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Evaluation of antifungal properties of some medicinal plants against *Aspergillus flavus* isolated from contaminated Corn *in vitro*

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Abstract: *Aspergillus* strains especially *A. flavus* and *A. parasiticus* are the most frequent grains molds producing carcinogenic aflatoxins, which is one of the main challenges in the agriculture and food industries. Chemical pesticides for control of the fungi have adverse effects on human health and environment; therefore, the necessity of finding acceptable substitutes for these substances seem apparent. In the present study, aqueous extracts of aerial parts of zataria (*Zataria multiflora* Boiss.), thyme (*Thymus vulgaris* L.), pennyroyal (*Mentha pulegium* L.), peppermint (*Mentha piperita* L.), senna (*Cassia senna* L.), and basil (*Ocimum basilicum* L.) along with flowers of safflower (*Carthamus tinctorius* L.) were examined against *A. flavus* isolated from contaminated corn. The extracts with different concentrations (100-600 ppm) and poly ethylene glycol (PEG) with equal osmotic potential of the plant extracts were added to potato dextrose agar (PDA) medium to evaluate fungus growth after 7 days and using agar dilution method. All concentrations of the extracts significantly inhibited the fungus growth in comparison with each other and the control, while the extracts of thyme and zataria were manifested to be the most effective prohibition with minimum inhibitory concentration (MIC) of 600 ppm. Potency of the plants' extracts on the growth of fungus was evaluated as follows: zataria> thymus> safflower> peppermint> pennyroyal> senna> basil. Results of this study presented aqueous extracts of thyme and zataria as effective preservatives against growth of *A. flavus* for corn products.

Keywords: *Aspergillus flavus*; *Carthamus tinctorius*; *Thymus vulgaris*; MIC; *Zataria multiflora*.

Introduction

Many various kinds of fungi can abundantly be found all around us especially in soil and air, contaminated grains on stored food commodities and agriculture products. *Aspergillus* strains especially *A. flavus* and *A. parasiticus* are the most frequent grains molds producing carcinogenic aflatoxins[1,2]. *Aspergillus flavus* is a saprophyte fungus with pathogenic property for both plants and animals[3]. Secretion of hydrolytic enzymes by the fungus causes strong affinity to colonize in various foods[4]. Aflatoxins, produced by *A. Flavus*, are structurally related to difuranocoumarin derivatives, among them aflatoxins B_1 is highly toxic, mutagenic and carcinogenic[5,6]. These toxins usually contaminate agricultural products including corn,

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peanuts, wheat, cotton and tree nuts causing economic loss all around the world[3,7]. Because of high toxicity of aflatoxin, prevention of the fungus growth on the agricultural and food products has become a great concern of the scientists. In the previous studies, inhibition activity of essential oils and extracts of some plants were examined toward *A. flavus* or other species of the fungi[8-12]. Zataria (*Z. multiflora*) grows widely in Iran, Afghanistan and Pakistan with traditional uses as an antiseptic, carminative, stimulant, diaphoretic, diuretic, anesthetic, anti-spasmodic and analgesic[13]. Thyme (*T. vulgaris*) is flowering plant used as expectorant, antitussive, antibroncholitic, antispasmodic, anthelmintic, carminative and diuretic properties in the folk medicine. Pennyroyal (*M. pulegium*), peppermint (*M. piperita*) and basil (*O. basilicum*) are widely cultivated in the most part of the world for their medicinal, ornamental properties as well as flavour and aroma for food[14,15]. Leaves and fruits of senna (*C. senna*) are well known laxative regarding to their anthraquinons[16]. Safflower (*C. tinctorius*) has reputation for curing several diseases and also for its oil reach seeds[17]. Eessential oils of some plants against *A. flavus* were tested and the results showed that oils of *Cinnamomum zeylanicum*, *M. piperita*, *O. basilicum*, *Origanum vulgare*, *Teloxys ambrosioides*, *Syzygium aromaticum*, and *T. vulgaris* totally inhibit the fungus growth[18]. The oil of *T. vulgaris* efficiently arrested aflatoxin B_1 production by *A. flavus* better than most of the synthetic fungicides[19]. According to the results of a study, strong antifungal activity of thyme oil could be attributed to thymol or synergism of other major components of the essential oil[20]. Antimicrobial effects of essential oils of *Z. multiflora* toward yeast, Gram-positive and Gram-negative bacteria as well as *A. flavus* and *A. parasiticus* have been well documented[21-23]. Protective effect of carvacrol, the main phenolic compound of *Z. multiflora* oil, has been evaluated against some fungi; therefore, it seems that the compound is responsible for inhibitory effect of the plant oil[24,25]. While, both polar and non-polar extracts of *C. senna* did not exhibit antifungal activity toward *A. niger*[26]. Inhibitory effect of oil of *M. piperita* was examined toward *A. fumigatus*, *A. flavus* and *A. ochraceus* during 10 days. The results indicated that the oil of *M. piperita* inhibits growth of all tested fungus species[27]. Menthol, the main organic component of *M. piperita* oil, effectively contributed to high antifungal activity of the oil[28]. In addition, oil of *M. pulegium*, which was reach in puelgone, had great potential for antibacterial and antifungal activities against tested microorganisms including *A. niger* and *A. flavus*[29]. Oil of *M. pulegium* from Iran, belongs to piperitone/piperitenone type, showed inhibition against *A. niger*[30]. Since chemical pesticides have adverse effects on human health and environment, the necessity of finding acceptable substitutes for these substances seems apparent. In the present study, aqueous extracts of some medicinal and edible plants including zataria, thyme, pennyroyal, peppermint, and basil belong to Lamiaceae and senna, a species of Fabaceae family, with safflower, a flowering plant of Asteraceae, were examined against *A. flavus* isolated from contaminated corn using agar dilution method to explore suitable natural preservative.

Results and Discussion

Using disk diffusion method, fungus growth diameter of *A. flavus* isolated from contaminated corn in the presence of aqueous extracts of some medicinal plants was successfully investigated in this study. The results of this study indicated that all the plants' extracts significantly inhibit *A. flavus* growth in comparison with each other and with the negative control (PEG) (Table 1).

Table 1: Effects of aqueous extracts of the plants on *A. flavus* growth in PDA medium at different concentrations expressed as fungus growth diameters ± SD and MIC (ppm) values.

	Fungus growth diameter (mm)						MIC
Conc. (ppm)	**100**	**200**	**300**	**400**	**500**	**600**	
T. vulgaris	24.6 ± 4.7	14.6 ± 3.7	12.3 ± 0.5	11.0 ± 1.0	1.1 ± 0.7	0 ± 0	600
Z. multiflora	29.6 ± 0.5	14.6 ± 1.5	13.0 ± 1.7	11.6 ± 0.5	6.3 ± 1.1	0 ± 0	600
C. tinctorius	31.3 ± 1.5	29.0 ± 1.0	25.6 ± 1.5	21.0 ± 1.7	17.3 ± 2.5	1.3 ± 0.5	> 600
M. piperita	31.3 ± 1.1	23.6 ± 2.3	17.0 ± 2.4	14.0 ± 1.0	11.0 ± 1.0	10.0 ± 0	> 600
M. pulegium	38.3 ± 2.8	27.6 ± 3.0	20.0 ± 0	18.6 ± 1.1	13.6 ± 1.1	10.0 ± 0.5	> 600
C. senna	34.6±1.5	27.0 ± 2.6	25.0 ± 0	20.3 ± 0.5	18.6 ± 1.5	10.0 ± 0	> 600
O. basilicum	40.0 ±0	26.6 ± 2.8	24.6 ± 1.2	20.6 ± 1.1	17.6 ± 2.0	11.6 ± 2.8	> 600
Control	60.0 ± 0	59.3 ± 0.5	58.6 ± 0.5	58.3 ± 0.5	58.3 ± 0.5	57.6 ± 0.5	-

Conc.: concentrations, MIC: minimum inhibitory concentration, ppm: part per million, control: polyethylene glycol.

Inhibition activity of all extracts was enhanced with an increase in their concentrations. Among them, the aqueous extracts of thyme (*T. vulgaris)* and zataria (*Z. multiflora*) with concentration of 600 ppm completely arrested the fungus growth and effectively prohibited the fungus growth even at lower concentrations (200-500 ppm) in comparison with other extracts and the negative control. Inhibition effects of the extracts of thyme and zataria in concentration of 500 ppm on the fungus growth were similar to safflower (*C. tinctorius*) in concentrations of 600 ppm ($p < 0.05$). The growth of *A. flavus* have been effectively inhibited by safflower extract at high concentration (600 ppm) with fungus growth diameter of 1.3 ± 0.5 mm, but the extracts of peppermint, pennyroyal, senna and basil exhibited lower activity against the fungus in comparison to thyme, zataria and safflower extracts.

In addition, MIC values of both, thyme and zataria were assessed as 600 ppm, while for other plants it was defined more than 600 ppm. Previous study, as it summarized in Table 2, has mostly examined antifungal activity of plants oils, for example essential oils of *T. vulgaris*, *Z. multiflora*, *M. piperita*, *M. pulegium* and *O. basilicum* demonstrated inhibitory activity against growth of *A. flavus* and other microorganisms[18,21-23,30]. However, in this investigation, aqueous extracts of *T. vulgaris* and *Z. multiflora* mostly arrested growth of the fungus related to their polar chemical constituents, which are water soluble. The results of our study are in consistency with previous investigation, which suggested that aqueous extracts of thyme and coriander mostly inhibit the isolated strain of *A. flavus* followed by dill and rose extracts[31]. Result of an experiment showed that safflower, which was wound-inoculated with *Phytophthora drechsleri* produces a polyacetylene compound that inhibits the growth of the mentioned fungus *in vitro*[32]. Both polar and non-polar extracts of *C. senna* did not exhibit antifungal activity toward *A. niger*[26]. While, antimicrobial activity of some *Senna* spp. against different microorganisms in the previous studies was demonstrated. For instance, aqueous extract of *S. obtusifolia* containing alkaloids and flavonoids prevented *A. niger* more than other extract of the plant[33]. Moreover, an unidentified flavonoid glycoside isolated from leaves of *S. alata* prohibited growth of *A. niger* with MIC value of 70 μg/mL[34]. Hairy root culture of *O. basilicum* produced rosmarinic acid that induced cytoskeleton damages with broken interseptas and convoluted cell surfaces in *A. niger*[35]. Based on the aforementioned studies, the antifungal activity of the plants attributed to the various kinds of secondary metabolites like flavonoids, alkaloids, phenolic acids and the essential oils[34,35].

Table 2: Antifungal activity of different plant samples against *Aspergillus* spp. reported in previous studies.

Plant sample	Extract	Microorganism	References
T. vulgaris, Satureja hortensis, Syzygium aromaticum	essential oil	*A. flavus*	[9]
Polymnia sonchifolia	aqueous extract	*A. flavus*	[10]
T. vulgaris	essential oil	*A. parasiticus, A. flavus*	[11,14]
T. vulgaris, T. tosevii, M. spicata, M. piperita	essential oil	*A. niger, A. ochraceus, A. versicolor, A. flavus, A. terreus*	[12]
Cinnamomum zeylanicum, M. piperita, O. basilicum, Origanum vulgare, Teloxys ambrosioides, Syzygium aromaticum, T. vulgaris	essential oil	*A. flavus*	[13]
Z. multiflora	essential oil	*A. parasiticus, A. flavus*	[17,18]
T. vulgaris	essential oil	*Aspergillus* spp.	[15]
M. piperita	essential oil	*A. fumigatus, A. flavus, A. ochraceus*	[22]
M. pulegium	essential oil	*A. niger, A. flavus*	[24,25]
T. vulgaris, Coriandrum sativum, Anethum graveoles, Rosa damascena	aqueous extract	*A. flavus*	[26]
S. obtusifolia	aqueous extract	*A. niger*	[28]

Antifungal activity of the tested plants in the present study also can be attributed to the various polar phytochemicals in their aqueous extracts.

Conclusion

Taking together, in our study the plants belong to Lamiaceae family, except for *O. basilicum*, along with safflower (Asteraceae) were more active against the fungus than species of senna (Fabaceae). Based on the results of this study, it can be proposed that aqueous extract of thyme, zataria and safflower effectively inhibit *A. flavus* growth attributed to their polar secondary metabolites and are suitable as natural antifungal agents to prevent the fungus activity. Hence, these three extracts inhibited fungus growth most effectively with concentration of 600 ppm in comparison with other tested extracts. While, fungus growth ranged between 10.0-11.6 mm in the presence of other extracts with concentration of 600 ppm. Evaluation of synergistic activity of the examined plants in prevention of the fungus growth for longer periods of time followed by organoleptic properties such as taste and smell of the processed food with those active extracts followed by isolation and identification of their active compounds are recommended for the further studies.

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Experimental Section

General

All chemicals used in this study were in analytical grades. Potato dextrose agar (PDA) medium was purchased from Merck Company (Darmstadt, Germany) and Millipore membrane filter (0.22 µm) from Membrane Solution (Ohio, USA).

Plant materials

Aerial parts of the plants including aerial parts of *Z. multiflora*, *T. vulgaris*, *M. pulegium*, *M. piperita*, *C. senna*, and *O. basilicum* along with flowers of safflower *C. tinctorius* were collected from Abarkuh city, Yazd province, Iran in spring of 2012. The samples of the plants were cleaned and dried at room temperature in the shade. Powdered plant materials (100 g) were extracted with distilled water (DW) three times by percolator apparatus each time 48 h. All the extracts were dried in the air flow. Extracts were consequently dissolved in DW to get different concentrations including 100, 200, 300, 400, 500 and 600 ppm.

Antifungal assay

The antifungal effects of the plants extracts were tested using agar dilution method. The fungus species was obtained from contaminated corn keeping in the storehouse of companies providing livestock food. Contaminated samples were stored in plastic bags, conveyed to the laboratory and stored in refrigerator at 4 ºC until analyzing. Samples were disinfected using hypochlorite sodium (2%) performed under laminar flow and consequently washed with DW for 30 seconds. Afterwards, the contaminated parts of the corns were transferred to PDA medium and stored at 25 ºC for 3 days. Colonies of the fungus with microscopic features of grown *A. flavus* were transferred to new PDA mediums. The plants extracts were filtered using Millipore membrane filter (0.22 µm) and mixed with culture medium to obtain concerned concentrations (100-600 ppm). The blank disks (6 mm) were subsequently impregnated in the fungus suspension (1×10^6 CFU/mL) and placed in the center of mediums. In order to eliminating osmotic effects of the extracts on the fungus growth, PEG with equal osmotic potential of the plants extracts were added to PDA medium as a negative control. All mediums were kept at 25 ºC and growth diameters of the fungi were measured at the end of 7 days. The tests were performed at least in triplicate and the mean diameters of the fungus growth were calculated as well. Minimum inhibitory concentration (MIC) values were also assessed as the lowest concentration of the plants extracts, which inhibited the fungus growth.

Statistical analysis

Growth diameters of the fungi at different concentrations of all the extracts and PEG were compared using SPSS software. All data were expressed as mean± standard deviation (SD) and statistical significances were assessed by analyzing of variance (ANOVA) along with Duncan post hoc test for multiple comparisons and $p < 0.05$ implies significance.

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