



Inhibitory Effect of *Echinophora platyloba* Essential Oil on *Aspergillus flavus* in Culture Media and Cheese

H. Hasanvand¹, H. Moshtaghi^{1*}, A. Heshmati², M. Boniadian¹, M. Abbasvali¹

1. Department of Food Hygiene, Faculty of Veterinary Medicine, University of Shahrekord, Chaharmahal and Bakhtiari, Iran

2. Nutrition Health Research Center, Hamadan University of Medical Sciences, Hamadan, Iran

HIGHLIGHTS

- *Echinophora platyloba* Essential Oil (EO) exhibited significant inhibition on growth of *Aspergillus flavus*.
- Inhibitory effect of EO on growth of *A. flavus* followed dose-dependent manner on the cheese.
- *E. platyloba* EO can be used as a mold inhibitor in dairy manufactures.

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Acronyms and abbreviations

EO=Essential Oil
PDA=Potato Dextrose Agar
MIC=Minimum Inhibitory Concentration
MFC=Minimum Fungicidal Concentration

ABSTRACT

Background: In order to avoid potential harm of synthetic additives food, development of novel functional foods containing natural ingredients is considered in current years. In this study, inhibitory effect of *Echinophora platyloba* Essential Oil (EO) on growth of *Aspergillus flavus* was evaluated in culture media and cheese.

Methods: *E. platyloba* EO was extracted by hydrodistillation method using a Clevenger-type system. Growth of *A. flavus* on Potato Dextrose Agar (PDA) culture media was assayed using an agar dilution method. The lowest concentration which inhibited the growth of *A. flavus* was considered as Minimum Inhibitory Concentration (MIC) and the lowest concentration of EO which eliminated the mold was taken as Minimum Fungicidal Concentration (MFC). Mold spore suspension was inoculated on Iranian ultra-filtered white cheese and the means of two perpendicular diameters of the fungal colony was calculated. Statistical analysis was carried out using SPSS, Inc, Chicago, IL software (v.16.0).

Results: All concentrations of EO exhibited significant inhibition ($p < 0.05$) of fungal growth in an agar medium. The concentration of 500 ppm of EO reduced the radial growth of *A. flavus* by 73.9% while higher concentration inhibited completely mold growth. MIC and MFC of the EO was 750 and 1500 ppm, respectively. All the levels of EO had an inhibitory effect ($p < 0.05$) against radial fungal growth in cheese. However, the highest inhibitory effect was seen in cheese group treated with 2000 ppm of EO in which radial growth was reduced by 75.53%.

Conclusion: This study suggested that the *E. platyloba* as a natural inhibitor was able to control the growth of molds in foods such as cheese.

Introduction

Occurrence of undesirable molds leads to reduction of quality as well as quantity of foodstuffs. The most important molds that are naturally found in foods include *Aspergillus*, *Fusarium*, and *Penicillium* spp. (Murphy et

al., 2006). Some *Aspergillus* species are responsible for many cases of food spoilage (Williams et al., 2004). These fungi are recognized as active agents in decay processes, cause of human and animal diseases, retarding food

* Corresponding author. ✉ hmoshtaghi@yahoo.com

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nutritive value, and producing mycotoxins (Kumar et al., 2007). Nowadays, processed foods are transported across large distances to reach consumers, therefore special requirements are needed to ensure products quality, mainly in prevention of spoilage (Carocho et al., 2014). One of the important biological contaminants during ripening and storage of cheese is *Aspergillus* spp. (Bullerman and Olivigni, 1974).

In recent years, novel functional foods containing health promoting natural ingredients include compounds or extracts from plants instead of synthetic additives have been intensively developed and commercialized by the food industry to avoid potential harmfulness of synthetic compound (Caleja et al., 2015; Carocho et al., 2014). Essential Oils (EOs) are especially recommended as one of the most promising groups of safe additives for the formulation of natural antifungal agents (Abdollahzadeh et al., 2014; Aminzare et al., 2016; Azhdarzadeh and Hojjati, 2016; Azizkhani et al., 2013; Varma and Dubey, 2001). Many EOs have been classified as safe preservatives, so these are potential targets for developing natural antifungals due to their safety on eukaryotic systems (Tolouee et al., 2010). In many studies, antifungal properties of medicinal plants against food spoilage as well as mycotoxigenic fungi have been investigated (Avijgan et al., 2006a; Avijgan et al., 2006b; Avijgan et al., 2010; Gandomi et al., 2009; Khorasany et al., 2016; Paranagama et al., 2003; Saei-Dehkordi et al., 2012; Singh et al., 2006; Velluti et al., 2003). Genus *Echinophora* is a member of Umbelliferae family, having ten different species. Among four native *Echinophora* species in Iran, *E. platyloba* DC. (with the Persian name Khosharizeh or Khosharouzeh) is the most important consumed traditional plant that use as antifungal preservative agent in pickled cauliflower, tomato paste, pickled cucumber, and traditional cheese by local people especially those who live in rural areas (Asghari et al., 2010; Mazloomifar et al., 2004; Pirbalouti, 2009). The goal of this study is to evaluate inhibitory effect of *E. platyloba* EOs on growth of *A. flavus* in culture media and cheese.

Materials and methods

Preparation of the EO

The wild growing *E. platyloba* from Hamadan province of Iran was collected in May 2016 and verified by a botanist at University of Shahrekord, Chaharmahal and Bakhtiari, Iran. Aerial parts of the plant were dried in an oven equipped with warm air circulation. Hundred grams of air-dried material was grounded and powdered. The EO was extracted from the powder by hydrodistillation method using a Clevenger-type system. The oil was kept at 2 to 4 °C in a sealed brown vial (Moosavy et al., 2008).

Microorganism

A. flavus ATCC 15546 strain was obtained from Department of Mycology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. At first, the strain was cultured on Potato Dextrose Agar (PDA) slant provided from Merck Darmstadt (Germany), for 10 days at 26±1 °C. Tween 80 solution (0.05%) was added to culture medium and gently scraping the mycelia with a sterile inoculating loop. Conidial concentration was determined by a haemocytometer to give a final content of 10⁶ ml (Nguefack et al., 2004).

Evaluation of antifungal effect of EO on culture media

Fungal growth on culture media was assayed using an agar dilution method. Appropriate volumes of EO were added to the sterilized molten PDA medium and then poured into sterilized plates to achieve EO concentrations including, 0, 100, 250, 500, 750, 1000, 1500, and 2000 ppm. Ten µl of spore suspension (10⁶ spores/ml) was inoculated on paper disk that placed at the center of each plate. After the incubation period (10 days at 26±1 °C), average of two perpendicular diameters of colony was calculated. To determine the fungistatic or fungicidal effect, disks showing no growth were transferred to PDA plates without EO and incubated. The lowest level of EO in which fungus grown on PDA was considered as Minimum Inhibitory Concentration (MIC) and the lowest concentration of EO in which no growth was shown on fresh medium, was taken as Minimum Fungicidal Concentration (MFC) (Gandomi et al., 2009).

Evaluation of antifungal effect of EO on cheese

For investigation of antifungal effect of *E. platyloba* in food model, Iranian ultra-filtered white cheese in brine was produced by a private company. After removing the outer 5 mm of cheese block with a sterile knife, cheese was cut into pieces with thickness of 8 mm and trimmed to fit into 10 cm diameter plates. Plates were exposed to germicidal UV light for 30 min. After that, different levels of EO (i.e., 100, 250, 500, 750, 1000, 1500, as well as 2000 ppm) prepared with ethanol (50%, v/v) was inoculated on the surface of cheese. Ethanol (50%) was considered as the control. After 10 min, 3 µl of spore suspension (10⁶ spores/ml) was inoculated and then each plate was sealed partially with an adhesive tape. The average of two perpendicular diameters of the fungal colony was calculated after incubation at 26 °C for 10 days (Nguefack et al., 2004).

Statistical analysis

Data were analyzed statistically based on normal confidence intervals as well as analysis of variance (one-way

ANOVA) using SPSS, Inc, Chicago, IL software (v.16.0). The levels were considered significantly different at $p < 0.05$. All experiments were carried out in triplicate and data were shown as mean \pm standard deviation.

Results

As shown in Table 1, all concentrations of EO exhibited significant inhibition ($p < 0.05$) of fungal growth in an agar medium. In concentrations ≥ 750 ppm, the growth of fungus was completely prevented. In the control group as well as concentration of 100 ppm, the growth was started from day 1 while no visual growth was recorded until day 3 at 250 ppm and until day 5 at 500 ppm. EO con-

centration of 750 ppm which inhibited the growth of the fungus was considered as MIC and the EO concentration of 1500 ppm which eliminated the fungus was taken as MFC.

The effect of *E. platyloba* EO on radial growth of *A. flavus* on Iranian ultra-filtered white cheese in brine as a food model is indicated in Table 2. All concentrations of EO had a significant inhibitory effect ($p < 0.05$) against radial fungal growth on the cheese. In the control group as well as concentrations 100, 250, and 500 ppm, the growth was started from day 1 while no visual growth was recorded until day 2 at 750 and 1000 ppm and until day 4 at 1500 ppm. No concentration of EO examined completely inhibited the growth of *A. flavus* on cheese.

Table 1: Effect of *E. platyloba* EO on radial growth of *A. flavus* ATCC 15546 in culture media

EO concentration (ppm)	Colony diameter (mm)	Inhibition (%)
0	119 \pm 1.5	0 ^a
100	91 \pm 2.1	22.7 ^b
250	73 \pm 2.5	41.2 ^c
500	32 \pm 3.5	73.9 ^d
750	NG	100 ^e
1000	NG	100 ^e
1500	NG	100 ^e
2000	NG	100 ^e

-Different letters in each column are significant at 5% level

-NG: No Growth

Table 2: Effect of *E. platyloba* EO on growth of *A. flavus* ATCC 15546 in cheese

EO concentration (ppm)	Colony diameter (mm)	Inhibition (%)
0	94 \pm 2.5	0.0 ^a
100	81 \pm 2.1	13.48 ^b
250	74 \pm 1.5	21.63 ^{bc}
500	66 \pm 1.0	29.79 ^{cd}
750	59 \pm 1.5	37.59 ^{de}
1000	48 \pm 1.0	48.84 ^{ef}
1500	37 \pm 1.0	60.64 ^{df}
2000	23 \pm 1.0	75.53 ^f

-Different letters in each column are significant at 5% level

Discussion

In this study, we found significant inhibitory effect of *E. platyloba* EO on growth of *A. flavus* in culture media and cheese. In recent years, consumer demand for foods regarding to replacement of the chemical synthetic additives with natural component has been increased. EOs, natural plant compounds, have been used as inhibitors of toxigenic molds and may be safer for consumption (Rahman and Kang, 2009; Tsigarida et al., 2009). The inhibitory effect of some EOs against growth of different microorganisms has been previously reported (Bahraminejad et al., 2013; Kuate et al., 2006; Misaghi and Basti, 2007; Moradi et al., 2014; Pawar and Thaker, 2006; Sadeghi et al., 2016). The presence of two major phenolic components such as thymol and carvacrol could

be considered as a very effective factor for antimicrobial properties of the *E. platyloba* EO. In a study by Saei-Dehkordi et al. (2012), phenolic compounds of *E. platyloba* inhibited growth of *P. citrinum* in some Galician cheeses. The influence of phenolic compounds on reduction of growth of different spoilage molds was previously documented (Wendorff and Wee, 1997). Wendorff et al. (1993) found that the phenolic components of wood smoke in cheese provided the antifungal properties. In previous researches, antimicrobial effect of *E. platyloba* has been shown on some pathogens such as *Trichophyton schenlaini*, *T. verucosum*, *T. rubrum*, *T. violaseum*, *T. mentagrophytes*, *Microsporum gypseum*, *M. canis*, and *Epidermophyton flucosum* by agar dilution

assay, *Candida albicans* by agar dilution technique (Avijgan et al., 2006a; Avijgan et al., 2006b), as well as *Listeria monocytogenes*, *Serratia marcescens* and *Providencia rettgeri* by agar dilution assay (Sharafati-Chaleshtori et al., 2012). Saei-Dehkordi et al. (2012) showed that the EO of *E. platyloba* could be used as natural antimicrobial agent for inhibition of food-borne pathogens growth. *E. platyloba* EO exhibited antimicrobial activity against *L. monocytogenes*, *Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *C. tropicalis*, *Rhodotorula rubra*, and *R. mucilaginosa* (Saei-Dehkordi et al., 2012). Also, synergistic effects of ethanol extract of *E. platyloba* and azole drugs against clinical isolates of *C. albicans* from women suffering recurrent vaginitis was reported by Avijgan et al. (2014). In the present study, all used concentrations of *E. platyloba* had inhibitory effect on radial growth of mold on PDA, and greater inhibitions were seen at higher concentrations. The MIC and MFC were estimated at 750 as well as 1500 ppm, respectively. Mahboubi et al. (2009) studied the anti-fungal activity of ethanol extract of *E. platyloba* against *C. albicans* ATCC 10231. In their study, The MIC and MFC values of ethanol extract of *E. platyloba* against *C. albicans* were 1.560 and 3.125 mg/ml, respectively. The synergistic activity of *E. platyloba* extract and amphotericin B has been documented so that the mentioned extract decreases the MIC and MFC of amphotericin B. The results of the present study are in agreement with these findings.

In this study, we also evaluated the potential application of *E. platyloba* EO on Iranian ultra-filtered white cheese as a food model. All used concentrations of EO showed inhibitory effect against mold growth on the cheese. No examined level of EO could completely inhibit the growth of *A. flavus* on cheese. However, greater reduction of the colony diameter was generally observed with increasing EO concentrations. In concentrations of, 100, 250, 500, 750, 1000, and 1500 ppm of EO, the radial growth of *A. flavus* on cheese was reduced by 13.48, 21.63, 29.79, 37.59, 48.84, and 60.64%, respectively, suggesting a dose-dependent pattern. The EO showed greater inhibition of fungal growth in culture than that in cheese. Bagamboula et al. (2004) has reported that higher levels of EOs are necessary to inhibit microbial growth in food than culture media because interaction between phenolic compounds and proteins and lipid in foods caused the potential loss of antimicrobial activity. In current study, we observed antimicrobial effect of EO increased during storage because of pH loss of cheese and the increment of hydrophobic properties of EOs (Holley and Patel, 2005). Antimicrobial effect of different EOs in some food models has been previously studied. In a study by Gandomi et al. (2009), evaluation of antifungal effect

of EO from *Zataria multiflora* Boiss. on *A. flavus* in cheese showed greater reduction of the colony diameter than *E. platyloba* in our study. The concentration of 1000 ppm from *Z. multiflora* Boiss. showed 75.4% inhibition in growth rate of *A. flavus* while the same concentration of *E. platyloba* caused the reduction of 48.84% in mold growth (Gandomi et al., 2009). This discrepancy is because of different levels of effective compounds of EOs in these plants. In another research, Darderafshi et al. (2014) revealed that EOs of *Ferulago angulata* had antibacterial effects on *S. aureus* in Iranian white cheese. Sadeghi et al. (2016) reported that EO of *Mentha pulegium* not only can improve sensory characteristics of cheese but also can inhibit the growth of *L. monocytogenes* in cheese. Raeisi et al. (2012) showed antibacterial effect of EO of otagaragon (*Artemisia dracunculus*) on *S. aureus* and *E. coli* in cheese that could be suggested as a preservative in foods. In order to improvement in sensorial effects of tarragon EO in foods, combination with the other preservative could be recommended to reduce effective dose of this EO (Raeisi et al., 2012).

Conclusion

Our results indicated that EO of *E. platyloba* had anti-fungal activity and can be used as a mold inhibitor in foods such as cheese. However, more studies are required to determine the economical costs of addition of EO from *E. platyloba* and its effects on organoleptic characteristics of products. In view of the growing interest of consumers for functional foods without chemical additives, it would be interesting to go further in the development of this novel formulation in order to increase the shelf life of food such as cheese.

Conflicts of interest

There are no conflicts of interest in this study.

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