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Effect of *Nigella sativa* Extract and Oil on Aflatoxin Production by *Aspergillus flavus*

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Abstract: : Crude extract of *Nigella sativa, N. sativa* oil, ground coffee beans and caffeine were tested for their effects on aflatoxin production by *Aspergillus flavus*. Crude extract of *N. sativa* inhibited the production of three types of aflatoxins (B1, B2 and G1) at 5% (w/v) concentration, while *N. sativa* oil inhibited all four types of aflatoxins using 3% (v/v) concentration. However, coffee inhibited the production of B1, G1 and G2 aflatoxins at 6% (w/v), while caffeine inhibited only G1 and G2 aflatoxin. Our results suggest the investigated ingredients may have a significant fungal antitoxic activity which can be exploited as food preservative to minimize mycotoxin effects.

Key Words: Aflatoxin, caffeine, coffee, *Nigella sativa*

Aspergillus flavus'un Aflatoksin Üretimi Üzerine *Nigella sativa* Özütü ve Yağının Etkisi

Özet: *Nigella sativa* özütü, *N. sativa* yağı, kahve çekirdeği ve kafeinin *Aspergillus flavus*'un aflatoksin üretimi üzerine etkisi incelenmiştir. *N. Sativa* özütü üç tip aflatoksinin (B1, B2 ve G1) üretimini %5 (w/v) derişimde engellerken *N. sativa* yağı tüm dört tip aflatoksinin üretimini %3 (v/v) derişimde engellemiştir. Yine de kahve B1, G1 ve G2 aflatoksinlerinin üretimini %6 (w/v) derişimde engellemiştir. Kafein sadece G1 ve G2 aflatoksini engellemiştir. Sonuçlarımız araştırılan içeriklerin; gıda koruyucu olarak mikotoksik etkileri azaltmakta kullanılabilecek düzeyde fungal antitoksik aktiviteye sahip olabileceklerini göstermektedir

Anahtar Sözcükler: Aflatoksin, kafein, kahve, *Nigella sativa*

Introduction

Aflatoxins are potent mycotoxins produced by certain *Aspergillus* species such as *A. flavus*, *A. parasiticus, A. nomius* and *A. niger*. These ubiquitous fungi are capable of infecting a wide variety of crops such as corn, cottonseed, peanuts and many others (1). There are four major known aflatoxins: B1, B2, G1 and G2, which are capable of inhibiting RNA synthesis (2). At present, a total of 18 aflatoxins are known. Of these, aflatoxin B1 is the most common and is a potent hepatocarcinogen, which can impair both non-specific and specific immunity. In addition, aflatoxin B1 was found to inhibit CD14-mediated nitric oxide production in murine peritoneal macrophages (3). These toxins at levels greater than 10 ppb can induce cancer and mycotoxicosis (4).

The medicinal properties of *N. sativa* include anti-inflammatory, analgesic and antipyretic activities (5). Moreover, *N. sativa* was found to play a role in the prevention of liver fibrosis and cirrhosis in the rabbit (6). The antimicrobial effects of crude extracts of *N. sativa* on multiple antibiotics-resistant bacteria have been reported (7).

High frequency contamination of coffee beans with *Aspergillus*, which may reach up to 80%, has been reported (8). However, caffeine was shown to inhibit the growth of the fungus and the production of aflatoxins (9). In view of these reports, the present study was undertaken to study the effects of *N. sativa* extract and oil on aflatoxin production by *Aspergillus flavus.* It was also of interest to investigate and confirm the reported

inhibitory action of caffeine and ground roasted coffee beans on the production of the toxins.

Materials and Methods

Isolation and identification of *Aspergillus flavus*

Aspergillus flavus was isolated locally from an infected culture medium containing white wheat. This was achieved by suspending 5 g of local white wheat in 5 ml water and incubating the mixture for 5-7 days at 25°C. Pure isolates of *A. flavus* were identified microscopically and cultured as previously described (10).

Growth conditions, production and purification of aflatoxins

The isolated strain of *A. flavus* was grown in conical flasks containing 100 ml brain-heart infusion (BHI) broth followed by addition of 10 g of peanuts to all flasks. Media were sterilized at 121°C for 15 min. Flasks were inoculated with 10^6 spores/ml of *A. flavus* and then incubated at 25±1°C for 5-7 days.

Infected peanut cultures were homogenized at high speed in a blender. The homogenate was further extracted. The extract was filtered and 2.5 g NaC1, 20 ml distilled water and 40 ml methanol were added to the filtrate in a separatory funnel. To this mixture, 45 ml of chloroform was added. Then 5 g of sodium sulfate was mixed with the lower layer, which was separated, filtered and then evaporated. The residue was suspended in 1 ml chloroform (11).

Detection of aflatoxin using thin layer chromatography (TLC)

The application, development and identification of the different aflatoxins in the extract by the TLC technique were carried out as previously described (12). The extracts were spotted on TLC plates. The separated spots of aflatoxins were detected using ultraviolet light at 365 nm. Standards of B1, B2, G1 and G2 aflatoxins were obtained from the Veterinary Laboratory, Jordanian Ministry of Agriculture.

Effect of crude extracts of *Nigella sativa* on aflatoxin production

Different weights of *N. sativa* (1.25 g, 2.5 g, and 5.0 g) were ground and added each to 100 ml BHI broth. Ten grams of powdered peanut was added to each flask and then sterilized at 121°C for 15 min. Culture flasks were incubated for 5-7 days at 25 ± 1°C after inoculation with 10^6 spores/ml of *A. flavus.* Purification and detection of aflatoxins were carried out as previously described (11).

Effect of *Nigella sativa* oil on aflatoxin production

Prof. Nizar Abu-harfeil (Department of Biotechnology & Genetic Engineering, Jordan University of Science and Technology) kindly provided *N. sativa* oil. To each 100 ml BHI broth, 1.0 ml, 2.0 ml and 3.0 ml sterile *N. sativa* oil was added. After inoculation with *A. flavus* culture (10^6 spores/ml), flasks were incubated at 25±1°C for 5-7 days. Purification and detection of aflatoxins were then done as mentioned previously.

Effect of coffee and caffeine on aflatoxin production

Different weights of caffeine (Sigma-Aldrich) (2.0, 4.0 and 6.0 mg) and locally roasted and ground coffee beans (2.0, 4.0 and 6.0 g) were added each to 100 ml BHI broth. Sterilization, inoculation, purification and detection were done as previously mentioned.

Results and Discussion

A. flavus was first isolated from local white wheat and then a pure culture was maintained on potato dextrose agar. It was grown in BHI medium that contained peanuts in 250 ml conical flasks. Aflatoxins were extracted and purified from cultures containing peanuts and identified on TLC plates, using standards of B1, B2, G1, G2 aflatoxins as shown in Figure 1. The effects of different concentrations of crude extract of *N. sativa* on the four types of aflatoxins are presented in Table 1 and Figure 2. Three types of aflatoxins (G1, B1, and B2) were not detected on the TLC plates after using 5% (w/v) crude extract of *N. sativa.* However, G1 was inhibited by 1.25% (w/v) and both G1 and B2 were inhibited by 2.5% (w/v) (Table 1).

Oil from *N. sativa* extract was also tested for its effect on the production of aflatoxin as presented in Table 2. Production of all aflatoxins was completely inhibited at 3% (v/v) concentration of black seed oil as shown in Table 2. However, B1 and B2 were not inhibited at 1% and 2% (v/v) concentrations, respectively.

The effects of different concentrations of coffee on the production of the four types of aflatoxin are presented in Table 3. Inhibition of three aflatoxins - B1, G1 and G2 - was observed using 6% (w/v) coffee, while

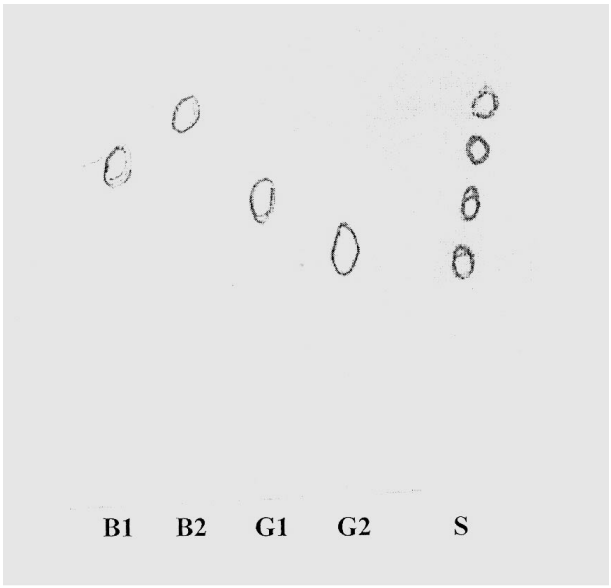

Figure 1. The TLC plate for the infected peanuts.

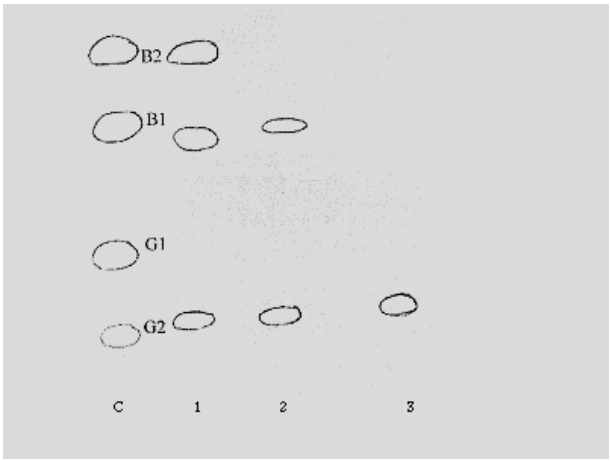

Figure 2. The TLC plate for the crude extracts of *Nigella sativa* with peanuts.
C: Control. 1: 1.25%. 2: 2.5%. 3: 5% (w/v).

2% and 4% (w/v) coffee inhibited only two types of aflatoxins, G1 and G2, as shown in Table 3. Likewise, caffeine inhibited only two types of aflatoxins, G1 and G2 (Table 4).

From the results of the present study, it is clear that *N. sativa* oil was the most efficient in inhibiting aflatoxin production, followed by *N. sativa* extract (Tables 1 and 2). Crude extracts of *N. sativa* have both antimicrobial (7,13-14) and antifungal activities (15).

Table1. Effect of different concentrations of *Nigella sativa* crude extract on aflatoxin production by *A. flavus*.

Concentration (w/v)	B1	B2	G1	G2
0.0	-	-	-	-
1.25%	-	-	+	-
2.5%	-	+	+	-
5.0%	+	+	+	-

- No effect
+ Inhibition in aflatoxin production

Table 2. Effect of different concentrations of *Nigella sativa* oil on aflatoxin production.

Concentration (v/v)	B1	B2	G1	G2
0.0	-	-	-	-
1.0%	-	-	+	+
2.0%	-	-	+	+
3.0%	+	+	+	+

- No effect
+ Inhibition in aflatoxin production

Table 3. Effect of different concentrations of coffee on aflatoxin production.

Concentration (w/v)	B1	B2	G1	G2
0.0	-	-	-	-
2.0%	-	-	+	+
4.0%	-	-	+	+
6.0%	+	-	+	+

- No effect
+ Inhibition in aflatoxin production

Table 4. Effect of different concentrations of caffeine on aflatoxin production.

Concentration (w/v)	B1	B2	G1	G2
0.0	-	-	-	-
2.0%	-	-	+	+
4.0%	-	-	+	+
6.0%	-	-	+	+

- No effect
+ Inhibition in aflatoxin production

Pharmacologically active constituents of *N. sativa* oil have been reported, and include thymoquinone, dithymoquinone, thymohydroquinone and thymol (16). Results found in this study were comparable to other studies using essential oils of certain medicinal plants such as thyme, basil, and cinnamon against aflatoxin production by *A. flavus* in maize kernel and wheat grain (17-18). This effect could be related to several components known to have biological activities, such as α-pinene and thymol (18). The anti-aflatoxin property of oils is mainly related to their high phenolic content as demonstrated previously (19). Moreover, in the present study, the observed results showed that *N. sativa* oil completely inhibited aflatoxin production at 3% (v/v) concentration, while crude extract of *N. sativa* inhibited three types of aflatoxins at 5% (w/v) concentration (Tables 1 and 2). In addition, coffee and caffeine appeared to contribute significantly to the inhibition of aflatoxin production. Coffee proved more effective than caffeine, as the highest effect of coffee was recorded at a concentration of 6% (w/v) inhibiting three types, while that of caffeine at 6% (w/v) inhibited only two types (Table 3 and 4). The higher activity of coffee beans, as compared to caffeine, probably indicates that other active ingredients in the coffee beans may have either additive or synergistic properties when concomitantly present with caffeine.

The findings in the present study indicate that the tested agents will probably be best used as food additives in the processed food industry to protect susceptible products contaminated with *A. flavus* from aflatoxin production. Moreover, following identification and illustration of the chemical structures of the active fungicides in the tested agents, such structures could be taken as starting material for the synthesis of safe fungal antitoxic agents with minimal toxic effects or impact on health.

Quantitative estimation of aflatoxins in response to different natural compounds and their effects as well on growth using high performance liquid chromatography (HPLC) and enzyme linked immunosorbent assay (ELISA) are in progress.

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