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Control of *Aspergillus flavus* Growth in Tomato Paste by *Cinnamomum zeylanicum* and *Origanum vulgare L.* Essential Oils

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ABSTRACT

This study was conducted to evaluate the antifungal activities of *cinnamon* (*Cinnamomum zeylanicum*) and *oregano* (*Origanum vulgare* L.) essential oils against *Aspergillus flavus* in culture media and tomato paste. The chemical compositions of the essential oils were determined by Gas Chromatography-Mass Spectroscopy (GC-MS). Trans- cinnamaldehyde was found to be the main constituent of *Cinnamomum zeylanicum* essential oil (CZEO), followed by methyl eugenol, δ- cadinene and γ- cadinene. The major components of *Origanum vulgare* L. essential oil (OVEO) were limonene, caryophyllene oxide, α-ionone, germacrene– D, γ- terpinene, β- pinene and terpinene-4-ol. For evaluating antifungal activities of CZEO and OVEO, *A. flavus* PTCC: 5006, was inoculated in Sabouraud Dextrose Broth (SDB) and tomato paste, then 0, 50, 100, 200, 300, 400, 500 and 600 ppm of essential oils were added to each sample and incubated at 25±0.5°C for 30 and 60 days, respectively. The antifungal activity was measured by Agar Dilution method. The EOs at all tested concentrations had inhibitory effect against *A. flavus* growth. 200 ppm of CZEO and 500 ppm of OVEO completely inhibited *A. flavus* growth in culture media, while in tomato paste 300 ppm of CZEO and 200 ppm of OVEO had the same effect. Test panel evaluations were carried out in tomato ketchup base and samples with 100 and 200 ppm CZEO were accepted by panelists. The results may suggest the potential replacement of antifungal chemicals by CZEO as natural inhibitor to control *A. flavus* growth in tomato paste.

Keywords: Essential oil, *Cinnamomum zeylanicum*, *Origanum vulgare* L. *Aspergillus flavus*, tomato paste

1. Introduction

Fungi are significant spoilage microorganisms that grow in fresh and processed foods during the storage. The presence and growth of fungi in food reduce its quality and also quantity. Some *Aspergillus* species are responsible for many cases of food and feed contamination. *Aspergillus flavus* and *Aspergillus parasiticus* are able to produce aflatoxin in food (Guo et al., 1996). These toxins are responsible for human hepatic and extra hepatic carcinogenesis (Massey et al., 1995; Kamkar, 2005).

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In recent years, consumers are more concerned about the processed foods they use. Demands for natural, high quality and preservative-free products that are safe and stable introduce a great challenge for the food industry (Lopez-Malo et al., 2007). Currently, there is a strong debate about safety aspects of chemical preservatives since they are considered responsible for many carcinogenic and teratogenic attributes as well as residual toxicity (Omidbeygi et al., 2007).

Essential oils and their constituents have been used broadly as flavor ingredients in a wide variety of foods, beverages and confectionery products. Many of such

compounds are classified as *Generally Recognized as Safe* (GRAS) (Kim et al., 1995). Simultaneously, several studies have reported the antifungal activity of spices and their essential oils (Nielsen et al., 2000; Lopez-Malo et al., 2007; Gangomi et al., 2009). In most cases, there is a relationship between the chemical structures of essential oils and their antifungal effects (Farag et al., 1989).

Recently considerable interest has developed on the preservation of food by use of essential oils to effectively retard fungal growth and mycotoxin production. Many investigators used essential oils such as *cinnamon*, *marigold, basil* and *spearmint* to assess their antifungal activity against *A. ochraceaus, A. flavus, A. parasiticus* and *F. moniliforme* (Soliman et al., 2002). Souza et al. (2007) reported the anti-yeast activity of *Origanum vulgare L.* essential oil against some yeasts recognized as potential food spoiling microorganisms. *Origanum vulgare* L., Laminaceae family, which is widely known as possessing therapeutic properties (diaphoretic, antiseptic and antispasmodic) is being used in traditional home remedy in many countries (Sagdic et al., 2002; Sahin et al., 2004). It has been widely used in agricultural, pharmaceutical and cosmetic industries as culinary herb, flavoring substances in food products, alcoholic beverages and perfumery for its spicy fragrance (Dorman et al., 2000; Aligianis et al., 2001). *Origanum Vulgare* L. is characterized by its high content of phenolic acids and flavonoids (Falerio et al., 2005). *Cinnamomum zeylanicum,* commonly known as *cinnamon,* refers to a tropical evergreen tree as well as a bark that is extracted from a plant. *Cinnamon* is classified in the botanical division Magnoliophyta, class Magnoliopsida, order Magnoliales and family Lauraceae (Thomas et al., 2000). Cinnamaldehyde is the major constituent of *cinnamon* leaf oil and provides the distinctive odor and flavor associated with *cinnamon*. In products with compatible flavor such as bakery products and tomato paste, where fungi are the most common spoiler, essential oils can be used as antifungal agents (Nielsen et al., 2000).

Tomato paste is a common ingredient in verity of foods and keeping its quality during the refrigerated storage is of high importance (Omidbeygi et al., 2007). Due to the danger of Aflatoxin produced by *Aspergillus flavus,* this study was conducted to assess the antifungal activity of *cinnamon* and *oregano* essential oils against *A. flavus* in culture media and tomato paste.

2. Materials and Methods

2.1. Essential Oils

500 g of dried *oregano* (*Origanum Vulgare* L.) or *cinnamon* (*Cinnamomum zeylanicum*) cultivated in Iran were obtained from Iranian Medical Plant Research Center (Karaj, Iran) and then these were extracted for 3 hours by distilled water, using a Clevenger type apparatus. The obtained essential oils were dried over anhydrous sodium sulphate and kept at 4 °C for later experiments.

2.2. Microorganism and chemicals

A. flavus (PTCC: 5006) was used as a test organism. Fungus was purchased from the National Scientific and Industrial Research Center of Iran (Tehran, Iran). All chemical and culture media (with the highest purity available) like PDA (Potato Dextrose Agar), SDB (Sabouraud Dextrose Broth) were purchased from Merck chemical Co (Darmstadt- Germany).

2.3. GC-MS analysis of essential oils

GC analysis was carried out on HP-6980 gas chromatograph equipped with a HP-5 capillary column (30m × 0.25 mm; 0.25 µm film thickness). The oven temperature was held at 40°C for 5 min, and then programmed to reach 240°C at a 3°C/min rate, than increasing the temperature up to 280°C at a rate of 15°C/min (held for 3 min), and finally increased to 340°C at 3°C/min. Other operating conditions were: carrier gas, Helium with flow rate of 0.8 ml/min and the splitter used a 1:10 ratio; injector and detector temperature were 290°C; Mass spectra were taken at 70 eV. Mass range was from m/z 35-375 amu. Quantitative data were obtained from the electronic integration of the FID peak areas. The components of the essential oils were identified by comparison of their mass spectra and retention indices with those published in the literature (Adams, 2007).

2.4. Cooler

As for sporulation in Petri dish *A. flavus* was cultured on Potato Dextrose Agar (PDA) medium during 5-7 days at 25±0.5°C. After spore formation, several sub cultures were prepared and stored in refrigerator. In order to determine CZEO and OVEO antifungal activity experimental test were organized in 2 stages: First, the volume of 10^6 CFU/ml of *A. flavus* was added to 10 ml of SDB media to obtain an inoculation level of 10^5 CFU/ml followed by addition of the 8 concentrations of CZEO and OVEO (0, 50, 100, 200, 300, 400, 500 and 600). The inoculated samples of SDB were incubated at 25± 0.5°C for 30 days. In the second stage, *A. flavus*, was inoculated (10^5 CFU/ml) in aseptic tomato paste (Brix =28°, pH= 4.4 and 1.0% salt), then different concentrations of essential oils (0, 50, 100, 200, 300, 400, 500 and 600 ppm) were added to each sample and incubated at 25±0.5°C for 60 days. The antifungal activity was measured by Agar Dilution method as described by Gul et al. (2002). (Diluents were 8.5 g NaCl, 1.0 g Peptone in one liter distilled water). Microbial tests were carried out on PDA medium. To evaluate the inhibition percent of each essential oil, the Petri dishes were incubated at 25°C for 3-5 days, and then numbers of colonies were enumerated and compared with the initial counts (10^5 CFU/ml). Each test was carried out in triplicate and averaged.

2.5. Test Panel

For sensory evaluation, 25 trained panelists used hedonic test to assess tomato paste treated with 100

and 200 ppm of *cinnamon*, *oregano* and without essential oil. Four ketchup samples were prepared by mixing tomato paste with 100 and 200 ppm of CZEO and OVEO and other ingredients such as sugar, vinegar, salt and pepper. The blank sample was prepared without adding essential oil, and then ketchups were given to panelist to eat with potato chips. The panelists assessed flavor and odor of ketchup samples on the scale from 1 to 5 indicating increasing flavor and odor. The scores of all 25 panelists were pooled; then the mean values and standard deviations were calculated.

2.6. Statistical analysis

All tests were performed in triplicate and data were displayed as mean values with standard deviations. Statistical analysis was carried out using SAS software. Mean values were compared by LSD (least significant difference) test and significance was determined at $P \leq 0.01$.

3. Result and Discussion

3.1. Compositions of essential oils

A summary of main component of CZEO and OVEO identified by GC-MS are listed in Tables 1 and 2, respectively. The major components of *cinnamomum zeylanicum* were: *trans*-cinnamaldehyde (47.25%), methyl eugenol (6.75%), δ-cadinene (4.68%) and γ-cadinene (3.13%). The major components of *origanum vulgare* L. were: limonene (13.29%), caryophyllene oxide (8.89%), α-ionone (8.17%), germacrene- D (7.72%), γ- terpinen (7.63%) and β- pinene (6.8%). Recently Singh et al. *(23)* reported (E)-cinnamaldehyde (97.7%), δ-cadinene (0.9%), α-copaene (0.8%) and α-amorphene (0.5%) as major components of cinnamon bark volatile oil. Mockute et al. *(17)* results showed that, sabinene (19.2%), β-caryophyllene (18.2%), germacerene- D (9.6%), caryophllene oxide (8.7%) and γ- terpinene (3.2%) were the main components of OVEO.

Table 1. Chemical constituent of CZEO determined by GC-MS.

Compound	t_R (min)	Amount (%)
α-pinene	16.43	0.27
terpin-4-ol	29.17	1.24
linalool acetate	31.97	1.2
geraniol	32.84	1.97
cinnamaldehyde(trans)	33.49	47.25
cinnamyl alchol	35.62	0.9
eugenol	37.72	0.16
β-elemene	39.43	2.72
methyl eugenol	39.67	6.75
ethyl cinnamate	42.38	2.55
coumarin(3-methyl-)	43.66	1.91
γ-cadinene	44.64	3.13
δ-cadinene	45.65	4.68
humulene epoxide	48.32	1
turmerone	50.34	1.05

Table 2. Chemical constituents of OVEO determined by GC-MS.

Compound	t_R (min)	Amount (%)
myrcene	14.33	1.15
β- pinene	13.48	6.8
limonene	16.16	13.29
1,8- cineole	16.27	1.51
trans- β- ocimene	16.85	4.88
bergamal	17.37	3.80
γ- terpinen	17.93	7.63
β- pinene- oxide	22.55	3.47
δ-terpineol	22.96	1.24
terpinen-4-ol	23.63	5.12
carvacrol	29.15	1.03
geranyl acetate	33.25	2.03
α- ionone	34.84	8.17
germacrene- D	37.42	7.72
γ- cadinen	38.39	2.60
caryophyllene oxide	40.07	8.89

3.2. Screening antifungal activity in culture media

Both CZEO and OVEO have significant antifungal activity against *A. flavus* in culture media (Figure 1). Our statistical analysis showed that the type and amount of essential oil have a significant effect on antifungal activity ($P \leq 0.01$). The results were the same for storage time. According Figure 1, it could be seen that as the EO concentration increases the inhibitory effect increases. CZEO had stronger antifungal activity than OVEO. Complete inhibition of *A. flavus* growth was observed at 200 ppm of CZEO, while 300 and 400 ppm of OVEO had inhibition percent of 97 and 97.3 respectively. Complete inhibition of *A. flavus* growth was observed at 500 ppm of OVEO. *Cinnamon* and *oregano* in comparison with other essential oils could inhibit the growth of *A. flavus* in lower concentrations. Soliman and Badeaa (2002) showed that *cinnamon* completely inhibited the growth of *A. flavus* in culture media at 1000 ppm, while *spearmint, basil* and *marigold* had the same effect at 3000 ppm.

In culture media the CZEO and OVEO inhibitory percent at 50 ppm were 76.17 and 71.65 percent respectively. It is obvious from Figure 2 that in the final day of storage, antifungal activity of CZEO and OVEO slightly decreases. However, inhibitory percent of *cinnamon* and *oregano* in the 30th day of storage were 88.14 (10.7% reduction) and 82.49% (13.7% reduction), respectively. Maintaining *cinnamon* and *oregano* essential oils antifungal activity against *A. flavus* in low concentrations and final day of storage indicates that CZEO and OVEO can be used as mold inhibitor in foods such as tomato paste.

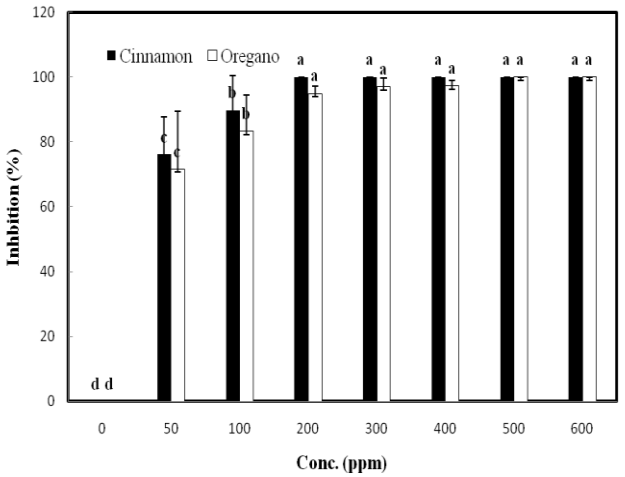

Fig 1. Effect of different concentrations of CZEO and OVEO on *A. flavus* growth in culture media.

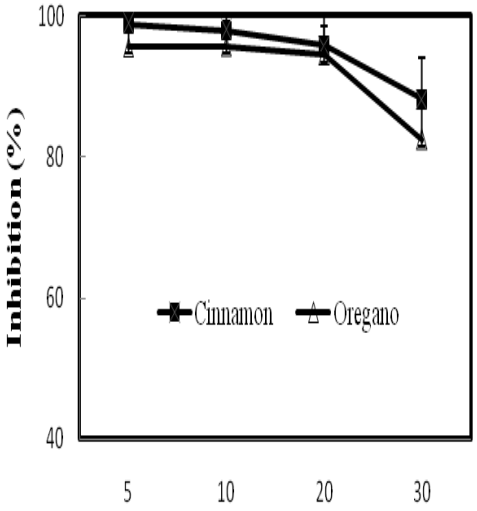

Fig 2. Effect of storage time on *A. flavus* growth in culture media.

3.3. Screening antifungal activity of CZEO and OVEO in tomato paste

In this study we also evaluated the potential application of CZEO and OVEO in tomato paste as food model. The EOs at all concentrations tested, had inhibitory effect against *A. flavus* growth, 300 ppm of CZEO and 200 ppm of OVEO could inhibit *A. flavus* growth completely in tomato paste (Figure 3), but in control samples lots of colonies were observed that were not countable. Similar to culture media experiments, the storage time, kind and the amount of essential oils had significant effect on antifungal activity ($P \leq 0.01$). As observed in Figure 3, the inhibitory effect of essential oils on *A. flavus* increased by increasing their concentration. This effect was considerable even at low concentrations; 50 ppm of CZEO and OVEO could inhibit *A. flavus* growth 82 and 79 percent respectively. Figure 4 shows antifungal activity of CZEO and OVEO during the storage period. In the last day of storage, the inhibitory percent of CZEO and OVEO reduced but it was still high (73% for CZEO and 90.4% for OVEO). *Cinnamon* and *oregano* constituents play the main role in their antifungal activities. *Trans*-cinnamaldehyde as main component of CZEO (47.25%) seems to be responsible for *cinnamon* inhibitory effect. Since its protective effect against various microorganisms have been reported by others (Cheng et al., 2006; Singh et al., 2007; Baskaran et al., 2010; Singh et al., 2010), *Cinnamon* antifungal activity can be attributed to this component. Although the mechanism of action of these compounds have not completely elucidated, but Wang et al. (2005) reported that a compound having a conjugated double bond and a long CH chain outside the ring, i.e. cinnamaldehyde, possesses strong antifungal activity.

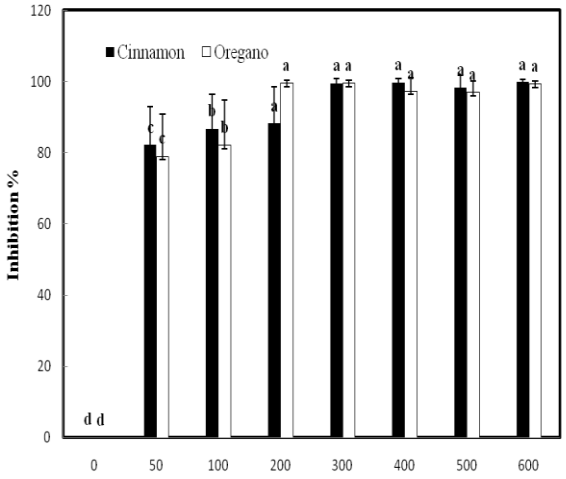

Fig 3. Effect of different concentrations of CZEO and OVEO on *A. flavus* growth in tomato paste.

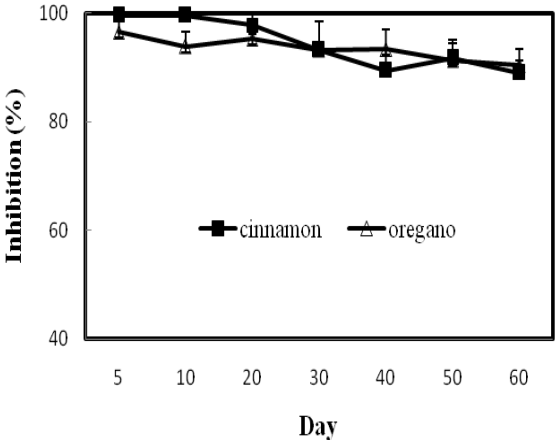

Fig 4 Effect of storage time on *A. flavus* growth in tomato paste.

Singh et al. (2010) claimed that DL-limonene, the major component of *citrus sinensis,* has strong antifungal and antiaflatoxigenic properties. These results are in agreement with those published by Ruberto and Baratta (2000) and Tyagi and Malik (2011) who suggested that monoterpenes (like limonene) in essential oils are the main reason for their antifungal activity. In view of the fact that limonene is the major component of OVEO, *oregano* antifungal activity can be attributed to this component.

3.4. Sensory evaluation

Several studies reported that addition of essential oils (as antimicrobial agents) impart strong flavor in foods (Harpez et al., 2003; Omidbeygi et al., 2007). Although the majority of the essential oils are classified

as GRAS, their use in foods as preservatives is often limited due to flavor considerations (Lambert et al., 2001). Careful selection of EOs according to the type of the food can moderate these effects. Our sensory evaluation showed that there was no significant differences ($p \leq 0.01$) between samples with 100 and 200 ppm of CZEO and the control (without essential oil), while samples with 100 and 200 ppm of OVEO had significant difference with its control (Figure 5).

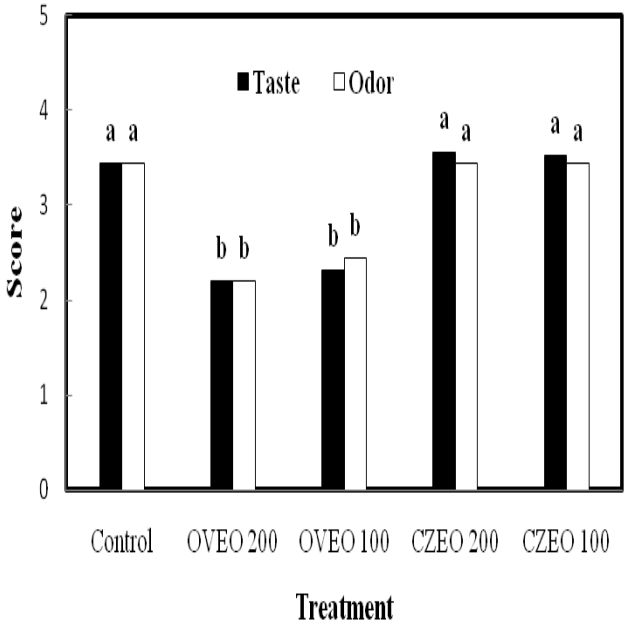

Fig 5. Taste panel results of five Ketchup sauces prepared by adding 100 and 200 ppm of CZEO and OVEO. Columns with different letters are significantly different at $p \leq 0.01$.

Present study indicates that *cinnamon* can be added to tomato paste without any effect on organoleptic attributes. In conclusion essential oils of *cinnamon* and *oregano* have significant antifungal activity against *A. flavus* in vitro and tomato paste. The minimum concentration of essential oils that inhibit the growth of microorganisms is a key factor. CZEO and OVEO in comparison with other plant EOs inhibit *A. flavus* growth in lower concentrations. Furthermore, CZEO did not change tomato paste sensory attributes. As a result, CZEO can be introduced as a natural preservative for tomato paste.

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