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# Antifungal Activity of *Pistacia eurycarpa* Yalt. Essential Oil on *Aspergillus flavus* by Direct Addition and Vapor Contact

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## ABSTRACT

**The inhibitory activity of *Pistacia eurycarpa* Yalt. Essential Oil (EO) toward *Aspergillus flavus* (PTCC: 5004) was evaluated by agar dilution method and vapor phase method. For the first method, the EO was added to agar medium to obtain concentrations of 0, 5,000, 6,000, 7,000, 8,000, 9,000, and 10,000 µL $L^{-1}$. For the vapor contact assays, uncovered inoculated potato dextrose agar plates and maize plates were placed inside airtight containers whose headspace was saturated by EO vapor. The results showed that at agar dilution test, the EO completely inhibited the growth of the tested mold at ≥ 10,000 µL $L^{-1}$. In vapor contact method, mycelia growth was inhibited 60.87% in PDA plates and no growth was observed within twenty days of incubation in maize plates.**

**Keywords**: Agar dilution method, Fungi, Maize, Potato dextrose agar.

## INTRODUCTION

Agricultural commodities such as maize can be contaminated by toxigenic fungi like *Aspergillus flavus* before, during, and after harvest (Bluma and Etcheverry, 2008; Gibriel *et al*., 2011; Menniti *et al*., 2010). This contamination often makes these commodities unfit for consumption by retarding their nutritive value and producing mycotoxines such as Aflatoxins, leading to significant economic losses (Gandomi *et al*., 2009; Rasooli and Owlia, 2005; Razzaghi-Abyaneh *et al*., 2008). Aflatoxins are mutagenic, carcinogenic, teratogenic and hepatotoxic mycotoxins, so, ingestion of contaminated food may cause chronic and acute toxicity (Li *et al*., 2016; Tian *et al*., 2011; Vilela *et al*., 2009).Various chemical treatments, physical and biological methods were investigated to inhibit mold growth and aflatoxin production (Atanda *et al*., 2007). To produce eco-friendly and safe products, there has been increased interest to explore natural substances with bioactivity against fungi (Passone *et al*., 2012). Essential Oils (EOs) are secondary metabolites of most plants, which are produced as part of their normal program of growth or in response to stress (Bluma and Etcheverry, 2008). They are a good source of several bioactive compounds, with antifungal, antibacterial, and antioxidant properties (Alves-Silva *et al*., 2013; Tongnuanchan and Benjakul, 2014). Numerous studies have documented the antifungal effects of plant EOs against *A. flavus.* EOs of basil (*Ocimum basilicum*), cinnamon (*Cinnamomum zeylanicum*), thyme (*Thymus vulgaris*), peppermint (*Mentha piperita*), origanum (*Origanum vulgare*), clove (*Syzygium aromaticum*), Boldo (*Pëumus boldus* Mol.), poleo (*Lippia turbinata* var. integrifolia Griseb.), anise (*Pimpinella anisum*), mountain thyme (*Hedeoma multiflora*), eucalyptus (*Eucalyptus* spp.) and *Zanthoxylum molle* Rehd. were found to be effective in controlling *A. flavus* (Bluma *et al*., 2009;

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Montes-Belmont and Carvajal, 1998; Rajkovic *et al*., 2015; Tian *et al*., 2014).

*Pistacia eurycarpa* Yalt. (Synonym: *Pistacia atlantica* subsp. kurdica) from Ancardiaceae family is a tree with an important source of gum. EO makes up 20% of the crude weight of gum (Hatamnia *et al*., 2014; Sharifi and Hazell, 2011). It is reported that the EO has antimicrobial and antioxidant properties (Bartosz, 2014; Sharifi and Hazell, 2011). According to literature survey, there are no reports on its antifungal activities against *A. flavus*. Therefore, in this study, the potential application of *Pistacia eurycarpa* Yalt. gum EO to control *A. flavus* growth on Potato Dextrose Agar (PDA) medium and maize grains was evaluated by volatile exposure and direct contact assays.

## MATERIALS AND METHODS

### Essential Oil

The EO was provided from trunk exudates of *Pistacia eurycarpa* Yalt. by steam distillation method in Saghez Sazi (Van Company) (Sanandaj, Kurdistan, Iran). In previous studies, the composition of the EO were determined by gas chromatography–mass spectrometry analysis (Sharifi and Hazell, 2011; Hesami *et al.*, 2014). The EO was sterilized by 0.45 µm pore size filters (Biofil) and was kept in a sterile airtight container at 4°C.

### Preparation and Activation of Mold

*Aspergillus flavus* (PTCC: 5004) was obtained from fungal collection center of Iranian Research Organization for Science and Technology (IROST). It was cultured on Potato Dextrose Agar (PDA, Merck, Darmstadt, Germany) slope for 10 days at 25±1°C. Conidia were harvested by 0.05% Tween 80 solution (Merck, Darmstadt, Germany). Conidial concentration was adjusted at $10^6$ $mL^{-1}$ by using hemocytometer and Tween 80 solution (Gandomi *et al*., 2009).

### Antifungal Activity of EO in Direct Addition to PDA Medium

The EO was added to sterilized PDA medium (at 45°C) to obtain final concentrations of 5,000, 6,000, 7,000, 8,000, 9,000 and 10,000 µL $L^{-1}$. After distribution in plates and solidification, Whatman No. 1 filter paper disc (6 mm diameter) was placed at the center of each PDA plates. Filter papers were inoculated with 10 µL of spore suspension ($10^6$ $mL^{-1}$). All of the above steps, except the addition of EO, were performed for the control. Plates were incubated at 25±1°C. The end of the trial was when the control mold covered the entire surface of the plate (Gandomi *et al*., 2009; Elsherbiny *et al.*, 2016).

### Agar Plate Method in the Vapor Atmosphere of EOs

Ten µL of the spore suspension ($10^6$ spores $mL^{-1}$) was inoculated on Whatman No. 1 filter paper disc (6 mm diameter) and placed at the center of each PDA plate. Inoculated PDA plates without lids were placed inside hermetically closed glass jars with transparent lids. A glass plate containing the essential oil (500, 4,000, 8,000, 12,000 mg of EO $L^{-1}$ of air) was located under the perforated plastic sheet in the jar. Jars were incubated at 25±1°C. A growth control with the same condition but without EO was prepared in parallel. Radial growth was measured every 24 hours during 9 days (Velazquez-Nunez *et al*., 2013).

### Application of EO Vapor in Maize Grain

Maize grain (Single Cross 704) was provided from Seed and Plant Improvement





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Institute of Alborz province, Iran. Moisture content of grains was determined by weighing them before and after drying at 105°C. In each 250-mL conical flasks, 100 g of maize was weighed and sterilized at 121°C for 15 minutes. Then, the moisture content of maize grains was adjusted to 20% with sterilized distilled water in accordance to the following formula (Gibriel *et al*., 2011):

$$S = \frac{B - A}{100 - B} \times 100$$

Where, *S* is the volume of water required for 100 g of corn sample to reach the required level of moisture content, *B* is required moisture content, *A* is initial moisture content.

Flasks were placed at 4°C for 48 hours and stirred at regular intervals. Then, grains were distributed in one layer in sterile Petri dishes. A 5 mm fungal disc was cut from the periphery of a seven-day-old culture and inoculated on maize grains in the center of each Petri dish. Uncovered inoculated plates with a glass plate containing the EO were placed inside the hermetically closed glass jars. The amount of EO weighed per unit volume of jars space were 0, 300, 400 and 500 mg $L^{-1}$. The jars were incubated at 25±1°C for 20 days. Every test was performed by triplicate (Bluma and Etcheverry, 2008; Gibriel *et al*., 2011; Marin *et al*., 2004; Velluti *et al*., 2004).

## RESULTS

The results of mixing the EO with PDA medium (Figure 1) showed that by increasing the concentration of EO, fungal growth was considerably reduced. Growth started after 3, 4, 4, 5 and 8 days of incubation at concentrations of 0, 5,000, 6,000, 7,000, 8,000 and 9,000 µL $L^{-1}$, respectively. At concentration of 10,000 µL EO per liter of PDA medium (T6) and more, mold growth was stopped completely (Figure 2).

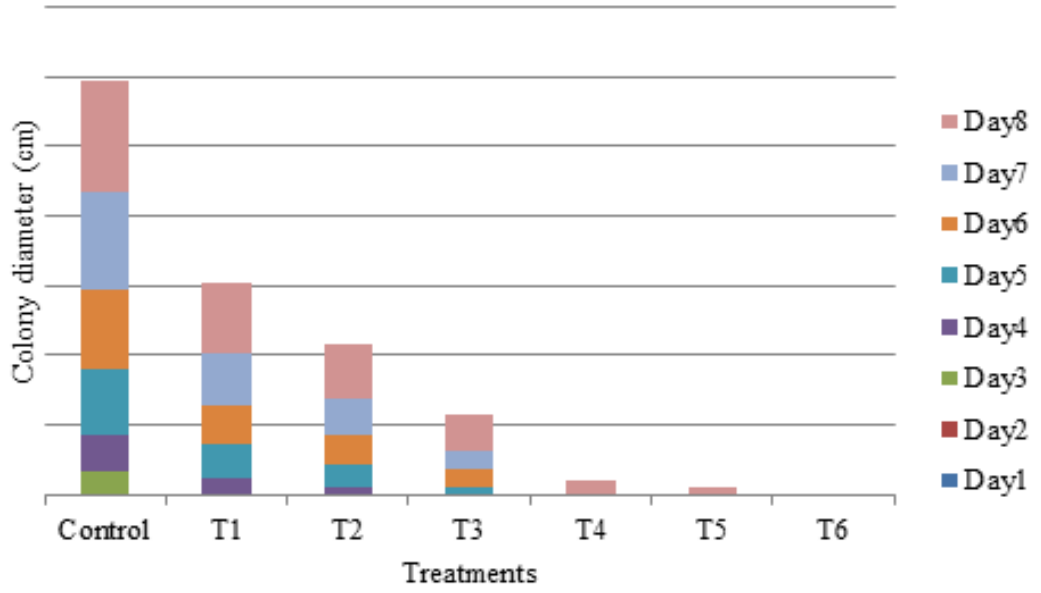


**Figure 1.** Effect of *Pistacia eurycarpa* Yalt. EO on radial growth of *A. flavus* PTCC 5004 in agar dilution method. EO concentrations (µL/ L) in treatments: Control (0); T1 (5,000); T2 (6,000); T3 (7,000); T4 (8,000); T5 (9000); T6 (10,000).





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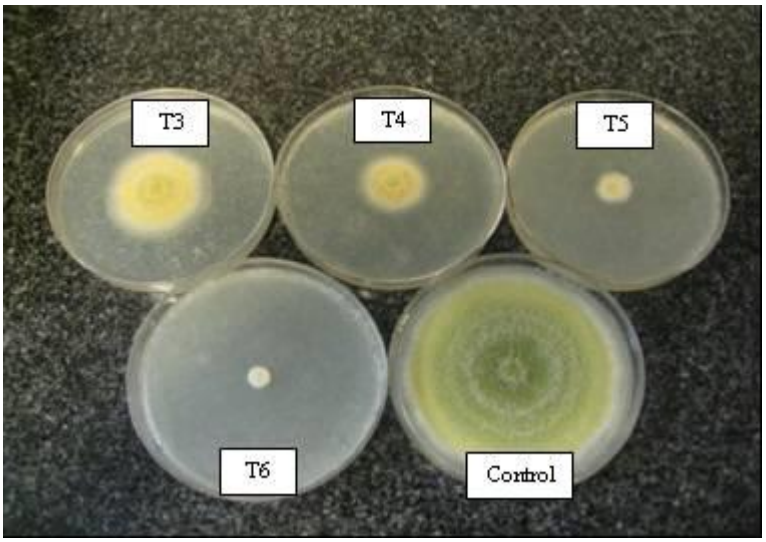


**Figure 2.** Image showing growth of *A. flavus* PTCC 5004 in agar medium with different EO concentrations (µL/ L) including: 0 (control); 7,000 (T3); 8,000 (T4); 9,000 (T5); 10,000 (T6) at the end of the 8 days.

At the second part of the experiment, EO was used at vapor phase instead of its direct addition. Based on the results, for all amount of the EO weighed per unit volume of jar space, the mean concentration of evaporated oil in jar atmosphere was 320 mg $L^{-1}$ (Table 1). Therefore, this concentration (320 mg $L^{-1}$) was evaluated on fungal growth eight times by triplicate.

The means of fungal growth diameter in treated and control samples during 9 days are presented in Table 2. In both groups, mycelial growth increased with incubation time, with the rate of mold growth in the control group being higher than the treated one.

The effect of EO vapors on fungal growth in inoculated maize grains (Table 3) showed that in the control group (without EO) mold growth started from the sixth day, and over 20 days of incubation, all plates diameter (8 cm) were covered by the fungus. In amount of 300 mg of EO per liter of jar space, mold growth started from the tenth day, and after twenty days, its diameter reached 3.28 cm. Similarly, with increase in the amount of weighed EO to 400 mg $L^{-1}$ of jar space, mold growth began with further delay from the thirteenth day, and on the 20$^{th}$ day, the diameter reached 1.73 cm. During twenty

**Table 1**. The amount of weighed and evaporated essential oil per unit volume of jar space (mg L-1).

| | | | | |
|---|---|---|---|---|
| Weighed (mg L-1) | 500 | 4000 | 8000 | 12000 |
| Evaporated (mg L-1) | 320 | 310 | 300 | 350 |

**Table 2.** Comparison of mold growth diameter in the treated and control samples during nine days incubation at 25±1°C.*a*

| Colony diameter (cm) | Incubation time (day) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Control | 0 | 0 | 0.6±0.1 | 2±0.1 | 4.5±0.2 | 5.7±0.1 | 6.4±0.2 | 6.8±0.2 | 8±0.1 |
| Treatment | 0 | 0 | 0 | 0.51±0.2 | 0.81±0.2 | 1.58±0.2 | 1.91±0.1 | 2.54±0.44 | 3.13±0.58 |

[a] Values are mean (n=24)±standard deviations.





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**Table 3.** Average daily radial growth of mold on maize kernels at different concentrations of essential oil.[a]

| Incubation time (day) | Average daily diameter of mold (cm) Essential oil concentration (mg L-1) 0 | 300 | 400 | 500 |
|---|---|---|---|---|
| 6 | 0.2±0 | 0 | 0 | 0 |
| 7 | 0.5±0 | 0 | 0 | 0 |
| 8 | 0.66 ±0.06 | 0 | 0 | 0 |
| 9 | 1.03±0.06 | 0 | 0 | 0 |
| 10 | 1.23±0.15 | 0.23±0.06 | 0 | 0 |
| 11 | 2.3±0 | 0.36±0.06 | 0 | 0 |
| 12 | 2.46±0.06 | 0.56±0.06 | 0 | 0 |
| 13 | 2.63±0.06 | 0.7±0 | 0.26 ±0.06 | 0 |
| 14 | 3±0 | 0.76± 0.04 | 0.46 ±0.06 | 0 |
| 15 | 4.06±0.06 | 1.06± 0.06 | 0.63±0.06 | 0 |
| 16 | 5±0.1 | 1.43± 0.1 | 1±0 | 0 |
| 17 | 5.73±0.06 | 1.83± 0.1 | 1.16±0.06 | 0 |
| 18 | 6.63±0.1 | 2.2 ± 0.1 | 1.43±0.06 | 0 |
| 19 | 7.63±0.1 | 2.96± 0.06 | 1.73± 0.06 | 0 |
| 20 | 8±0 | 3.28± 0.1 | 1.73±0.43 | 0 |

[a] Values are mean (n=3)±standard deviations.

days of incubation, in 500 mg of weighed EO per liter of jar space, equivalent to 320 mg of evaporated EO per liter of jar space, no mold growth was observed. Therefore, by increasing the EO concentration until the jar space was saturated with its vapors, the mold growth decreased to zero.

## DISCUSSION

As a strategy to enhance shelf life of foods and agri-commodities, development of safe plant-based preservatives like Eos, instead of synthetic ones, is a target of many studies (Kedia *et al*., 2014). The antimicrobial activity of plants EOs is well recognized (Bluma *et al*., 2009). EOs are composed of multiple chemical groups that have different mechanisms to destroy microorganisms. The most important feature of these groups is that they are hydrophobic. These materials enter into cells membrane and mitochondrial lipids. This disturbs the normal structure and creates more permeability, causing ions and other contents to exit the cell (Burt, 2004). The difference between EOs antimicrobial activities are related to their chemical composition , their concentration, microbial species, and methods used to investigate their activity (Bakkali *et al*., 2008; Bluma and Etcheverry, 2008; Tajkarimi *et al*., 2010).

Most studies have surveyed a direct impact of EOs on a wide range of microorganisms, while unlike many other antimicrobial materials, the vapor phase of EOs has a high biological activity inherently. So, in general, smaller concentrations of them are required by fumigation compared with direct contact application (Boukaew *et al.*, 2017). For example, Velazquez-Nunez *et al.* (2013) found that antifungal activity of orange peel EO vapor on *A. flavus* was more than its direct addition and, in this condition, lower concentrations were required to achieve the same antifungal effect. Li *et al.* (2016) also reported the same result for inhibition of *A. flavus* in licorice by using *Litsea cubeba* essential oil in direct contact and fumigation assays. The diminished activity of EO in direct addition may be due to the presence of lipophilic materials (Velazquez-Nunez *et al*., 2013). In fact, in direct contact application, some of the hydrophobic and volatile active substances of EOs are bound by food





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components and others, according to their affinity with water, are partitioned through the product (Boukaew *et al.*, 2017). Bioactivity of EOs in the vapour phase makes them attractive as possible fumigants for stored grain (Bluma *et al*., 2009). Also, lower concentrations of EO in vapor phase decreases its impact on sensory attributes of products (Velazquez-Nunez *et al*., 2013).

In the present study, both methods, i.e. vapor and direct contact of EO, confirmed its inhibitory effect on *A. flavus* growth, but at different concentrations. In mixing EO with PDA medium, with increasing the concentration of EO from 5,000 to 9,000 µL $L^{-1}$, mold growth was decreased gradually, and was inhibited completely at concentration of 1,0000 µL $L^{-1}$. Gandomi *et al.* (2009) also showed the inhibitory effect of *Zataria multiflora* Boiss. EO on growth and sporulation of *A. flavus* by a dose-dependent pattern. By using EO vapor for inhibition of mold growth on PDA medium, the interior jar space was saturated with 320 mg EO $L^{-1}$ (at 25±1°C) and on the ninth day of study, mold growth was inhibited about 60.87% compared to the control sample. By using the EO vapor on maize samples, when jar space was saturated with EO, no growth was observed within twenty days of incubation. This study shows the higher impact of EO on fungal growth in vapor phase than in direct addition.

Alpha–pinene is a major constituent (> 91%) of *Pistacia eurycarpa* Yalt. EO (Sharifi and Hazell, 2011). Alpha-pinene and beta-pinene are effective in anti-fungal activity in some of the EOs (Bajpai *et al*., 2007).

Studies have shown that smaller compounds such as monoterpenes (of which α-pinene is one of the principal species) are most efficient when used as headspace volatiles (Vilela *et al*., 2009).

The mechanism through which pinenes are active against microorganisms lies mainly in their capacity to induce toxic effects on the membrane structure and functions (Andrews *et al*., 1980).

Therefore, these results show potential replacement of antifungal chemicals by *Pistacia eurycarpa* Yalt. EO (especially in vapor phase) as a natural inhibitor to control *A. flavus* growth in food materials such as maize.

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## فعالیت ضدقارچی اسانس *Pistacia eurycarpa* Yalt. بر *آسپرژیلوس فلاووس* به روش تماس مستقیم و فاز بخار

**خ. امینی، و س. بهرامیان**

### چکیده

فعالیت بازدارندگی اسانس *Pistacia eurycarpa* Yalt. علیه *آسپرژیلوس فلاووس* (PTCC: 5004)به روش رقیق سازی در آگار و روش فاز بخار مورد ارزیابی قرار گرفت. در روش اول اسانس به محیط آگار اضافه گردید تا غلظت های ۰، ۵۰۰۰، ۶۰۰۰، ۷۰۰۰، ۸۰۰۰، ۹۰۰۰ و ۱۰۰۰۰ میکرولیتر در لیتر حاصل گردد. در روش ارزیابی بخار پلیت های حاوی محیط کشت پوتیتو دکستروز آگار و نیز پلیت‌های حاوی ذرت، تلقیح شده با کپک و بدون درپوش، در جارهای نفوذناپذیر به هوا که فضایشان با بخارات اسانس اشباع شده بود قرار داده شدند. نتایج نشان دادند که در آزمون رقیق سازی در آگار، اسانس در غلظت های بالاتر از ۱۰۰۰۰ میکرولیتر در لیتر به شکل کامل از رشد کپک مورد آزمون جلوگیری می نماید. در روش تماس با فاز بخار، در پلیت های پی دی آ رشد میسلیوم‌ها ۶۰/۸۷٪ مهار شد و در پلیت های حاوی ذرت طی ۲۰ روز گرمخانه گذاری هیچگونه رشدی مشاهده نگردید.





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