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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 *Cinnamomum zeylanicum* and *Origanum vulgare* L. essential oil against *Aspergillus flavus* in culture media and tomato paste. 200 ppm of cinnamon and 500 ppm of oregano completely inhibited *A. flavus* growth in culture media, while in tomato paste 300 ppm of cinnamon and 200 ppm of oregano had the same effect. Test panel evaluations revealed that samples with 100 and 200 ppm cinnamon were acceptable. The results may suggest the potential use of *Cinnamomum zeylanicum* essential oil as natural preservative in tomato paste.

***Keywords*—**Antimicrobial, Food safety, GC/MS, Natural preservative

I. 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 [1]. These toxins are responsible for human hepatic and extra hepatic carcinogenesis [2].

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 [3]. 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 [4].

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) [5]. Simultaneously, several studies have reported the antifungal activity of spices and their essential oils [4, 6]. In most cases, there is a relationship between the chemical structures of essential oils and their antifungal effects [7].

Recently considerable interest has developed on the preservation of food by use of essential oils to effectively retard fungal growth and mycotoxin production.

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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* [8]. The anti-yeast activity of *Origanum vulgare L.* essential oil against some yeasts have been recognized as potential food spoiling microorganisms [9]. *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 [10]. 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 [11]. *Origanum Vulgare* L. is characterized by its high content of phenolic acids and flavonoids [12]. *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. This herb has been used medicinally for thousands of years to fight toothache, clear up urinary tract infections and soothe stomach irritation. It has a broad range of historical uses in different cultures including the treatment of diarrhea, arthritis and various menstrual disorders. The large number of medicinal applications for cinnamon indicates the widespread appreciation of folk herbalists for its healing properties [13].

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 [6].

Tomato paste is usually considered as semi finished food because it is commonly used as ingredient in other foods, so keeping its quality during the refrigerated storage is of high importance. 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.

II. Materials and Methods

500 g of dried oregano (Origanum Vulgare L.) or cinnamon (Cinnamomum zeylanicum) cultivated in Iran were purchased 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.

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).

GC analysis was carried out on HP-6980 gas chromatograph equipped with a HP-5 capillary column (30m $\times$ 0.25 mm; 0.25 μm film thickness). The oven temperature was held at $40^{o}C$ for 5 min, and then programmed to reach $240^{o}C$ at a $3^{o}C$/min rate, than increasing the temperature up to $280^{o}C$ at a rate of $15^{o}C$/min (held for 3 min), and finally increased to $340^{o}C$ at $3^{o}C$/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 $290^{o}C$; 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 [14].

As for sporulation in Petri dish A. flavus was cultured on Potato Dextrose Agar (PDA) medium during 5-7 days at $25\pm0.5^{o}C$. After spore formation, several sub cultures were prepared and stored in refrigerator. In order to determine CZEO (Cinnamomum zeylanicum essential oil) and OVEO (Origanum vulgare L. essential oil) 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 $25\pm 0.5^{o}C$ for 30 days. In the second stage, A. flavus, was inoculated (10^{5} CFU/ml) in aseptic tomato paste (Brix $=28^{o}$, 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 $25\pm0.5^{o}C$ for 60 days (since the typical shelf life that food technologists suggest is 2 month). The antifungal activity was measured by Agar Dilution method as described by [15]. (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 $25^{o}C$ 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.

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.

All tests were performed in triplicate and data were displayed as mean values ± S.D. (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 $P \leq 0.01$.

III. Results and discussion

A summary of main component of CZEO and OVEO identified by GC-MS are listed in Tables I and II, 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 it has been reported that (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 [16]. The results reported by Mockute et al., 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 [17].

Both CZEO and OVEO have significant antifungal activity against A. flavus in culture media (Fig. 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 Fig. 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.

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TABLE I
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 II
CHEMICAL CONSTITUENT 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

In culture media the CZEO and OVEO inhibitory percent at 50 ppm were 76.17 and 71.65 percent respectively. It is obvious from Fig. 2 that in the final day of storage, antifungal activity of CZEO and OVEO slightly decreases.

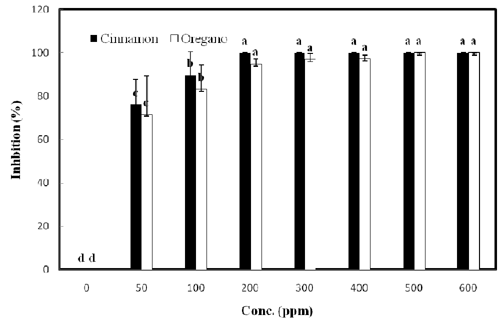

Fig. 1 Effect of different concentrations of CZEO and OVEO on *A. flavus* growth in culture media. Columns with different letters are significantly different at $p \leq 0.01$.

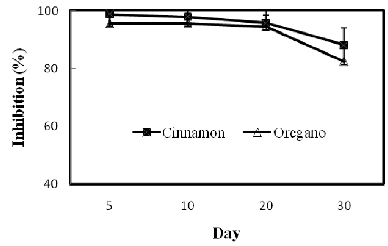

Fig. 2 Effect of storage time on *A. flavus* growth in culture media

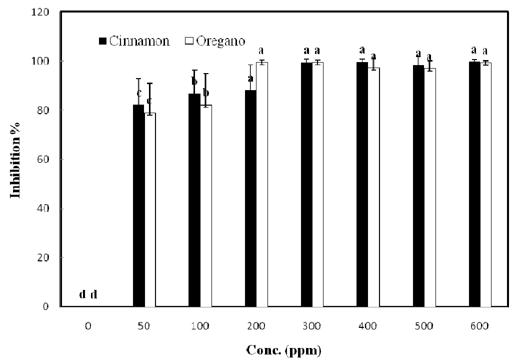

Fig. 3 Effect of different concentrations of CZEO and OVEO on *A. flavus* growth in tomato paste. Columns with different letters are significantly different at $p \leq 0.01$

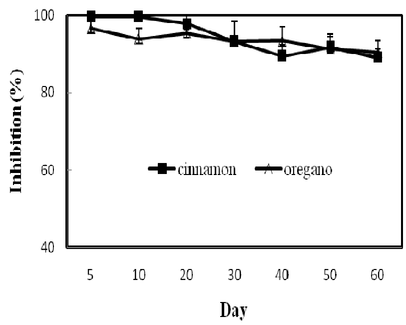

Fig. 4 Effect of storage time on *A. flavus* growth in tomato paste

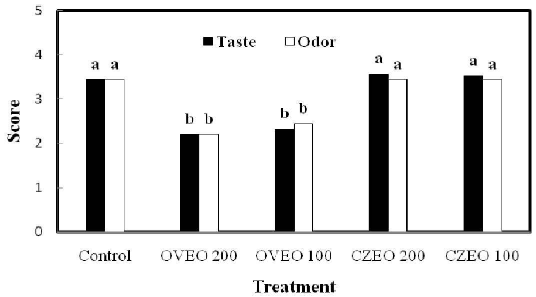

Fig. 5 Taste panel results of five Ketchup sauces prepared by adding 100 and 200 ppm of CZEO and OVEO. Columns with different letters are significantly different at $p \leq 0.01$

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. In this study we also evaluated the potential application of CZEO and OVEO in tomato paste as food model. The essential oils 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 (Fig. 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 Fig. 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. Fig. 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 [16, 18]. Cinnamon antifungal activity can be attributed to this component. Although the mechanism of action of these compounds have not completely elucidated, but it has been found that a compound having a conjugated double bond and a long CH chain outside the ring, i.e. cinnamaldehyde, possesses strong antifungal activity.

Singh et al. claimed that, the major component of *citrus sinensis*, DL-limonene, has strong antifungal and antiaflatoxigenic properties [18]. These results are in agreement with those published by Ruberto and Baratta and Tyagi and Malik who suggested that monoterpenes (like limonene) in essential oils are the main reason for their antifungal activity [19, 20]. In view of the fact that limonene is the major component of OVEO, oregano antifungal activity can be attributed to this component.

Several studies reported that addition of essential oils (as antimicrobial agents) impart strong flavor in foods [4]. Although the majority of the essential oils are classified as GRAS, their use in foods as preservatives is often limited due to flavor considerations [21]. 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 (Fig. 5). Present study indicates that cinnamon can be added to tomato paste without any effect on organoleptic attributes.

IV. CONCLUSION

The 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 essential oils 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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