Jundishapur Journal of Microbiology. 2013 Sep; 6(7); e7210. DOI: 10.5812/jjm.7210

Published Online 2013 September 10. **Research Article**

Stimulato-Inhibitory Response to Cumin Oil in Aflatoxin B1 Production of *Aspergillus* Species

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Received: July 11, 2012; **Revised:** August 14, 2012; **Accepted:** September 04, 2012

Background: *Aspergillus* species produce the highly toxic and carcinogenic metabolite, Aflatoxin B1 (AFB1), on food and agricultural commodities. Some natural products are known to inhibit aflatoxin production.
Objectives: With the aim of controlling aflatoxin production, the essential oils of *Cuminum cyminum* L. from the best known regions of Iran i.e. Alborz Mountain and Kerman region, were obtained by hydrodistillation.
Materials and Methods: Antifungal activities of the oils to inhibit growth and aflatoxin productivity of *A. flavus* PICC-AF39, *A. flavus* PICC-AF24, and *A. parasiticus* NRRL-2999 were studied. Minimal inhibitory (MIC) and minimal fungicidal (MFC) concentrations of the oil were determined. Sub-MIC was selected for the measurement of aflatoxins B and G concentration. Samples were analyzed either using a high performance liquid chromatography (HPLC) method with some minor modifications. Aflatoxins (AFs) were determined by reverse-phase HPLC and fluorescence detector with post column derivatization (PCD) involving bromination.
Results: A significant reduction in Aflatoxin production was noted which was not due to the inhibitory effect but because of antifungal property of the oil. Interestingly, the oil promoted toxin production for the reasons yet to be investigated. The extent of aflatoxin production was dependent on the concentration of essential oil used. All toxin-producing fungi in this study produced higher amount of aflatoxin at low concentrations of the oil. 400 ppm concentration of *C. cyminum* L. from Alborz Mountain increased aflatoxin production to over fourfold. Aflatoxin productivity was declined at high concentration of the oil.
Conclusions: Antimicrobial and antitoxigenic properties of natural products need a firmly established criterion before they could be offered to application.

Keywords: Aflatoxin; *Aspergillus Flavus*; *A. Parasiticus*, Antifungal; Essential Oil; *Cuminum Cyminum L.*

1. Background

The presence and growth of fungi in food may cause spoilage and reduce its quality and quantity. Some *Aspergillus* species are responsible for contamination of food and feed (1, 2). *Aspergillus flavus*, *A. parasiticus* and rarely *A. nomius* are able to produce aflatoxins in food and feed stuff. *A. flavus* produces only B aflatoxins. Aflatoxin B and G are produced by *A. parasiticus*. However aflatoxin B1 (AFB1) is the most potent carcinogen known in mammals, the risk assessment of which is very well established (3-5). Each year many compounds are introduced to global markets as preservatives. Herbal products are of the most popular preservatives. The use of medicinal plants and spices in the food and pharmaceutical industries are increasing. Scientific evidence is that the spices do have medicinal properties which alleviate symptoms or prevent disease.

Cuminum cyminum is one of the popular spices often used as a flavoring agent. China is an important exporter of this commodity besides its use in traditional medicine in the treatment of some ailments. *C. cyminum* is widely used in food (6). The use of natural antimicrobial compounds is important in the preservation of food and in the control of human and plant diseases of microbial origin (7). Today, many natural compounds have been identified to have antimicrobial activity (8-10), essential

Implication for health policy/practice/research/medical education:
Nowadays, the use of essential oils and herbs as flavorings and preservatives is growingin the food industry. Some essential oils have antimicrobial properties. Cumin oil is one of the essential oils that have antimicrobial properties. In our studies, Cuminum cyminum L. stimulated aflatoxins production by *Aspergillus* species at sub MIC. Therefore, serious attention is needed toward standardization of the essential oils used in food industry to prevent food spoilage and toxin production.

oils being the most important of these compounds (11). *C. cyminum* essential oils possess useful antimicrobial and antioxidant properties. (12-14). Unfortunately, in the food industry, many preservatives were used for their pleasant flavors and antimicrobial properties. Toxicity and adverse side effects are important factors which need attention in the preservatives.

2. Objectives

In this study attempts were made to evaluate the response in aflatoxin B and G production of *Aspergillus* species to the essential oil of *C. cyminum*.

3.Materials and Methods

3.1. Fungal Strains and Cultures

The microorganisms used were *A. flavus* PICC-AF39, *A. flavus* PICC-AF24, and *A. parasiticus* NRRL-2999. All microorganisms are toxigenic. The microorganisms were maintained on Sabouraud Dextrose Agar (Merck, Germany) at 4 °C. Spore suspensions were prepared and diluted in sterile yeast extract sucrose (YES) broth to a concentration of approximately 106 spores/ml. Spore population was counted using a haemocytometer. Subsequent dilutions were made from the above suspension, which were then used in the tests. YES broth also served as aflatoxin production medium (15).

3.2. Chemicals

Aflatoxin (AF) standards were from Sigma Chemical Company, The USA. A stock standard solution of AF at 10000 μg/ml in methanol was prepared and kept wrapped in aluminum foil at -20 °C. AF working solutions were prepared by dilution in the same solvent and stored in glass-stoppered tubes at zero °C. All solvents used for the experiments (methanol, acetonitrile, deionized water) were HPLC grade.Aflatest immunoaffinity columns (IAC) were purchased from the Romer Company. HPLC column (C 18) was from the Waters, The USA.

3.3. Determination of Mycelial Weight and Antifungal Analysis

Various concentrations (200 ppm, 400 ppm, 600 ppm and 800 ppm) of the oil were added to 100 ml of YES broth (Merck, Germany) containing 106spore/ml. The flasks were incubated at 28±2 °C for 12 days on an incubator. Flasks containing mycelia were filtered through preweighed Whatman filter No. 1 and were then washed with distilled water. The mycelia were placed on preweighed Petri plates and were allowed to dry at 50 °C for 6 h and then at 40 °C over night. The net dry weight of mycelia was then determined. Three replicates were used for each treatment. The percentage mycelia inhibition was calculated by the equation: $I = 1 - T/C \times 100$, where I is inhibition (%), C is the colony weight of mycelium from a control flask (mg), and T is the colony weight of mycelium from a test flask (mg) (16).

3.4. Measurement of Aflatoxins

3.4.1. Aflatoxins Extraction

Erlenmeyer flasks containing 50 ml of Yeast Extract Sucrose (YES) broth and *C. cyminum* oil from the Alborz Mountain at concentrations lower than the MIC (200 ppm, 400 ppm, 600 ppm and 800 ppm) were inoculated with fungal spores to give 106 spores /ml. The flasks were then incubated at 28±2 °C for 12 days. Aflatoxin B1, B2, G1 and G2 were determined at the end of the incubation period. Aflatoxin extraction was performed routinely with solvent extraction as described by Razzaghi et al. (17) and Allameh et al. (18).

3.4.2. Extraction and Clean Up

Samples were analyzed either using a high performance liquid chromatography (HPLC) method (19) with some minor modifications.In HPLC analysis, the test portion was extracted with 200 ml of methanol/water (80 ml/20 ml). After filtration, the extract was diluted with water and filtered through glass microfiber filter. For clean-up of samples, Aflatest IACs were used. 10 ml phosphate buffer saline (PBS) was passed through the IAC. 75 ml of the filtrate was passed through the IAC at flow rate of ca. 1 drop/s. The column was washed with 15 ml water and dried by applying little vacuum. Finally, AFs were evaluated with methanol by the following procedure. First, 0.5 ml methanol was applied on the column which passed through by gravity. After 1 min, the second portion of one ml methanol was applied and collected. The collection was diluted with water and analyzed by HPLC.

3.4.3. AF Standards

After preparation of standard solutions of individual AF, the concentration of each one was determined using UV spectrophotometer. These standards were used to prepare mixed working standards for HPLC.

3.4.4. Analysis of AFs Using HPLC

AFs were determined by reverse-phase HPLC and fluorescence detector with postcolumnderivatization (PCD) involving bromination (20). HPLC system (pump 1525, florescence detector 2475, analytical column, Nova-pack-C18 250 ×4.6 mm: 4 μm) PCD (postcolumnderivatization) was achieved with a Kobra cell and addition of bromide to the mobile phase. After dilution of AF evaluate with water, 100 μl was injected into HPLC. Mobile phase was water: methanol: acetonitrile (600:300:200, v/v/v) and 350 micro liter from nitric acid 4 mole/lit and 12 mg of potassium bromide with a flow rate of 1 ml/min. The fluores-

cence detector was operated at an excitation wavelength of 365 nm and emission wavelength of 435 nm. Each working day, a five-point calibration curve was built for each individual AF including aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) checked for the linearity and used for quantification of AFs in samples.

3.4.5. Quality Assurance

For evaluation of the reliability of the results of AF analysis, in addition to using validated methods, internal and external quality control experiments were performed. Regarding internal quality control, the accuracy and precision of the methods were verified. In this regard, recoveries of AFB1 and AFB2 were recorded by analyzing a blank sample spiked at 5ng/g for each AFB1, 1 ng/g for each AFB2, 1 ng/g for each AFG1, and 1 ng/g for each AFG2. According to the recovery values, AF levels were corrected for recoveries.

4. Results

The effect of *C. cyminum* L. oil from Alborz Mountain on mycelial mass and aflatoxin production is shown in Tables 1 - 4.

Table 1. Effect of the *C. cyminum* L. Essential Oil From the Alborz Mountain on Growth of *A. flavus* PICC-AF24 and Its Aflatoxin Productivity Against 106 CFU/Ml of Fungal Suspensions

Oilconcentration, ppm	Dry weight of mycelium, mg	Aflatoxin B1, ng/mL	Aflatoxin B1 (ng) Production Per (mg) Mycelial Weight	Aflatoxin B2, ng/mL	Aflatoxin B2 (ng) Production Per (mg) Mycelial Weight	Total Aflatoxin (ng/ml)	Total Aflatoxin Ratio(ng) Production Per (mg) Mycelial Weight
0	1390	160.18	0.115237	212.4	0.152806	372.58	0.268043
200	1580	492.51	0.311715	639.39	0.404677	1131.9	0.716392
400	1490	781.2	0.524295	605.36	0.406282	1386.56	0.930577
600	1530	264.22	0.172693	81.79	0.053457	346.1	0.226209
800	1000	9.08	0.00908	4.2	0.004200	13.28	0.013280

Table 2. Effect of the .*C. cyminum* L. Essential Oil From the Alborz Mountain on Growth of *A. flavus* PICC-AF39 and Its Aflatoxin Productivity Against 106 CFU/mL of Fungal Suspensions

Oilconcentration, ppm	Dry Weight of Mycelium, mg	Aflatoxin B1, ng/mL	Aflatoxin B1 (ng) Production Per (mg) Mycelial Weight	Aflatoxin B2, ng/mL	Aflatoxin B2 (ng) Production Per (mg) Mycelial Weight	Total Aflatoxin, ng/mL	Total Aflatoxin Ratio(ng) Production Per (mg) Mycelial Weight
0	410	1.05	0.002561	0.06	0.000146	1.11	0.002708
200	350	2.28	0.006514	0.48	0.001371	2.76	0.007886
400	330	4.33	0.013121	1.51	0.004576	5.84	0.017697
600	210	3.1	0.014762	0.99	0.004714	4.09	0.019476
800	180	1.56	0.008667	0	0.000000	1.56	0.008667

Table 3. Effect of the *C. cyminum* L. Essential Oil From the Alborz Mountain on Growth of *A. Parasiticus* NRRL-2999 and Its Aflatoxin B Productivity Against 106 CFU/mL of fungal Suspensions

Oilconcentration, ppm	Dry Weight of Mycelium, mg	Aflatoxin B1, ng/mL	Aflatoxin B1(ng) Production Per (mg) Mycelial Weight	Aflatoxin B2, ng/mL	Aflatoxin B2(ng) Production Per (mg) Mycelial Weight	Total Aflatoxin, ng/mL	Total Aflatoxin ratio(ng) Production per (mg) Mycelial Weight
0	630	31.70	0.0503175	0.83	0.0013175	46.61	0.0739841
200	500	139.97	0.27994	4.48	0.00896	226.71	0.45342
400	410	116.76	0.2847805	6.85	0.0167073	158.58	0.3867804
600	400	113.21	0.283025	10.41	0.026025	193.66	0.48415
800	350	141.65	0.4047143	4.83	0.0138	233.11	0.6660285

Table 4. Effect of the *C. cyminum* L. Essential Oil From the Alborz Mountain on Growth of *A. Parasiticus* NRRL-2999 and its Aflatoxin G Productivity Against 106 CFU/mL of Fungal Suspensions

Oilconcentration, ppm	Dry Weight of Mycelium, mg	Aflatoxin G1(ng/mL)	Aflatoxin G1 (ng) Production Per (mg) Mycelial Weight	Aflatoxin G2, ng/mL	Aflatoxin G2 (ng) Production Per (mg) Mycelial Weight
0	630	14.08	0.0223492	0.00	0.00
200	500	82.00	0.164	0.26	0.00052
400	410	34.09	0.0831463	0.88	0.0021463
600	400	69.25	0.173125	0.79	0.001975
800	350	84.95	0.2427143	1.68	0.0048

The scores related to AFB1, AFB2, AFG1 and AFG2 were used for a linear regression in the standard. HPLC analysis of spiked samples gave a good correlation with spiked values (R2 > 0.992). The scores regression coefficient (R/R2) for AFB1, AFB2, AFG1 and AFG2 were0.9965/0.9930, 0.9963/0.9926, 0.9981/0.9962 and 0.9996/0.9992 respectively.

The concentration of essential oil to inhibit production of aflatoxin (B1, B2 and total) for *A. flavus* PICC-AF39, *A. flavus* PICC-AF24, and (B1, B2, G1, G2 and total) for *A. parasiticus* NRRL-2999 was determined to be in the range of sub-MIC (200 ppm, 400 ppm, 600 ppm and 800 ppm). Mycelia weight declined with increasing concentration of essential oil showed in Tables 1 - 3. We can clearly show an increase in aflatoxin production by all three species of toxin-producing *Aspergillus* (Tables 1, 4). Whereas dry weight of mycelium(mg) of *A. flavus* PICC-AF39, *A. flavus* PICC-AF24 and *A. parasiticus* NRRL-2999 was declined (Tables 1, 3).

Dry weight of mycelium (mg) of *A. flavus* PICC-AF24 in 800 ppm concentration declined 28.06% compared to control without oil (Table 1). In this Table, at 400 ppm concentration of *C. cyminum* L. essential oil from Alborz Mountain, the concentration of aflatoxin B1, B2 and total aflatoxin were 4.55, 2.66 and 3.47 fold respectively. The same happened in *A. flavus* PICC-AF39 at 800 ppm and 400ppm for dry weight of mycelium and aflatoxin production (B1 and B2) respectively. At 800 ppm oil concentration the mycelium weight reduced to 56.1%. At 400 ppm, aflatoxin B1, B2 and total production were increased 5.76, 2.66 and 6.54 fold respectively (Table 2).

Tables 3 and 4 show oil concentration of the Alborz Mountain *C. cyminum* L. (ppm), dry weight of mycelium, aflatoxins (B1, B2, G1, G2 and total) (ng/mL) and Aflatoxins production (B1, B2, G1, G2 and total) (ng) per (mg) mycelia weight for *A. parasiticus* NRRL-2999. The mycelial dry weight compared to control without oil calculated at 800 ppm was lost (Table 3). Toxin production was increased by *A. parasiticus* NRRL-2999 at 800 ppm of the oil.Aflatoxin production (B1, B2, G1and total)(ng) per (mg) mycelia weight were increased to 8, 19, 10.86, and 9 fold respectively.

A. parasiticus NRRL-2999 does not produce G2 toxin production under normal conditions. When oil was added to the flask containing fungal spores of *A. parasiticus* , AFG1 was produced and increased to about 10-fold (Table 4). The extent of aflatoxin production was dependent on the concentration of essential oil used (Tables 2, 3). All toxin-producing fungi in this study produced higher amount of aflatoxin at low concentrations of the oil. Aflatoxin productivity was declined at high concentration of the oil (Table 1, 4).

5. Discussion

Antifungal activities of *C. cyminum* L. essential oilfrom the Alborz Mountain against *A. flavus* PICC-AF39, *A. flavus* PICC-AF24 and *A. parasiticus* NRRL-2999 was determined (14). Minimal inhibitory (MIC) and minimal fungicidal (MFC) concentrations (ppm) of the *C. cyminum* L. essential oil from the Alborz mountain of Iran against 106 spores/ml of *A. flavus* PICC-AF24, PICC-AF39 and *A. parasiticus* NRRL-2999 were 1000/3000, 1000/2500 and 750/3000 ppm respectively (14). Our results of antifungal properties are in agreement with those reported by Mohammadpour et al. (14). Mycelia weight declined with increasing concentration of *C. cyminum* L. from the Alborz Mountain (Tables 1 - 3).

Antimicrobial activity of Cumin oil on some bacteria and fungi has been reported (21, 22). Although expression of antifungal activity is often very clear, the mechanism of action is ambiguously understood. Therefore, further research is to be performed to determine the antimicrobial activity of *C. cyminum* L. The oils exhibited significant inhibition of fungal growth and aflatoxin B1 production. Turmeric leaf oil exhibited 95.3% and 100% inhibition of aflatoxin production respectively at 1.0% and 1.5% concentrations in YES broth (23). Bhanu Prakash et al.,(24) showed antifungal properties of *Piper betle* L. essential oil (EO) in fungal contamination on some dry fruits. MIC of *P. betle* L. was found to be 0.7 µl/ml against *A. flavus*. The EO reduced AFB1 production at 0.6 µl/ml. They reported efficacy of *P. betle* EO as aflatoxin suppressor.

A finding showed that aflatoxin production can be controlled by costoring whole sweet basil leaves (*Ocimum basilicum*) with aflatoxin infected foods (25). There are other studies that have confirmed above (15, 26-28).

On the other hand, the compounds are also stimulates for aflatoxin production by toxin-producing fungi. Our research showed that Cumin essential oil from Alborz Mountain had stimulating effect on toxin productivity of *A. flavus* PICC-AF24, *A. flavus* PICC-AF39 and *A. parasiticus* NRRL-2999. This strange phenomenon is being reported for the first time. Since most essential oils have been found to be cytotoxic rather than mutagenic, it is likely that most of them are noncarcinogenic. Some metabolites are genetically induced by some essential oils. Lower concentrations of the cytotoxic effect can induce the production of secondary metabolites in Saccharomyces cerevisiae (29-31).

Aflatoxins are also secondary metabolites. However, some essential oils or rather some of their constituents may be considered as secondary carcinogens after metabolic activation. For example, essential oils like those from Salvia sclarea and Melaleuca quinquenervia provoke estrogen secretions which can induce estrogen-dependent cancers (32, 33). In conclusions serious attention is needed toward standardization of the essential oils used in food industry to prevent food spoilage and toxin production.

Acknowledgements

This study was fully supported by the Vice chancellor for Research of Hidaj, Azad University.

Authors' Contribution

Study concept, design and the protocol: Fakoor, Rasooli and Owlia; Reverse-phase HPLC and analysis of HPLC for toxin assay: Fakoor, Rasooli, Owlia, Mazaheri and Shokrollahi; Manual techniques for microbiology: Fakoor, Shehni, Jalilli and Mohammadpour; Wrote the manuscript: Fakoor and is guarantor

Financial Disclosure

None declared.

Funding/Support

This study was financially supported by Azad University (Hidaj branch).

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