–MS analysis

GC/MS (Thermo Trace GC Ultra/ThermoTrace ISQ, Courtaboeuf, France) was employed to analyze the EOs. Helium was used as the carrier gas at a constant flow of 1.2 mL/min. The injection port was held at 230°C and used in the split mode (split ratio: 1/50). The transfer line was maintained at 250°C. The separations were carried out on an Ultra Alloy capillary column (5% phenyl methyl polysiloxane; 30 m x 0.25 mm i.d. and 0.25 µm film thickness). The oven temperature was programmed from 50°C to 300°C at 15°C/min; the final temperature was maintained for 1 min. The mass spectrometer was used in electronic ionization mode (70 eV) with an acquisition mass range from m/z 30 to m/z 800. The relative percentage of the components was calculated from their peak area in the total ion chromatogram by the Excalibur 2.1 software of the device. Compounds were identified by comparison of their mass spectral fragmentation patterns with those present in the NIST 2.0f (2008) library. The most likely structures were retained.

Preparation of tomato paste

Tomato paste was prepared in a laboratory by concentrating tomato juice to 27°Brix with a laboratory evaporator at 85°C for 3h.

Preparation of the conidial suspension

A. niger (PTCC 5298) was obtained from the culture collection at Iran Institute of Industrial and Scientific Research (Tehran, Iran). Preparation of fungal suspensions was done as described by continue. Briefly, the fungal stock culture was kept frozen (-25°C) in potato dextrose broth supplemented with 30% glycerol. The fungal strain was inoculated on sabouraud dextrose agar (SDA) and incubated at 25°C until sporulation. Then the spores were re-suspended in physiological water with 0.1% Tween 80. The spores were counted by using a Neubauer hemocytometer (Simax Kavalier, Germany) and the concentration was adjusted to10^6 spores/mL using physiological water.

Determination of the minimum inhibitory concentration (MIC)

The MIC values of three EOs were obtained by using the disc diffusion test. Three EOs were diluted in a sterile solution of Tween 80 at 0.001% and evaluated in final concentrations that ranged from 200, 400, 800, 1200, 2400 and 3000 ppm. 15 mL of warm SDA medium was poured into a 90 mm sterile plastic petri dishes and after solidification the surface of medium was inoculated with 100 µL of spore suspension (10^6CFU/mL), and then was spread gently. Afterward, a 6 mm diameter sterilized paper disc (Whatman, No 1) was deposited on the medium surface, then 10 µL of the serial EOs dilutions was added to each petri dish. The petri dishes were then sealed using sterile laboratory Parafilm® to avoid evaporation of the EOs. Blank discs with and without 0.001% Tween 80 solution served as negative control. The positive control was performed in the medium containing only the conidia suspension. After incubation at 25°C for 5 days, the MICs were recorded based on the inhibitory activity against fungal growth. The lowest concentration which completely inhibited fungal growth after a specified incubation period was considered as the MIC (Dikbas *et al*., 2008).

Disc-diffusion assay

The antifungal activity of all EOs against *A.niger* was determined by the disc diffusion method. SDA was poured into the petri dishes and after solidification, the fungal suspension (100 µL) was spread on the culture media. Then, three EOs were dissolved in 0.001% Tween 80, then sterilized by filtration through 0.45 µm syringe filter. A 6 mm diameter paper disc (Whatman, No 1) deposited on the medium surface in the center of plates and 10 µL of different concentrations of each EOs poured on the disc blank. Blank was made by adding 10 µL of water on the disc blank. The petri dishes were sealed using sterile laboratory Parafilm® to avoid evaporation of the EOs, followed by incubation at 25°C. Antifungal activity was evaluated by measuring the mycelial growth of the test fungi. All tests were performed in triplicate. The percentage of mycelial inhibition was calculated, using the following formula (Pandey *et al.*, 1982):

$$\text{Percentage of mycelial inhibition} = \frac{dc - dt}{dc} \times 100$$

where, d_c is the mean colony diameter for the control and d_t is the mean colony diameter for the treatment.

Effects of EOs against A. niger *in tomato paste*

In order toevaluate the antifungal effects of three EOs against *A.niger* in tomato paste, 15 grams of tomato paste was added to each container, then different concentrations of three EOs (500, 1000, 1500 ppm) were added to the tomato paste, and mixed gently. Afterward, all of the samples were inoculated with 100 µL (10^6CFU/mL) of fungal suspension. All of the containers were kept at 25°C until visual observation of fungal development. In addition, some tomato paste samples were remained without fungal inoculation, in order to investigate the effect of all tested EOs on the natural microflora of tomato paste.

Determination of the number of yeasts and molds

The number of yeasts and molds was evaluated at days of 0, 15, 24, 30, 45 and 60 according to the Guttirez *et al.*(2009) with some modification. 10 g of tomato paste was aseptically weighted and poured into a sterilized flask include 90 mL physiologic water (NaCl 0.9% W/V) and was mixed under aseptic condition for 5 min at 260 rpm. Afterwards, serial dilutions (10^{-1}-10^{-5}) were prepared, and 1 mL of each dilution was inoculated into the petri dishes which includes about 15 mL solidified SDA medium. The plates were incubated at 25°C for 5 days, then the numbers of yeasts and molds were counted and expressed in a logarithm scale (log CFU/g).

Sensory analysis

Sensory evaluation of tomato paste samples (pure and were in pizza) performed by a 50 member sensory panels composed of staff from the laboratory. Each attribute was scored on a 5-point hedonic scale where 1= very bad, 2= bad, 3= not bad, not good, 4= good, 5= very good. The blank sample prepared without adding EOs. Also, commercial tomato paste was used to compare with all samples. The scores of all 50 panelists were pooled; then the mean values and standard deviations were calculated. Pizzas were prepared by tomato paste, mozzarella cheese, oregano as seasoning agent, and industrial freeze pizza paste.

Statistical analysis

This experiment was set up as factorial based on completely randomized design (CRD), all tests were performed in triplicate and data displayed as mean values with standard deviations. Also, the effects of different treatments over the time were analyzed by Split Plot design. Statistical analysis was carried out using SAS software (V9.1).

Results and Discussion

Chemical composition of EOs

Thyme, savory and sage EOs were analyzed by GC and GC-MS and their compounds are presented in Table 1. As seen, major components of thyme, savory and sage EOs were: thymol (30.91%), ρ-cymene (14.66%), carvacrol (11.96%), borneol (11.39%), linalool (7.19%) and, γ-terpinene (5.53%); carvacrol (31.32%), γ-terpinene (30.03%), thymol (20.56%) and ρ-cymene (4.28%); carvacrol (46.11%), camphor (9.77%), α-thujene (9.09%), 1,8-cineole (6.14%) and α-pinene (3.83%), respectively.

These results are in agreement with those of Ozcan and Chalchat. (2004); Arraiza *et al.*(2009); Fei *et al.* (2011); and Ivanivic *et al.* (2012) found thymol (36.3-48.49%), p-cymene (9.9-27.8%) and γ-terpinene (5.3-16.2%) were the main components of thyme (*Thymus vulgaris* L.). Similarly, the major components found in sage EO by Pinto *et al.* (2007); Fasses *et al.* (2008); Hayuoni *et al.* (2008);and Ivanivic *et al*.(2012) were, 1, 8- cineole, (9.11-33.27%), α-thujone (13.45-33.55%), camphor (8.5 - 20.81%) and borneol (4-7.39%). Also, Ozturk. (2012) proposed carvacrol (34.6), thymol (12.8%), p-cymene (13%) and γ-terpinene (22.9%) were the main components of savory (*Saturejathymbra* L.). Obtained yields of the EOs (w/w on dry weight basis) extracted by hydrodistillation method are presented in Table 1. The yield of thyme, sage and savory EOs were 1.09%, 0.52% and 0.86%, respectively. Thyme showed the highest yield of EO extraction significantly (P<0.01) among all EOs; however the lowest extraction yield was obtained for sage. Our results are in agreement with the study of Rasooli *et al.*(2006), indicated the yields of 1.2% and 1.0% w/w for two species of thyme namely, *T. eriocalyx* and *T. x-porlock*, respectively.

Minimum inhibitory concentration (MIC)

MIC values were obtained by disc diffusion method are shown in Table 1. The lowest MIC value (200 ppm) was obtained for thyme, followed by 400 ppm for savory and 2400 ppm for sage. The results indicated the highest antifungal properties against *A. niger f*or thyme EO among all three studied EOs. Our observations are in good agreement with the study of Stevic *et al.* (2014) that showed lower MIC for thyme (0.07 mg/mL) than savory (0.28 mg/mL). In another study, Soliman and Badeaa (2002) showed that thyme EO inhibited *A. flavus, A. parasiticus, A. ochraceus* and *Fusarium monili* for meat a concentration lower than 500 ppm. In addition, Rassoli *et al*.(2006), reported the MIC of two species of thyme (*T. eriocalyx* and *T. x-porlock*) EOs at 125 ppm and 250 ppm against *A. niger*.

Table 1. Chemical composition, obtained yields (mass %) and MIC (ppm) of savory, thyme, and sage EOs

	Savory	Thyme	Sage
Chemical composition			
Thymol	20.56	30.91	-
ρ-Cymene	4.28	14.66	0.52
Carvacrol	31.32	11.96	46.11
Linalool	1.07	7.19	1.00
γ-Terpinene	30.03	5.53	0.24
Methyl thymylether	-	4.4	-
α-Pinene	1.28	2.09	3.83
α-Caryophyllene	-	1.36	2.8
β-Pinene	0.69	0.45	2.38
α-Terpinene	2.06	0.3	-
Limonene	1.6	0.8	1.31
Myrecene	1.53	3.01	0.87
α-Phellandrene	0.42	-	-
Camphene	1.17	0.65	2.92
1,8-cineole	-	3.1	6.14
β-thujone	-	-	3.16
camphor	-	0.24	9.77
Borneol	2.16	11.39	4.05
Bornyl acetate	-	-	1.98
α-Humulene	0.35	-	2.39
Viridiflorol	-	-	1.13
Total content	99.66	98.24	99.69
Yield of essential oil (%)	0.86^{b}	1.09^{a}	0.52^{c}
MIC (ppm)	400^{B}	200^{C}	2400^{A}

*: The means of yield of EO and MIC at two later rows have the same letters are not significantly different at *P*< 0.01

Evaluation of the effects of three EOs in culture media (in vitro antifungal assay)

In vitro antifungal activities of thyme, savory, and sage EOs against *A. niger* are shown in Figure 1.The results of disc diffusion test during 16 days of storage (Figure 1A) represented increase in fungal development in all samples during the incubation time. However, the control sample showed more fungal growth in comparison to the other samples. The results indicated significant antifungal properties for all three EOs at all concentrations, and the control significantly showed the highest fungal growth at all 16 days of storage. The results from statistical analysis (Table S1) showed that the type and the amount of EO as well as the time of incubation had a significant effect on the antifungal activity (P< 0.01). Also the mutual effects of the treatment and the time were significant on the fungal growth (P< 0.01).

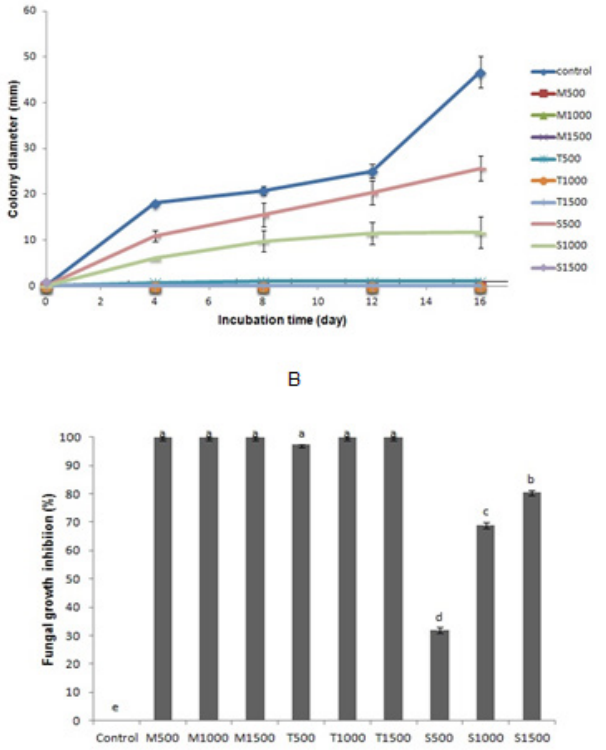

Figure 1. Effects of different concentrations (500, 1000 and 1500 ppm) of savory (M), thyme (T) and sage (S) EOs on colony growth of *A.niger* at 25°C for 16 days using disc diffusion method (A) and percentage of fungal growth after 16 days of storage (B). Values are mean (N=3) standard deviation, *-: The means that have the same letters are not significantly different at P <0.01.

Comparison of fungal growth inhibition (Figure 1B) showed the strongest antifungal properties for thyme EO, followed by savory and sage EOs. These results are in good agreement with the results from MIC. In addition, the fungal growth decreased in all samples, when the concentration of EOs were increased. Our findings are similar to those of Rasooli *et al*.(2006); and Tian *et al*.(2011) that showed the dependence of fungal growth inhibition to the concentration of EO. At all tested concentrations for savory, and 1000 and 1500 ppm for thyme EOs, the fungal development was completely inhibited after 16 days of incubation. The percentage of fungal growth inhibition for thyme and savory EOs was significantly (P< 0.01) higher than that of sage EO. These concentrations were found to be fungicidal. Sage EO did not exhibit fungicidal effect at tested concentrations. However, the inhibition of fungal growth in sage EO was increased with its concentration and the highest fungal inhibition in samples contain 1500 ppm sage EO was seen by 80.53%.

The antifungal properties of EOs may be associated to several compounds, known to have biological activities. Stevic *et al*. (2014) related the antimicrobial activity of thyme and savory to

Table 2. Evaluation of visual observation of yeasts and molds in different samples stored at 25°C (savory (M), thyme (T), sage (S))

Treatment	Growth delay (days)		Intensity of fungal growth in non-inoculated samples*			
	Inoculated	Non inoculated	Day 22	Day 30	Day 35	Day 50
Control	8±1.2^{a}	23.33± 1.15^{c}	+	++	++	+++
M500	19±2.01^{bc}	29.66 ± 0.57^{b}	-	-	-	++
M1000	22±1.55^{b}	37.33 ± 1.52^{b}	-	+	+	+
M1500	24±2.34^{a}	48.33± 1.15^{a}	-	-	-	+
T500	20±1.09^{b}	32.33± 2.51^{b}	-	-	+	+
T1000	21±2.4^{b}	44.66± 1.15^{a}	-	-	-	+
T1500	26±3.4^{a}	48.66± 0.57^{a}	-	-	-	+
S500	15±2.45^{c}	30.00± 1.03^{b}	-	+	+	++
S1000	18±1.3^{bc}	32.5± 0.98^{b}	-	-	+	+
S1500	21±1.65^{b}	34.00± 1.01^{b}	-	-	+	+

* - : No fungal growth, + fungal growth lower than 25% of plate, ++ fungal growth between 25 to 50% of plate surface, +++ fungal growth around 75% of plate surface.
**-: Columns with different letters are significantly different at P< 0.01.

the phenolic compounds such as carvacrol and thymol, and also, the synergistic activity between them which is an important factor in antimicrobial activity. γ-terpinene and ρ-cymene are biological precursors of phenolic components, have important effects on biological properties of EOs. The results of GC-MS in our study, showed substantial content of both components (30.3% and 5.53% for γ-terpinene and 4.28% and 14.66% for ρ-cymene) in savory and thyme, respectively, which is the main reason corresponded to high antifungal effects of them. Whereas, the concentration of these compounds (γ-terpinene (0.24%) and ρ-cymene (0.52%)) were almost low in sage EO. Despite to have the highest concentration of carvacrol in compared to thyme and savory, sage EO showed the lowest antifungal activity, probably due to the absence of thymol, where the synergistic effect between thymol and carvacrol is the main mechanism for its antifungal property.

Effectiveness of three EOs in tomato paste (in vivo antifungal assay)

The results from the effectiveness of all EOs in tomato paste are presented in Table 2. The inoculated control sample showed fungal development after 8 days, however this time increased after incorporation of tested EOs in tomato paste to 21 days (sage), followed by 24 days (savory), and 26 days (thyme) at the highest concentration of all EOs (1500 ppm). The results from non-inoculated samples exhibited the earliest fungal observation (23 days) for the

Table 3. Numbers of yeasts and molds (log CFU/g) for 60 days of incubation at 25°C (savory (M), thyme (T), sage (S))

Time (day)	control	M500	M1000	M1500	T500	T1000	T1500	S500	S1000	S1500
0	N.D±0.0	N.D±0.0	N.D±0.0	N.D±0.0	N.D±0.0	N.D±0.0	N.D±0.0	N.D±0.0	N.D±0.0	N.D ± 0.0
15	1.69±0.03	N.D±0.0	N.D±0.0	N.D±0.0	N.D±0.0	N.D±0.0	N.D±0.0	N.D±0.0	N.D±0.0	N.D ± 0.0
24	2.69±0.03	N.D±0.0	N.D±0.0	N.D±0.0	N.D±0.0	N.D±0.0	N.D±0.0	1.69±0.01	1.39±0.02	N.D ± 0.0
31	3.33±0.04	N.D±0.0	N.D±0.0	N.D±0.0	N.D±0.0	N.D±0.0	N.D±0.0	1.87±0.02	1.69±0.01	1.44 ±0.01
45	3.53±0.03	2.65±0.02	2.6±0.02	2.47±0.04	2.54±0.01	2.39±0.04	2.39±0.02	2.83±0.03	2.73±0.02	2.5±0.02
60	3.71±0.04	2.71±0.01	2.65±0.03	2.54±0.01	2.69±0.02	2.65±0.03	2.59±0.03	2.96±0.02	2.89±0.01	2.74±0.02

N.D: No total counts detected.

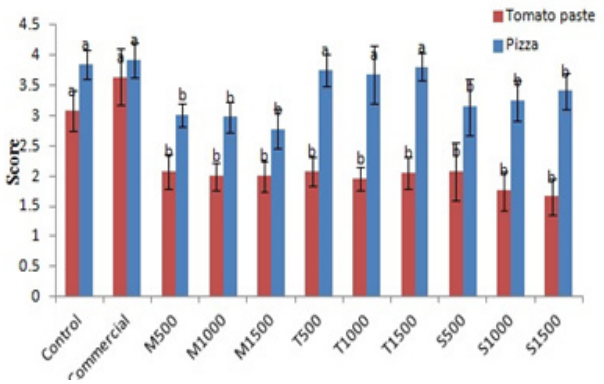

Figure 2. Sensory evaluation of tomato paste pure (red) and in pizza (blue) for different concentrations of EOs: M: savory, T: thyme and S: sage. All data shown are averages and standard deviation for three replications of experiment. Significant difference ($P < 0.01$) between means are indicated by letters above the histogram bars.

control sample ($P < 0.01$). The comparison between all samples incorporated with EOs, indicated an increase in the shelf life of tomato paste by increasing the concentration of EOs. As can be seen, thyme in 1000 and 1500 ppm and savory EO in 1500 ppm showed the highest time of observation in mold growth significantly ($P < 0.01$) by 48, 44 and 48 days, respectively. The sage EO showed the weakest antifungal effect in comparison to the thyme and savory EOs, where fungal growth was observed after 34 days of storage. These results are in correspondence with MIC and disc diffusion results.

According to the Table 3, determination of the numbers of yeasts and molds during 60 days of storage at 25°C for non-inoculated samples, exhibited increasing the number of yeasts and molds over the time. However, this increase was higher for the control compare to the other samples. The statistical analysis (Table S1) showed that the type and the amount of EOs as well as the time of incubation had a significant effect on antifungal activity ($P < 0.01$). Also, the mutual effects of treatment and time were significant on the fungal growth ($P < 0.01$). Thyme and savory EOs showed lower numbers of yeasts and molds than sage EO. Additionally, by EOs concentration increasing in all samples, fungal development decreased. Our findings are in agreement with the results from MIC and, disc diffusion tests.

Sensory evaluation

Results from the sensory evaluation of pure tomato paste and pizza are shown in Figure 2. The commercial sample showed the highest score among all other samples, followed by control, probably due to the absence of salt in the formulation of control and other samples, which has a significant effect in flavor. As can be seen in Figure 2, there was no significant differences between all samples with different concentrations of three EOs, and all samples contain EOs showed significantly ($P < 0.01$) lower scores than those of commercial and control samples. The results showed that three tested EOs had negative effects on organoleptic properties of tomato paste, due to have strong odorous.

Although the majority of the EOs are classified as GRAS, their use in foods as preservatives is often limited due to have adverse effects on flavor, since effective antimicrobial doses may exceed organoleptically acceptable levels (Lambert *et al*., 2001). Because tomato paste is always using with different spices into the food, maybe its odorous became negligible, when it is used with seasoning agents in the foods. Therefore, the effects of all three EOs in a model food (pizza) was evaluated. The results showed increase in the score of panelists, when the three EOs were used in pizza, which confirmed seasoning agents were masked the adverse odorous of EOs. The results showed, no significant differences between the scores of commercial, control and thyme EOs at all concentrations ($P < 0.01$). However, savory and sage showed lower scores in comparison to the other samples. Our results are in agreement with Omidbeygi *et al.* (2007) reported no significant difference between samples contain 500 ppm of savory, clove and thyme EOs and control in ketchup souse. They reported addition of tomato paste in ketchup souse with different spices were masked the high organoleptic properties of EOs. Also they mentioned that the application of EO should consider not only for their antimicrobial activity but also for their flavoring effect. According to the obtained results from our study, it seems that application of EOs in foods with high content of seasoning agents,

is a good solution to overcome their negative effects on organoleptic properties of food.

Conclusion

Tomato paste is a common food flavoring in Iran, which is contaminated with molds when its packages opens. Considering the prohibition of chemical preservatives addition in tomato paste in Iran, and consumers demanding for safe foods, finding new ways to increase the shelf life of tomato paste is necessary. In this study, the antifungal properties of three EOs, were studied *in vitro* and *in vivo*. The results of the MIC, disc diffusion test and *in vivo* tests in tomato paste showed three EOs were highly effective in retarding the microbial growth, however the antifungal activity was higher for thyme and savory compared to the sage EO. The presence of high amount of γ-terpinene and ρ-cymene in both thyme and savory EOs and the synergistic effects between those compounds was the main probable reason for the strong antifungal effects of thyme and savory EOs. Our results confirmed a good antifungal potential for thyme and savory to use as a natural preservative in tomato paste. Sensory evaluation tests showed adverse effects of all EOs on the organoleptic properties of tomato paste, however application of EOs in to the food were masked the strong odorous of thyme EO. The results exhibited that thyme EO can use as a good alternative to chemical preservatives. However, more studies are required to recommendation of these EOs as a commercial and natural antifungal agent.

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