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ORIGINAL ARTICLE

Study on Anti Fungal Effects of Herbal Essences on *Aspergillus flavus*

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ABSTRACT
*Aflatoxins are generated in pistachio as the secondary fungal metabolites by Aspergillus Flavus and Parasiticus fungi. These compounds are extremely toxic and cancerous. Essences of different medicinal plants were used to study the antifungal (inhibitory) effect of herbal essences on prevention from growth of A. Flavus fungus. Among the herbal essences, 7 cases namely thymus vulgaris (thyme), mint, eucalyptus, fennel, galbanum, rosemary, and satureja were studied. Solid and liquid environments were used for analyzing mycelium fungus. Results of statistical analyses indicated that thyme and fennel essences exhibited maximal inhibitory effect. In the next step, the aqueous essence was sprinkled over 4 pistachio cultivars in order to study the impact of these essences at the concentrations of 400 and 600 ppm on reduction of fungal growth and its spore contamination. In the current research, factorial test was applied in basic completely randomized design for assessing the individual and mutual impacts. The final results suggested that the effects of type (cultivar) and essence concentration caused significant differences on A. Flavus colonization percent .Also, interaction effects (cultivar*essence concentration) was significant on colonization percentage of this fungus but other treatments did not leave any significant effect on the colonization percentage. Results showed that thyme at the concentration of 600 ppm exhibited the maximal inhibitory effect on the fungal growth while fennel essence at the concentration of 400 ppm had the minimal inhibition.*
***Key Words**: Essence Herbal Plants, Pistachio, Aspergillus flavus.*

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INTRODUCTION

Toxin-generative fungi and bacteria are among the most important factors that produce wastes in agricultural and gardening crops, and, application of bactericide and fungicide chemicals for reducing activities and wastes caused by micro organisms is one of the most common ways to preserve agricultural crops. In this regard, microbiology and alimentary industries researchers have deployed various compounds but application of these mainly synthetic compounds could have many undesirable side effects for humans in addition to their high cost production costs [15].During the recent years, the researchers have devoted attention to a group of natural herbal compounds referred to as essences which are natural and lowly risky substances for human health and environment besides featuring antifungal properties. Seemingly, application of herbal essences in all of the treatment stages is capable of affecting population of toxin-generative isolates of A. Flavus fungus, and ultimately, could help mitigating crop contamination to Aflatoxin[15].Aspergillus Flavus grows on a wide range of agricultural crops and foods and contaminates them through secretion of Aflatoxin. Aflatoxin is obnoxious and cancerous chemical and its value in agricultural crops was rigorously investigated; the permissible value in most countries ranged between 5 to 15 ppm [6]. Essentially, plant essences are potential sources of bactericidal compounds and greatly useful and effective for this purpose. It is very difficult to compare the results reported about bactericidal properties of different essences. The reasons might be attributed to different evaluation methods of these properties and source of preparation as well as planting conditions, variant microbial isolates and even different bacteria and fungus concentrations used as inoculum [1].
Some researchers believe there is a correlation between chemical structure of these compounds and their active ingredient content. Normally, the essence rich in phenol compounds have high

antimicrobial properties. These compounds can both penetrate into cellular membrane and play role in clotting the cell ingredients [4,3,2]. The conducted studies also showed that essences of basil, club, caraway, and rosemary at the concentration of 1000 ppm completely inhibit growth of A. Flavus and the aforementioned essences at the concentrations of 500, 700, and 1000 ppm prevent the respective fungus from generating Aflatoxin B1[7]. In another research, effects of DENA thyme, Shiraz thyme, black caraway, and hyssop essences were studied on different Aspergillus including: A. Niger, A Parasiticus, and A. Flavus.

The results implied that essences of DENA thyme and hyssop can be applied as fungicides against A.Fumigatus, A.Niger and A.Flavus species[10].Antifungal effect of rosemary essence against A.Flavus fungus was studied and the results demonstrated that rosemary essence as compared to gentamycin antibiotich as strong antifungal activity against A.Flavus at the concentrations of 0.5 and 0.25%. A large portion of this antifungal activity of rosemary essence is attributed to α-Pinenemonotropein [12].

In the present research, effects of different herbal essences were studied and analyzed on growth and spore contamination level of A.Flavus fungus. Additionally, the research investigates application of some natural substances and products having no detrimental side effects such as herbal essences as substitute of chemical fungicides for control and/or reduction of diseases and wastes following the pistachio harvest.

MATERIALS AND METHODS

The current research was evaluated in the Pathology Laboratory of Islamic Azad University, DAMGHAN Branch, Iran. The essences of garden thyme, satureja, eucalyptus, rosemary, galbanum, and mint obtained through water distillation and in industrial scale were supplied by KASHAN Galbanum Essence Company. The aforementioned essences were kept at 4° C inside a fridge until analysis and application time; the isolates used in the present research were AspergillusFlavus prepared from the laboratory of DAMGHAN's Pistachio Research Station. The respective fungi were planted on Sabouraud Dextrose Agar (SDA)slants.The samples were preserved in incubator at 28° C to grow and produce spores. Spore suspension was then prepared by distilled water and spore concentration was counted using hemocytometric technique. YES planting environment was used for studying the antifungal effects of the thyme, fennel, mint eucalyptus, rosemary, galbanum, and satureja essences on growth of Aspergillus Flavus. The essences were added to the planting environment together with Tween at the concentrations of 200, 400, 600, 800, and 1000 ppm.For the control treatment, the same volume of distilled water and Tween80 (0.05)were used instead of essences. In the next step, 0.5 cc of 10^6 spore/ml suspension was added to the tubes which were subsequently shaken. The tubes underwent vortex current for 60 seconds in order to have uniform suspension and essence.

The tubes were filtered after 12 days using Whatman filter paper and dry weight of mycelium was measured by means of 0.0001 g SARTORIOUS balance. Subsequently, 12 gr of pistachio nut of each cultivar (in completely randomized design with three replications) were soaked in distilled water for approximately 10 minutes using 0.5% sodium hypochlorite for ensuring surface disinfection and absorption of initial moisture. The pistachio nuts of different cultivars were then placed in sterile Petri dishes and inoculated by one milliliter of fungus suspension (at a concentration of $2*10^6$ "spores/ml"). The Petri dishes were then positioned inside plastic vessels in whose bottoms sterile distilled water had been already poured to provide moisture at the saturated level [11]. Then, thyme and fennel essences were used at the concentrations of 400 and 600 ppm as the best and most effective treatments in comparison with a control treatment. The pistachio surfaces were sprayed such that the whole suspension-immersed area of pistachio was covered by essences, and, the Petri dishes were placed inside plastic vessels in whose bottom sterile distilled water had been already poured to provide moisture at saturated level. The Petri dishes were then kept in incubator at 26° C for 7 days. Visual scoring was carried out in the 3rd and 5th days and the spores were counted in the 7th day using hemocytometer lam. Factorial test based on completely randomized design was used in the current study to assess the individual and mutual impacts. Also, SAS software and Duncan's multi-domain's test were applied for analysis of variances (ANOVA) and grouping the mean values of treatments.

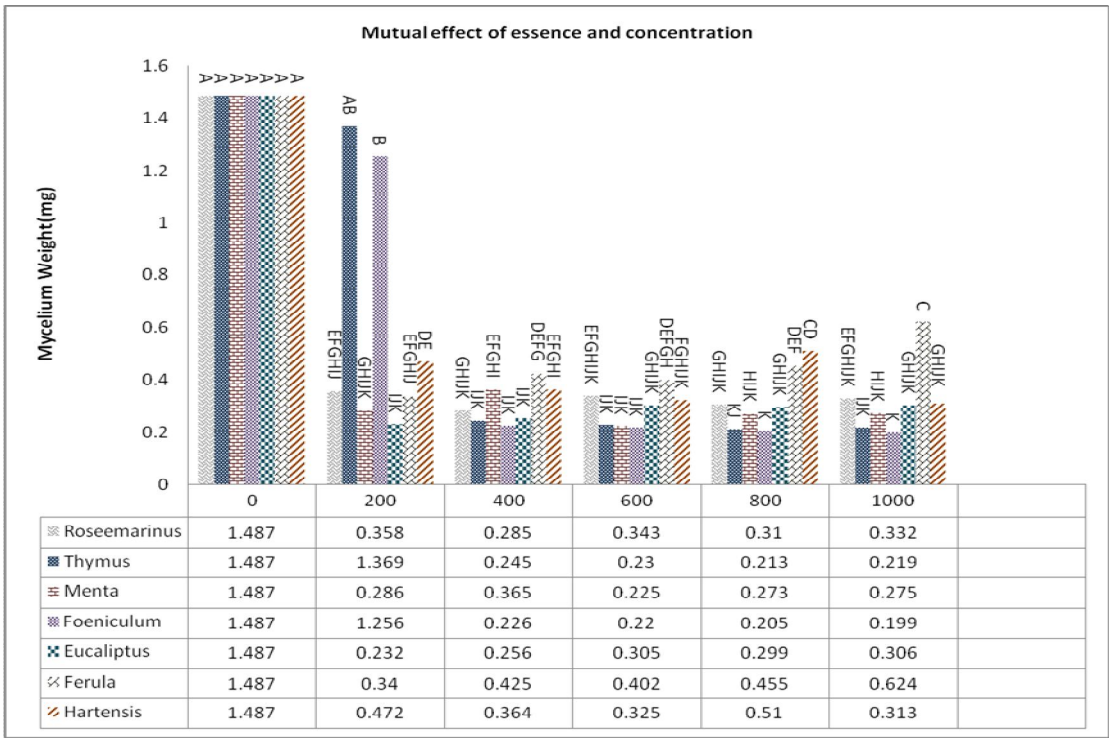
RESULTS

Table 1. Analysis of Variance (ANOVA) for effects of different plant essences and concentration on mycelium weight in YES medium

Soures of Variations	Degree of Freedom	Mean of Squares
Essence Type	6	0.071**
Concentration of Essence	5	4.651**
Essence Concentration* Essence Type	30	0.152**
Error	84	0.005**

Ns, *,** respectively mean insignificant, and significant at p-values of 1 and 5 percent

ANOVA results for the data relatedto the impacts of different concentrations and types of plant essences on mycelium weight of A.Flavus in YES environment after 12 days indicated that essence type and concentration effects as well as their mutual impact are significant at p-value = 0.01. Comparison of mean values revealed that the least weight belonged to 600 ppm concentration and was equal to 0.292 mg. This concentration was classified in the same statistical group together with 400, 600, 800, and 1000 ppm. The maximal weight was observed for the zero concentration sterilized with distilled water (1.486 mg). It is consequently inferred that the concentrations above 400 ppm had significant impact on the weight value(table 1).
Comparison of mean values of mycelium weight for different essences and concentrations in liquid planting environment.



	0	200	400	600	800	1000
Roseemarinus	1.487	0.358	0.285	0.343	0.31	0.332
Thymus	1.487	1.369	0.245	0.23	0.213	0.219
Menta	1.487	0.286	0.365	0.225	0.273	0.275
Foeniculum	1.487	1.256	0.226	0.22	0.205	0.199
Eucaliptus	1.487	0.232	0.256	0.305	0.299	0.306
Ferula	1.487	0.34	0.425	0.402	0.455	0.624
Hartensis	1.487	0.472	0.364	0.325	0.51	0.313

Figure1.Mutual effect of essence and concentration

Results of comparison of mean values showed that effects of essence type and concentration on colonization percentage of A.Flavus fungus in the 7th day yielded significant difference at p-value=0.01. Also, mutual impact (cultivar*essence concenrtation) on colonization percentage of this fungus was significant at p-value of 5% but other treatments did not significantly affected

colonization percentage of this fungus in the seventh day. (table 2).It was also observed that zero concentration of essence yielded the maximal colonization percentage for this fungus in the 7th day with an average value of 5.72% and the next ranks for this parameter respectively belonged to concentrations of 600 and 400 ppm. Concerning the mutual effects, AKBARI and SHAHPASAND cultivars and zero-concentration essences with average values of 6.398 and 6.312 were ranked in the superior statisrical group as compared to other treatments.

Table2: Analysis of Variance (ANOVA) for effects of different different treatments of A.flavus colonization in 7 days after incubation

Source of Variations	Degree of Freedom	Mean of Squares
Cultivars	3	0.866ns
Essence Type	1	55.757**
Cultivar*Essence Type	3	0.47ns
Essence Concentration	2	13.43**
Cultivar*Essence Concentration	6	5.683*
Essence Type * Essence Concentration	2	3.516ns
Essence Type* Concentration*Cultivar	6	1.057ns
Error	48	1.864

Ns, *,** respectively mean insignificant, and significant at p-values of 1 and 5 percent

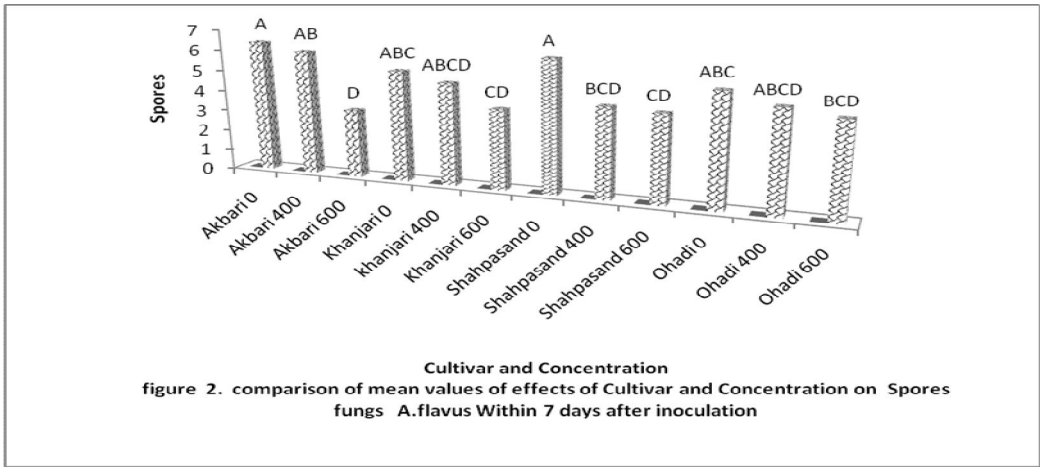


figure 2. comparison of mean values of effects of Cultivar and Concentration on Spores fungs A.flavus Within 7 days after inoculation

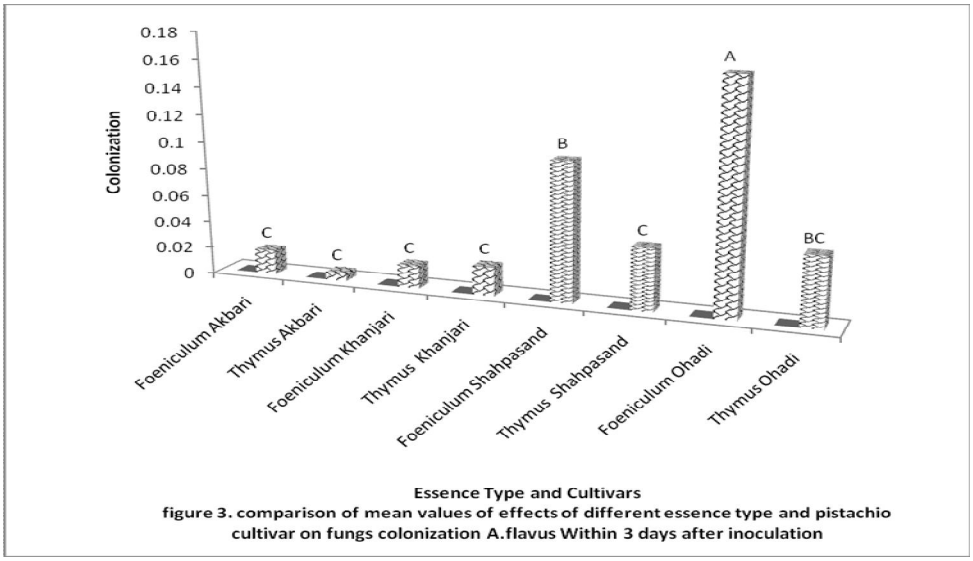


figure 3. comparison of mean values of effects of different essence type and pistachio cultivar on fungs colonization A.flavus Within 3 days after inoculation

DISCUSSION

The conducted experiments revealed the fact that the highest and lowest fungal growth inhibition levels were observed for concentrations of 600 and 200 ppm. Results of Aspergillus Flavus were assessed after24 hours during 7 days (the 3rd, 5th, and 7th) following inoculation. No growth occurred in any of the treatments in the first day. There was significant difference between essence-free pistachio nut and the immersed pistachio at both p-values because growth inhibition extent increased with exceeding concentration i.e. concentration of 600 ppm had greater inhibitory effect than concentration of 400 ppm.

Consequently, the difference in antifungal activity of essences depends on their compositions. A compound might result in antifungal activity of the essence either ,individually or as the intensifier of other compounds[14]. As demonstrated by inhibitory strength of thyme essence improves with increase in its concentration, and thereby, thyme essence at the concentration of 10% led to prevention from A.Flavus; this result is in agreement with the findings of the present study [8]. GANDOMI et al. showed through a research that thyme essence at a concentration of 400-1000 ppm causes prevention from fungal growth and morphological alterations of A. Flavus fungus [13].

Sensitivity of fungal species relies on their essence type and different concentrations. The difference in antifungal activity of herbal essences is associated with their constituents such that a compound might result in antifungal activity of the essence either individually or as the intensifier of other compounds to have acceptable effect at a certain concentration. As demonstrated by RAD et al, thyme and satureja essences with respective concentrations of 200 and 400 ppm managed to control growth of AspergillusParasiticus fungus through measuring the dry weight [15]. Through another study realized that thyme essence at the concentration of 350 microliter per ml in liquid environment leads to 100% inhibition of fungal growth of A. Flavus [13]. They also concluded in another research that thyme essence exhibits suitable antifungal property against A. Flavus fungus thanks to its phenol compounds (thymol, carvacrol, borneol). This essence also leads to granulation of cytoplasmic content and destruction of cytoplasmic membranes, and ultimately, prevention from mycelium germination [5]. In another study, they found out that thyme essence contributes to reduction of mycelium growth at the concentration of 8 “µl/ml”. It was shown that increase in the thyme concentration causes denaturation of enzyme protein, which in turn results from presence of phenol compounds (thymol, carvacrol, eugenol)[16].

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