Phytoprevention of aflatoxin production

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Abstract

Background: Consumption of mycotoxic foods is associated with several cases of human poisoning, or mycotoxicosis, sometimes resulting in death.

Objective: Phytopreventive inhibition of *Aspergillus parasiticus* growth and its aflatoxin production by the essential oils extracted from *Thymus kotschyanus* Boiss & Hohen and *Zataria multiflora* Boiss. is reported in this study.

Methods: Minimal inhibitory concentration (MIC), minimal fungicidal concentration (MFC) and fungicidal kinetics of the oils were determined and compared with each other.

Results: The oils from the above mentioned plants were found to be strongly fungicidal and inhibitory to aflatoxin production. Both oils inhibited aflatoxin B_1 (AFB_1) production by *A.parasiticus*. *T. kotschyanus* and *Z. multiflora* oils at 25 ppm concentration, reduced AFB_1 levels by 100% and 47.87% respectively. Aflatoxin production was significantly inhibited at lower than fungistatic concentration of both oils. The analysis of oils by GC and GC/MS led to identification of 27 and 22 components in *T. kotschyanus* and *Z. multiflora* Boiss. respectively which were very similar to each other.

Conclusion: Prevention of fungal growth and aflatoxin production by natural compounds is recommended.

Keywords: *Thymus kotschyanus*, *Zataria multiflora*, Essential oil, Antifungal, Aflatoxin

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Introduction

Aflatoxins are secondary metabolites produced by certain strains of *A. flavus*, *A. parasiticus* and *A. nomius* which is phenotypically similar to *A. flavus* but with a distinctive bullet shaped sclerotia [1]. Other species that produce aflatoxins in minute quantities include *A. peudotamarii*, *A. bonbysis* and *A. ochraceoroseus* [1]. These fungi grow rapidly on a variety of natural substrates and consumption of contaminated food can pose serious health hazards to human and animals. Aflatoxin B_1 (AFB_1) is a highly toxic and carcinogenic metabolite produced by Aspergillus species on agricultural commodities [2]. Natural products may regulate the cellular effects of aflatoxins and evidence suggests that aromatic organic compounds of spices can control the production of aflatoxins [3]. Spices occupy a prominent place in the traditional culinary practices and are indispensable part of daily diets of millions of people all over the world. They are essentially flavouring agents used in small amounts and are reported to have both beneficial effect and antimicrobial properties, if properly stored [4]. Their antimicrobial properties have been found to be mostly due to the presence of alkaloids, phenols, glycosides, steroids, essential oils, coumarins and tannins [5, 6]. Allameh et al. [7] reported more than 50% inhibition of aflatoxin production at 50% (v/v) neem extract concentration. Bhatnagar and McCormic [8] have demonstrated that addition of neem leaf extract above 10% (v/v) effectively inhibited aflatoxin production by *A. parasiticus* and *A. flavus*. Under such conditions the mycelial dry weight was not affected [8, 9]. Thyme oils present high antimicrobial effect [10, 11, 12] compared to the oils of other plants [13]. Thyme oils may be utilized in the food industry and as a dietary supplement. Various species of thyme have been reported to possess antifungal properties [14, 15]. The aim of this study was to investigate the effect of essential oils of *Thymus kotschyanus* and *Zataria multiflora* Boiss. on *A. parasiticus* growth and AFB_1 production. Moreover the chemical compositions of the oils were studied as well.

Materials and methods

Chemicals, Cultures and Media

A. parasiticus (NRRL-2999) was maintained on Sabouraud Dextrose Agar (E. Merck) slants. Spore suspensions were prepared and diluted in sterile yeast extract sucrose (YES) broth to a concentration of approximately 10^6 spores/ml. Spore population was counted using haemocytometer. YES broth also served as aflatoxin production medium. Subsequent dilutions were made from the above suspension, which were then used in the tests. Incubation temperature was 28 ± 2^oC. Aflatoxin standards were the products of Sigma Chemical Co. St. Louis, Mo. USA. TLC silica gel 60 F254 were purchased from E. Merck, Germany. All other solvents and reagents were of analytical grade obtained from E. Merck, Germany.

Oil extraction

The plants were collected from National Botanical Garden of Iran in May 2006. The fresh aerial parts i.e. the leaves, were subjected to hydrodistillation for 90 min using a Clevenger. The extraction was carried out for 2 h after a 4-h maceration in 500 ml of water. The oils were stored in dark glass bottles in a freezer until they were used.

Oil analysis

GC analyses were performed using a Shimadzu-9A gas chromatograph equipped

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with a flame ionization detector, and quantitation was carried out on Euro Chrom 2000 from Knauer by the area normalization method neglecting response factors. The analysis was carried out using a DB-5 fused-silica column (30 m × 0.25 mm, film thickness 0.25 μm, J & W Scientific Inc., Rancho Cordova, CA, USA). The operating conditions were as follows: injector and detector temperature, 250°C and 265°C, respectively; carrier gas, Helium. Oven temperature program was 40 - 250°C at the rate of 4°C/min. The GC/MS unit consisted of a Varian Model 3400 gas chromatograph coupled to a Saturn II ion trap detector was used. The column was same as GC, and the GC conditions were as above. Mass spectrometer conditions were: ionization potential 70 eV; electron multiplier energy 2000 V. The identities of the oil components were established from their GC retention indices, relative to C7- C25 n-alkanes, by comparison of their MS spectra with those reported in the literature [16], and by computer matching with the Wiley 5 mass spectra library, whenever possible, by co-injection with standards available in the laboratories.

Antifungal analysis

The fresh oils were tested for their antifungal activities. The disc diffusion method was used for antifungal screening as follows: Sterile Sabouraud dextrose agar medium (Merck) was prepared and distributed into Petri plates of 70 mm diameter. This medium was used for antibiogram assays. The disc size used was 6 mm (Whattman no. 1) paper. The spore suspension was streaked over the surface of the Sabouraud dextrose agar plates using a sterile cotton swab in order to get a uniform microbial growth on both control and test plates. Under aseptic conditions, the discs were placed on the agar plates and then 5 and 20 μl from each of the oils were put on the discs. The plates were then incubated at 28 $\pm$ 2°C for 10 days in order to get reliable microbial growth. Diameters of microbial inhibition zones were measured using vernier calipers. The minimal inhibitory concentration (MIC) was determined by a broth dilution method in test tubes as follows: 50μL from each of various dilutions of the oils was added to 5mL of nutrient broth tubes containing 10^6 CFU/mL of spores. The tubes were then incubated on an incubator shaker as to evenly disperse the oil throughout the broth in tubes. The highest dilution (lowest concentration), showing no visible growth, was regarded as MIC. Cells from the tubes showing no growth were subcultured on Sabouraud dextrose agar plates to determine if the inhibition was reversible or permanent. The minimal fungicidal concentration (MFC) was determined as the highest dilution (lowest concentration) at which no growth occurred on the plates.

Fungicidal kinetics of the oils

MFC concentrations of the each oil was added to 5 ml of each spore suspension containing 10^6 spores/ml and were then incubated at 28 $\pm$ 2 °C for 5 - 60 min at increments of 5 min in an incubator shaker. Samples were taken after the time intervals and were cultured on Sabouraud dextrose agar for 48 h at 28 $\pm$ 2°C. The control tube had not essential oils. Microbial colonies were counted after incubation period and the total number of viable spores per ml was calculated. The calculation was converted to percent dead spores using routine mathematical formulae.

Determination of mycelial weight

Flasks containing mycelia were filtered through Whatman filter no. 1 and then were washed with distilled water. The mycelia were

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placed on pre weighed petri plates and were allowed to dry at 50°C for 6 h and then at 40°C over night. The flasks containing dry mycelia were weighed. The net mycelial dry weight was then determined.

Measurement of aflatoxin B_1

10^6 spores/ml were added to 100-ml Erlenmeyer flasks containing 20 ml of YES broth containing the essential oils at concentrations lower than the MIC levels (6.25 ppm, 12.5 ppm, 25 ppm and 50 ppm) of *T. kotschyanus* and *Z. multiflora Boiss.* Aflatoxin B_1 measurement was determined at the end of the incubation period (28 ± 2 °C for 7 days). Aflatoxin extraction was performed routinely with solvent extraction [7, 17]. Silica gel 60-F precoated TLC sheets (E Merck, Germany) were used for analysis of aflatoxin B_1 produced by the fungal strain. The relative percentage of aflatoxin B_1 was estimated by TLC scanner III (Desaga densitometer CD60, Germany) at 365 nm using thin layer chromatography (TLC) method [18].

Results and Discussion

The inhibitory effects of the two kinds of Thyme essential oils against *Aspergillus parasiticus* were tested. Preliminary experiments were carried out in vitro using the disc diffusion and broth dilution methods to investigate antifungal action of the essential oils. Two concentrations of essential oils from *T. kotschyanus* and *Z. multiflora* Boiss. tested on Sabouraud dextrose agar plates showed a very strong antifungal property. 5 and 20µl from each of the oils caused complete inhibition of the growth of toxigenic *A. parasiticus* strain for 10 days. Many spices and herbs, such as cassia, basil, sweet basil, cloves, anise, star anise seeds, thyme, cinnamon, marigold, spearmint and caraway have been reported to inhibit toxigenic and foodborne moulds [15, 19 -22]. MIC and MFC techniques were employed to assess fungistatic and fungicidal properties of the oils. It was found that both oils had static effect at 250 ppm. *T. kotschyanus* oil at 600 ppm and *Z. multiflora* Boiss. oil at 750 ppm concentrations exhibited fungicidal property. Nguefack et al. [23] reported complete inhibition of *Aspergillus flavus*, *Aspergillus fumigatus* and *Fusarium moniliform* by the oils of *Ocimum gratissimum*, *thymus vulgaris* and *Cymbopogon citratus* at 800, 1000 and 1200 ppm respectively. Study of fungicidal kinetics of essential oils revealed more than 50% spore death in 15 minutes (Fig. 1). Complete lethal effects by *T. kotschyanus* and *Z. multiflora* Boiss. were observed within 30 and 50 minutes respectively (Fig. 1). Both oils inhibited aflatoxin B_1 (AFB_1) production by *A. parasiticus*. The order of inhibitory activity was *T. kotschyanus* > *Z. multiflora* Boiss. A concentration of 12.5 ppm *T. kotschyanus* in the YES broth resulted in 10.16% reduction of AFB_1 levels. Increasing *T. kotschyanus* concentration to 25 ppm, reduced AFB_1 levels by 100% limits. In the case of *Z. multiflora* Boiss., 3.34% inhibition of aflatoxin B_1 production was observed at a concentration of 12.5 ppm in the YES broth. When the concentration of *Z. multiflora* Boiss. was raised to 25 ppm in the YES broth, an inhibition of 47.87% was achieved (Table 1). Aflatoxin production was significantly inhibited at lower than fungistatic concentration of both oils (Table 1). Chemical analysis of the oils led to identification of 27 and 22 compounds in *T. kotschyanus* and *Z. multiflora* Boiss. oils respectively (Table 2). The profile of the oil components from *T. kotschyanus* was similar to that of *Z. multiflora* Boiss. in almost all the

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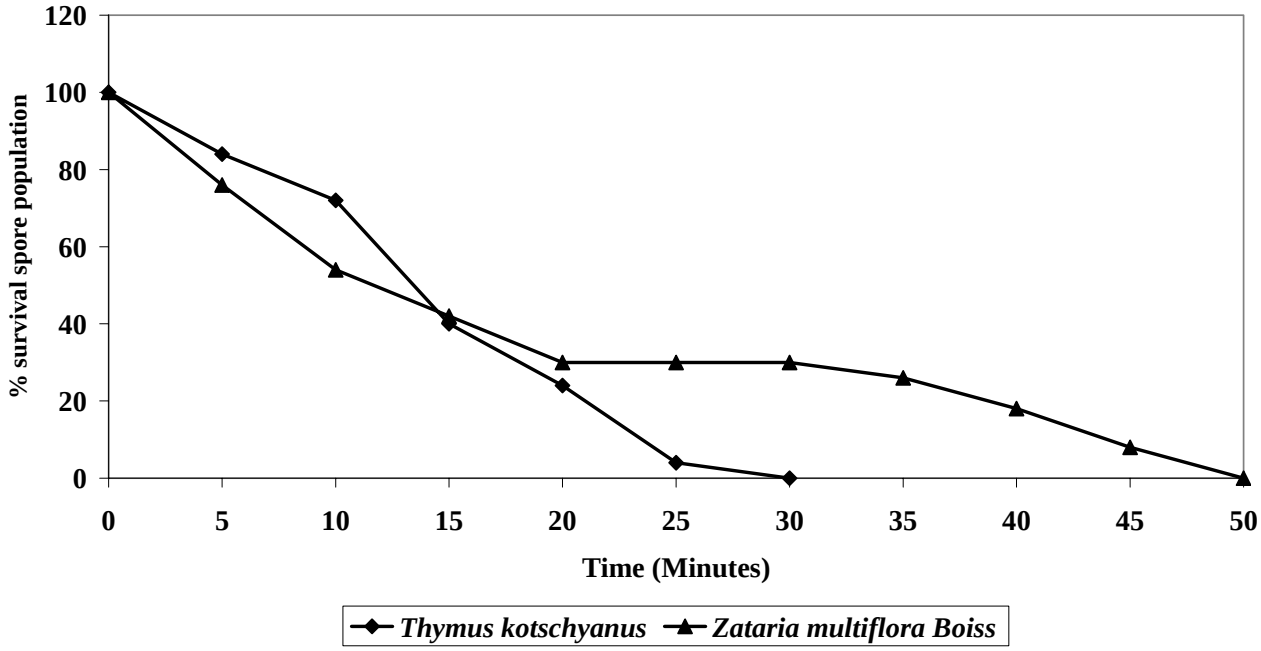

Fig. 1- Kinetics of *A. parasiticus* spore destruction at MFC levels of the fresh essential oil from *Thymus kotschyanus* and *Zataria multiflora* Boiss., Initial spore concentration: 10^6/ml

Table 1: Effect of essential oils on growth of and aflatoxin production by *Aspergillus parasiticus* NRRL 2999

Oil and concentration (ppm)	Total Mycelial dry weight (mg)	Aflatoxin (mg/ml)	Aflatoxin (ng):dry weight (mg) ratio per ml	% Aflatoxin inhibition
Control (0)	450 ± 20	1036	46.04	0
T. kotschyanus (6.25)	450 ± 20	960	42.66	7.34
T. kotschyanus (12.5)	440 ± 30	910	41.36	10.16
T. kotschyanus (25)	450 ± 10	ND	0	100
T. kotschyanus (50)	430 ± 30	ND	0	100
Z. multiflora Boiss. (6.25)	470 ± 10	1050	44.68	2.95
Z. multiflora Boiss. (12.5)	400 ± 30	890	44.50	3.34
Z. multiflora Boiss. (25)	410 ± 30	492	24	47.87
Z. multiflora Boiss. (50)	430 ± 20	486	22.60	50.91

ND: Not detectable

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Table 2: Chemical composition of essential oils from *Thymus kotschyanus* and *Zataria multiflora* Boiss.

No.	*T.kotschyanus*	RI*	%	*Z. multiflora*	RI*	%
1	α – Thujene	930	1.43	α – Thujene	925	0.9
2	α – Pinene	943	2.02	α – Pinene	933	5.0
3	Camphene	952	0.93	Camphene	947	0.3
4	Sabinene	979	0.39	Sabinene	970	0.6
5	β-Pinene	981	1.83	Myrcene	986	1.6
6	α – Phellandrene	1002	0.25	Decane	996	3.9
7	3-Carene	1007	1.5	α – Terpinene	1013	1.4
8	*p*-Cymene	1018	5.25	*p*-Cymene	1018	15.0
9	1,8-Cineole	1028	1.5	Limonene	1026	0.8
10	Limonene	1030	0.8	1,8-Cineole	1027	0.7
11	γ-Terpinene	1050	10.34	γ-Terpinene	1057	6.5
12	Transe-Sabinene hydrate	1056	1.78	Sabinene hydrate	1064	0.3
13	*Cis*-Sabinene hydrate	1066	0.43	Terpinolene	1086	0.2
14	Camphor	1126	0.18	Undecane	1098	3.8
15	Borneol	1155	2.32	Dodecane	1198	8.9
16	4-Terpineol	1160	0.25	Thymol (Methyl ether)	1232	0.5
17	α – Terpineol	1165	0.3	Carvacrol (Methyl ether)	1242	5.2
18	Carvacrol (Methyl ether)	1212	0.3	Thymol	1289	3.3
19	Thymol (Methyl ether)	1221	0.47	Carvacrol	1298	37.0
20	Thymol	1287	19.55	Thymol acetate	1349	0.2
21	Carvacrol	1297	41.49	Tetradecane	1394	1.9
22	Thymol acetate	1340	0.24	β-Caryophylene	1418	1.9
23	Geranyl acetate	1354	0.3	–	–	–
24	β-Caryophyllene	1414	2.5	–	–	–
25	Germacrane D	1484	1.52	–	–	–
26	β-Bisabolene	1497	0.73	–	–	–
27	Germacrane B	1513	1.3	–	–	–

*RI: Retention index in reference to C_7-C_{25} n-alkanes identified 99.9% in *T. kotschyanus* and *Z. multiflora*

compounds but at different concentrations. The major components of *T. kotschyanus* and *Z. multiflora* Boiss. oils were Carvacrol (41.49, 37%), Thymol (19.55, 3.3%), γ-Terpinene (10.34, 6.5%) and P-Cymene (5.25, 15%) respectively (Table 2). The effectiveness of *T. kotschyanus* and *Z. multiflora* Boiss. on the growth of the *A. parasiticus* strain and aflatoxin production are probably due to major substances such as thymol and carvacrol showing antifungal effects [24-26] and completely suppressing aflatoxin synthesis [27]. It is well known that a phenolic-OH group is very reactive and can easily form hydrogen bonds with the active sites of enzymes [28].

The present study demonstrated the potentials of the essential oils from *Thymus kotschyanus* and *Zataria multiflora Boiss.* in the control of growth and aflatoxin productivity of *A. parasiticus*. The use of these aromatic plants commonly used as spices or in beverage formulations is feasible as they are considered safe. However, further studies on their stability, toxocity and public acceptability as food preservatives are needed.

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