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Full Length Research Paper

The study of antifungal effects of herbal essences on *Aspergillus parasiticus*, an aflatoxin producer in pistachio (*Pistacia vera*)

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Antifungal effects of 6 herbal essences (thyme, oregano, spearmint, lime, eucalyptus and galbanum) that seemed to have powerful antifungal characteristics on *Aspergillus parasiticus* were studied in this research. *A. parasiticus* is one of the most important species that produces aflatoxin in pistachios. The herbal essences of the used concentrations had no effects on the flavor of pistachios. We used pure lyophilized culture of *A. patasiticus* for laboratory experiments. After activation of fungus, a suspension containing 10^6 spore/ml was prepared and cultured in yeast extract sucrose medium, containing different concentrations of essences (between 200 to 1000 ppm). Then, the growth ratio of fungus was determined by weighting produced mycelium. The results were analyzed statistically to determine the most appropriate essence and the minimum inhibitory concentration (MIC) of each one. The results indicated that essences of thyme and oregano had the most inhibitory effect on *A. patasiticus* growth at concentrations of 200 and 400 ppm, respectively.

Key words: Aflatoxin, antifungal, *Aspergillus parasiticus*, essence, pistachio.

INTRODUCTION

One of the most important factors that increase agricultural and horticultural wastes is the activity of bacteria and toxin producing fungi. Application of chemical antifungal and antibacterial substances is now common for preserving agricultural products. In this regard, microbiologists and food technologists have applied different chemicals. However, most of these compounds are synthetic and expensive and have side effects on human beings. In recent years researchers have shown tendency toward natural herbal compounds named essences. Essences have little harm for human health and environment. In laboratory experiments they have shown great potency in preventing growth of fungi and bacteria and their toxin production (Falahati, 1986; Briozzo et al., 1996). Iran ranked first in the production and export of pistachios (Iran, US, Turkey, Italy and Greece). Doubtlessly, the most important issue in exporting pistachios has been aflatoxin in recent years. In the latest 36 years there have been some cases that Iranian pistachio has been sold cheaper or sent back as a result of its aflatoxin. Pistachio is a strategic product and has economic importance. As a result, it is necessary to develop appropriate methods for reducing microbial contamination and especially fungal contamination of pistachios and retaining its quality for export (Deans and Ritchie, 1987). Herbal essences have antifungal effects and fortunately we can produce a large variety of them in our country. In this research we observed antifungal effects of some of these essences (including thyme, oregano, spearmint, lime, eucalyptus and galbanum) on *Aspergillus patasiticus* (which is one of the most important aflatoxin producers in pistachios) and determined their minimum inhibitory concentration (MIC). Bullerman et al. (1997) compared antifungal effects of some herbal essences with antifungal compounds and indicated that essences have the same antifungal

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potency of synthetic compounds. Karapinar (1990) studied the inhibitory effects of two essences, Thymol and Eugnol, on the growth and toxin production of *A. parasiticus* and indicated that these compounds inhibited the growth of this microorganism. Evandro De Souza et al. (1981) investigated the inhibitory action of some essential oils and phytochemicals on toxin production by various moulds isolated from foods. Omidbeygi et al. (2006) added 50, 200, 350 and 500 ppm of thyme, summer savory and clove essential oils to culture media and ketchup. They showed that thyme and cloves had the highest inhibitory effect on *Aspergillus flavus*. Also they do not affect the flavor of the product at the studied concentrations. Mahdavi et al. (2010) investigated the effects of thyme, pennyroyal and lemon essential oils at concentrations of 0.002 to 4% on *candida* species. They showed that thyme was most effective in inhibiting the fungus. Davidson (2001) stated that thymol, carvacrol, eugenol may inactivate some essential enzymes and the high inhibitory effect of thyme may relate to its high content of thymol. Rasooli and Razzaghi (2004) studied on the inhibition of *A. parasiticus* growth and its aflatoxin production exposed to the essential oils extracted from two varieties of Thyme. In their research, they found that the oils from the above plants were strongly fungicidal and inhibitory to aflatoxin production. They recommended the substitution of currently used antifungal and aflatoxin inhibiting chemicals by natural compounds. In our study we investigated the possibility of the application of some natural antifungal substances to inhibit the growth of aflaoxin producer fungi.

MATERIALS AND METHODS

A. patasiticus used in the experiments was obtained in the form of lyophilized pure culture from Fungus and Bacteria Collection Center affiliated to Biotechnology Research Institute of Iran. All chemicals including medium cultures were of Merck brand and the herbal essences were obtained from a local market in Mashhad (Iran).

Preparing standardized suspension from fungus spore

To activate lyophilized culture, a tube containing fungus was broken in sterile conditions and was put into Erlenmeyer containing 500 cc of Sabouraud Dextrose Broth. After 20 days of incubation period at 25°C, the fungus was activated and started to grow in culture medium. After completing the growth of the fungus, the Erlenmeyer was put on a shaker. The fungus mycelia were broken and scattered in culture environment due to the stirring action of the shaker. Then about 1 ml of the suspension was taken and cultured in test tubes containing PDA and its concentration was measured. Then tubes were incubated at 25°C for 10 days in order to let the fungus grow and produce spore.

Sterile glass pearls and 10 ml of sterile Tween 80 (0.05% solution) were used for getting the spores. Shaking the tubes caused the spores to enter the solution and clash with pearls. Then the solution was filtered by sintered glass filter in sterile conditions to separate the pieces of fungus mycelia and culture medium from spore suspension. The suspension containing *A. patasiticus* spores was cultured on PDA culture medium and was diluted to obtain a suspension with concentration of 10^6 spore/ml. This suspension was used later to study the effect of essences on mycelia growth (James, 1993; Falahati, 1986; Evandro et al., 2005).

Fungus growth in the medium containing essence

YES (yeast extract sucrose) culture medium was used to evaluate the effect of essences on fungus growth. In each tube we poured 19 ml of culture medium containing essences with the concentrations of 200,400,600,800 and 1000 ppm. One milliliter of the suspension containing 10^6 spore/ml was inoculated into the tubes. Then tubes were incubated at 25°C for 12 days to let spores germinate and the fungus grows in culture medium (Alonso and Croleau, 1991). We used 4 replications for each treatment (kind of essence and its concentration).

Measurement of fungus growth in culture medium

In our experiments we used statistical software SPSS 8 and a completely randomized design for analysis of the results. To determine the fungus growth ratio in the culture medium, the dry mass of fungus mycelia was measured. So, culture medium containing grown fungus passed through the dry sintered glass filter and was then washed 3 times by distilled water, after which the filter containing mycelia mass was put in the oven at 70°C for 24 h to dry. At the end, the filter was weighted by precision scale and the empty dry filter weight of the pre-experiment was reduced for it to obtain the weight of dry mycelia mass (Falahati, 1986; Arras and Grella, 1992; Saeid Mahdavi et al., 2010).

RESULTS AND DISCUSSION

The statistical analysis of the obtained data, based on completely randomized design, showed that application of different essences had significant effects on *A. patasiticus* growth. As it is shown in Figure 1, thyme and oregano had the most effect, while eucalyptus and lime had the least inhibitory effect on *A. patasiticus* growth. Rasooli and Razzaghi (2004) obtained similar results about the fungicidal effect of thyme on *A. patasiticus* growth. Comparison of means showed that the least fungus growth observed in the cultures containing thyme and oregano, and the most fungus growth occurred in the cultures containing spearmint, lime and eucalyptus (Table 1). The results indicated that increasing the concentration of essences leads to significant decrease of fungus growth in culture medium (Figure 2). It can be seen from Table 2 that the highest fungus growth belongs to the control treatment (without essence) and the lowest growth can be seen at the essence concentrations of 600, 800 and 1000 ppm. There was no significant difference between the last 3 concentrations of essences.

Omidbeygi et al. (2006) in their similar research indicated that thyme oil showed the strongest inhibition at the concentration of 350 ppm. In our research, we found that the fungus was able to grow in all of the concentrations of spearmint, lime, eucalyptus and galbanum, but thyme and oregano essence in concentrations of 200 and 400 ppm, respectively,

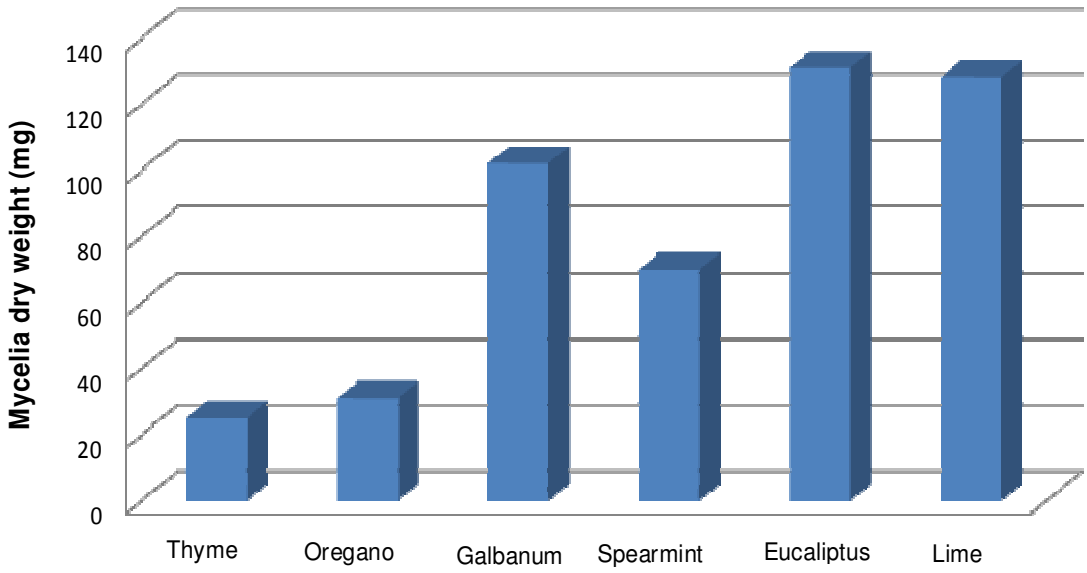

Figure 1. The effect of essences on *A. parasiticus* growth.

Table 1. The effect of essences on *A. parasiticus* growth.

Statistical ranking α = 0.05	Essences	Mycelia dry weight
a	Thyme	25
a	Oregano	31
b	Galbanum	102
bc	Spearmint	70
c	Eucaliptus	131
c	Lime	128

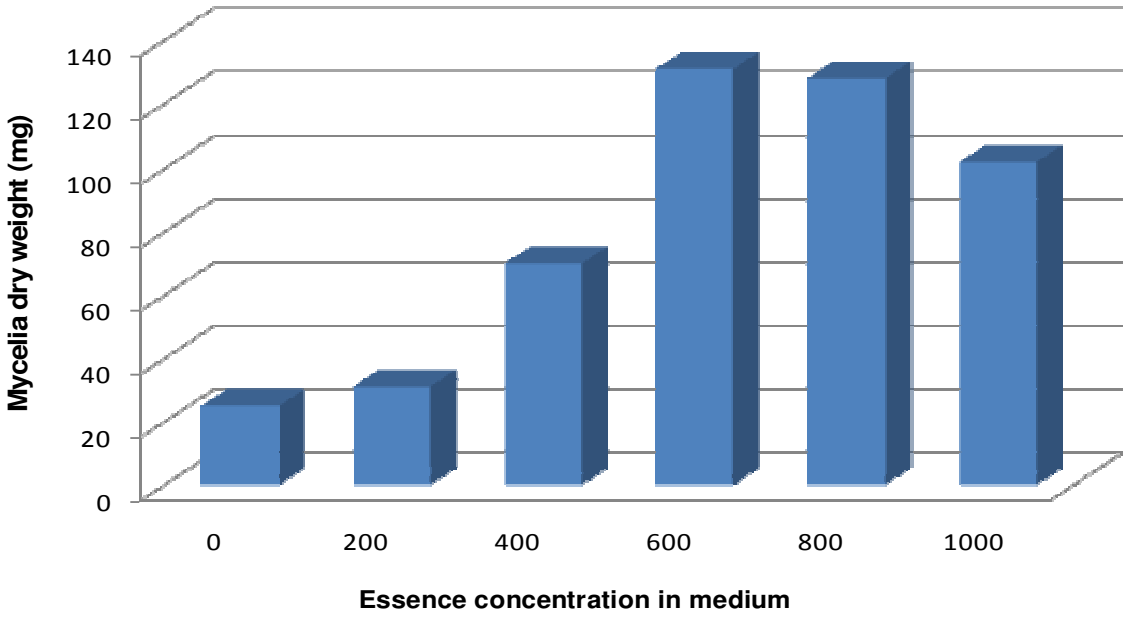

Figure 2. The effect of essence concentration on *A. parasiticus.*

Table 2. The effect of essence concentration on *A. parasiticus.*

Statistical grade α = 0.05	Essence concentration (ppm)	Mycelia dry weight
d	0	25
c	200	31
bc	400	70
abc	600	131
Ab	800	128
a	1000	102

Table 3. The effect of kind and concentration of essences on *A. patasiticus* growth.

Essence	Dose (ppm)					
	0	200	400	600	800	1000
Thyme	150.5	0	0	0	0	0
Oregano	150.5	37	0	0	0	0
Spearmint	150.5	132	121.5	105	94.25	76
Eucalyptus	150.5	139	133.75	139.5	120.75	107.75
Lime	150.5	175	173.25	136.25	84.75	49.75
Galbanum	150.5	123.5	106.75	95	74.75	62.75

completely stopped the fungus growth. This result is confirmed by many researchers (Nicola et al., 2005; Karapinar, 1990; Aras and Grella, 1992; Briozzo et al., 1996).

In general, the inhibitory action of natural products on mould cells involves granulation of cytoplasm, rupture of cytoplasmic membrane and inactivation and/or inhibition of synthesis of intra and extracellular enzymes. These actions can occur separately or concomitantly and culminate with the mycelium germination inhibition (Evandro De Souza et al., 2005). Occurrence of phytochemicals which did not show antifungal activity could suggest that probably, in some situations, certain phytochemicals shown their antimicrobial action only when acting in a synergistic way with other constituents when they are in the form of extracts, essential oils, decocts, etc. Moreover, resistance and invasion capability of the mould strains used as test microorganisms could be cited as important interfering factors to the antifungal efficiency of the essential oils and phytochemicals included in the antifungal assays (Evandro De Souza et al., 2005; Gueldner et al., 1985). The results obtained justify future researches and it emphasizes the antimicrobial properties of plant products and their possible uses as natural alternatives to control the microbial growth in foods. The comparison of results is shown in Table 3.

Conclusion

According to the results, we concluded that all the studied essences reduced the growth of *A. patasiticus*. Among the studied essences, thyme showed the most inhibitory effect. It stopped the growth of *A. patasiticus* at concentration of 200 ppm completely. Oregano ranked second in inhibitory effects after thyme. It stopped the growth at the concentrations of 400 to 1000 ppm. Other essences reduced the growth rate, but had no inhibitory effect on fungi.

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