Original Article

Evaluation of antifungal activity of aqueous extracts of some medicinal plants against *Aspergillus flavus*, pistachio aflatoxin producing fungus *in vitro*

Abstract

Background: Contamination with aflatoxin, by *Aspergillus flavus*, is one the major challenges in agriculture and food industry. Preparation of organic products using natural components is widely considered these days. **Aims:** In this study, effects of aqueous extracts of five medicinal herbs, including thyme, senna, mentha, basil, and safflower on the growth of the *A. flavus* were investigated. **Mterials and Methods:** The extracts with different concentrations (200–800 µg/mL) and polyethylene glycol with the equal osmotic potential of plant extracts were added to the potato dextrose agar medium to evaluate fungus growth after 7 days using agar dilution method. Benomyl, a fungicide, was used as a positive standard. The tests were performed in triplicate, and the mean diameters of fungus growth were calculated as well. **Results and Conclusion:** All concentrations of the plants extracts significantly inhibited the fungus growth in comparison with each other and control treatments, while the extracts of thyme and safflower manifested the most effective prohibition compared to benomyl with minimum inhibitory concentration of 200 and 400 µg/mL, respectively.

Key words:
Aspergillus flavus, minimum inhibitory concentration, safflower, thyme

Introduction

Iran with about 440 thousand acres of pistachio garden produce 57% of the world production and over 60% of the world export of pistachio.[1,2] Therefore, pistachio products play an important role in the development of national economy.[3,4] Since in the previous years, pistachio of Iran banned because of aflatoxin contamination, hence many studies carried out in the universities research centers to control contamination of this mycotoxin.[5] The fungus of *Aspergillus flavus* has a strong tendency to grow in oil seeds and nuts because of their high content of fat and carbohydrates, so they are considered the natural source of aflatoxin toxicity for a human.[6] The fungi contaminated the pistachio and peanut products mostly in high humidity leading to aflatoxin contamination, a potent carcinogen of the liver.[7] Furthermore, some fungi specious appeared, which are resistant to the known antifungal agents, therefore, finding new antifungal compounds is critical for researchers.[8] In the previous studies, the activity of essential oils and extracts of some plants were tested against *A. flavus* growth or other species of the fungi.[9-14] For instance, oils of thyme, peppermint and sweet basil along with their major constituents were suppressed the growth of plant pathogenic fungi.[14,15] Oils of basil and thyme were also prevented the *A. flavus* growth attributed to their antifungal constituents.[16] In addition, the activity of polar and nonpolar extracts of *Cassia senna* was examined against some microorganisms using disc diffusion method, which results revealed no antifungal effects toward *A. niger*.[17] In the present study, antifungal activity of aqueous extracts of some medicinal plants,

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including mentha (*Mentha pulegium*), senna (*C. senna*), basil (*Ocimum basilicum*) and thyme (*Thymus vulgaris*) along with flowers of safflower (*Carthamus tinctorius*) were successfully evaluated toward *A. flavus in vitro* and minimum inhibitory concentration (MIC) values were assessed as well.

Materials and Methods

General

All chemicals used in the study were of analytical grades. The potato dextrose agar (PDA) medium and Millipore membrane filter (0.22 μm) were purchased from Merck (Darmstadt, Germany) and Membrane Solution (Ohio, USA), respectively. Benomyl, was also prepared from Shenzhen company (Guangdong, China).

Plant materials

Plant materials including aerial parts of mentha (*M. pulegium*), senna (*C. senna*), basil (*O. basilicum*), and thyme (*T. vulgaris*) along with flowers of safflower (*C. tinctorius*) were gathered from Abarkuh city, Yazd province, Iran (spring of 2012). The samples were dried after cleaning in the shade at room temperature for 7–10 days. Aerial parts of the plants (250 g) were powdered and extracted with distilled water 3 times using percolator apparatus. The obtained extracts were dried in the airflow of fans. Dry extracts (3 g) were dissolved in 3 mL of distilled water to get 100% of concentration of each extracts, which used for preparation of other concentrations including 200, 400, 600, and 800 μg/mL using distilled water.

Antifungal assay

The antifungal properties of the extracts were examined in the culture medium using agar dilution method.[18] The fungus species was obtained from Pistachio Research Center of Rafsanjan, Kerman Province, Iran in winter 2012 (ASTCP: 1684 FERDOSIEH). The extracts were filtered using Millipore membrane filter (0.22 μm) and culture medium mixed with each extracts to obtain concerned concentrations (200–800 μg/mL). The blank disks (6 mm) were impregnated in the fungus suspension (1×10^6 CFU/mL) and placed in the center of mediums with different concentrations of the extracts. In order to eliminating osmotic effects of the extracts on the fungus growth, polyethylene glycol (PEG) with the equal osmotic potential of plant extracts were added to PDA medium as a negative control. Benomyl, a fungicide agent, were also applied as a positive control with concentrations of 200–600 μg/mL. The tests were performed at least in triplicate, and the mean diameters of fungus growth were calculated as well. All mediums were kept at 25°C and growth diameter of fungi were measured at the end of 7 days. The MIC values were also assessed as the lowest concentration of the plant extracts, which inhibited the fungi growth after 48 h.

Statistical analysis

Growth diameter of the fungus at different concentrations of all the extracts and PEG were compared using SPSS software 11.5 windows (SPSS Inc., Chicago, IL, USA). All data were expressed as mean ± standard deviation. Statistical significances were assessed by analyzing of variance along with Duncan *post-hoc* test for multiple comparisons and $P < 0.05$ implies significance.

Results and Discussion

All concentrations of the plants extracts as showed in Table 1 significantly inhibited the *A. flavus* growth ($P < 0.05$) in comparison with negative control (PEG) in dose-dependent manner. Thyme and safflower were the most active plants among others, in which the extract of thyme strongly suppressed the fungus growth even at low concentrations (200 and 400 μg/mL). The extract of thyme with concentrations of 600 and 800 μg/mL completely inhibited the tested microorganism. Moreover, the thyme extract even with low concentrations (200 μg/mL) prohibited the fungus growth until the day of 5, in 2 other days the fungus growth was much lower than PEG, negative control. Safflower extract also prevented the fungus growth in a higher level compared with the other extract except for thyme. The growth of *A. flavus* have been inhibited by senna extract with high concentrations (600 and 800 μg/mL), but the extracts of mentha and basil exhibited lower activity against the fungus in comparison to thyme, safflower and senna extracts. Benomyl, a fungicide agent, with all the tested concentrations of 200, 400, and 600 μg/mL displayed weaker activity in comparison with the thyme and safflower extracts. In addition, the antifungal activity of benomyl with a concentration of 200 μg/mL is much lower than thyme and safflower with the same concentration. MIC values of the extracts were assessed as the lowest concentration of the extracts, which inhibited the growth

Table 1: Effects of the plants extracts on *Aspergillus flavus* growth at different concentrations expressed as fungus growth diameters±SD after 7 days

Concentrations (μg/mL)	Fungus growth diameters (mm)			
	200	400	600	800
Thymus vulgaris	1.33±0.57	0.66±0.57	0.00±0.00	0.00±0.00
Carthamus tinctorius	2.66±1.52	0.66±0.57	0.66±1.15	0.33±0.57
Cassia senna	14.33±0.57	11.00±1.00	1.66±0.57	1.00±1.00
Mentha pulegium	16.33±0.57	14.33±0.57	12.00±1.00	8.66±0.57
Ocimum basilicum	20.00±1.00	16.00±1.00	14.33±0.57	8.66±1.52
PEG*	75.00±0.00	74.66±0.57	71.00±1.73	73.66±1.52
Benomyl**	8.00±0.10	1.20±0.44	1.00±0.00	ND

*PEG – Polyethylene glycol with equal osmotic potential of plant extracts (negative control); **Positive control; ND – Not determined; SD – Standard deviation

Table 2: MIC of the plants extracts against *Aspergillus flavus* calculated after 48 h

Samples	MIC (μg/mL)
Thymus vulgaris	200
Carthamus tinctorius	400
Cassia senna	600
Mentha pulegium	800
Ocimum basilicum	800

MIC – Minimum inhibitory concentration

of the fungi after 48 h [Table 2]. The MIC values of thyme, safflower, and senna were estimated as 200, 400, and 600 μg/mL, respectively. However, MIC values of both senna and basil extracts were defined as 800 μg/mL after 48 h. In the previous studies, the activity of essential oils of some plants was mostly tested against *A. flavus* or other species of the fungi.[9-12] While, in some other experiments, same as the present study, aqueous extracts of the plants were examined against the mentioned fungi or other organisms.[19] The results of our study are in consistency with the previous investigation, which suggested that aqueous extracts of thyme and coriander were mostly inhibited the isolated strain of *A. flavus* followed by dill and rose extracts.[20] Phenolic contents of aqueous extract of thyme were assessed in a survey, which confirmed the presence of phenolic compounds in the extract of the plant. Rosmarinic acid was determined the main phenolic constituent in the aqueous extract of thyme using high-performance liquid chromatography method.[21] Phenolic compounds can diffuse trough the microbial membrane and interfere with metabolic pathways like a synthesis of ergosterol, glucan, chitin, proteins, and glucosamine in fungi.[22] Previous qualitative tests of aqueous extract of thyme showed the presence of flavonoids, carbohydrates, condensed tannins, catechol, loquanthucyandin, saponins, and phenolic acids. The extract moderately inhibited the growth of *Staphylococcus aureus* and *Escherichia coli*.[23] While, essential oils are constituted of hydrophobic compounds in nature.[24] Essential oils of thyme and basil showed antifungal activity with MIC values of 625 and 5000 ppm attributed to thymol and linanol, respectively.[16] Results of an experiment showed that safflower, which wound-inoculated with *Phytophthoru drechsleri* produced polyacetylene compound that inhibited the mentioned fungus growth *in vitro*.[25] Antimicrobial property of aqueous extract of *C. tinctorius* against several bacteria and fungi were examined. It concluded that water-soluble compounds were extracted in aqueous extract of the plant responsible for the extract inhibition effect toward *Bacillus mycoides*, *Bacillus subtilis*, *Bacillus cereus*, *Geotrichum candidum*, *Aspergillus nigra*, and *Penicillium expamsum*.[26] Essential oils of basil, coriander, laurel, and senna were examined on the production of aflatoxins B1 and G1 suggesting that coriander oil was completely ineffective. While, laurel, and senna oils with a concentration of 10% exhibited anti-aflatoxin B1 and G1 activity and reduced 90% of mycotoxin production.[9] Antimicrobial activity of some *Senna* spp. were tested against different microorganisms in the previous studies. For instance, aqueous extract of *Senna obtusifolia*, containing alkaloids and flavonoids, was prevented *A. niger* more than other extract of the plant.[27] Besides, the methanol extract of *Senna alata* showed antifungal activity against *A. niger* with concentration >5 mg/mL and partially purified fractions were more potent toward examined microorganisms.[28] Moreover, an unidentified flavonoid glycoside isolated from leaves of *S. alata* with MIC value of 70 μg/mL suppressed growth of *A. niger*.[29]

On the basis of the aforementioned studies, the antifungal activity of the plants attributed to the various kind of secondary metabolites like flavonoids, tannins, saponins, phenolic acids, alkaloids, polyacetylene, and the essential oils constituents.[23,29] In the present study, the extracts of the examined plants were ecofriendly obtained with distilled water that solved many natural hydrophilic products, which make them appropriate for prevention of aflatoxine contamination of pistachio or other foods products. Based on the results of this study, it could be proposed that aqueous extracts of thyme and safflower effectively inhibited *A. flavus* growth even better than benomyl that could be attributed to their several polar secondary metabolites. 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 of the processed food with those active extracts is recommended for the further studies.

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