

In Vitro Inhibition of Growth and Aflatoxin B1 Production of *Aspergillus Flavus* Strain (ATCC 16872) by Various Medicinal Plant Essential Oils

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Abstract

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The hazardous nature of aflatoxins to human and animals necessitate the need for establishment of control measures. The objective of this study was to evaluate the inhibition of growth and aflatoxin production of *Aspergillus flavus* strain (ATCC 16872) by various essential oils in Yeast Extract Sucrose (YES) growth media at 25°C. Essential oils of basil, fennel, coriander, caraway, peppermint and rosemary were tested for their effects on mycelial growth and aflatoxin production. Aflatoxin B1 production was determined by high performance liquid chromatography (HPLC). The findings of this study revealed the antifungal efficacy of the all tested essential oils. The extent of inhibition of fungal growth and aflatoxin production was dependent on the type and concentration of essential oils used. The complete inhibition of *Aspergillus flavus* growth was observed at 1000 ppm concentrations of essential oils of basil, coriander, caraway and rosemary. While, essential oils of basil and coriander showed marked inhibition of aflatoxin B1 produced by *Aspergillus flavus* at all concentrations tested 500, 750 and 1000 ppm.

Introduction

Aflatoxins are biologically active secondary metabolites produced by certain strains of *Aspergillus parasiticus*, *Aspergillus nominus* and *Aspergillus flavus* [1]. The aflatoxin producing fungi are widely distributed in nature and can grow over a wide range of environmental conditions [2]. Aflatoxins have been detected in cereal grains, oil seeds, fermented beverages made from grains, milk, cheese, meat, nut products, fruit juice and numerous other agricultural commodities [3].

Aflatoxin B1 (AFB1) is the most prevalent and carcinogenic of the aflatoxins and the International Agency for Research on Cancer (IARC) classify AFB1 as a group I carcinogen (an agent that is carcinogenic to humans). Epidemiological studies also indicated that areas in the world with high levels of aflatoxins are correlated with high incidence of liver cancer [4].

AFB1 caused damage to cells by two different ways. Firstly, AFB1 (C17H12O6) is activated to AFB1-8,9-oxide and forms adduct primarily at N7 position of guanine and is responsible for its mutagenic and

carcinogenic effects [5, 6]. Secondly, aflatoxins especially AFB₁, produce reactive oxygen species (ROS) such as superoxide radical anion, hydrogen peroxide and lipid hydroperoxides; though these do not appear to interact with DNA, but they are precursors to the hydroxyl radical. The hydroxyl radicals interact with DNA and produces mutations [7].

Numerous diverse compounds and extracts containing inhibitory activity to aflatoxin biosynthesis have been reported. The most of these inhibitors are plant-derived such as phenylpropanoids, terpenoids and alkaloids [8]. A group of plant-derived inhibitors is essential oils that possess antimicrobial activities against *A. parasiticus* and/or *A. flavus* [9-12].

Several studies have documented the antifungal [13, 14] and antibacterial [15, 16] effects of plant essential oils. Screening experiments with 13–52 essential oils and major active components against 5–25 microorganisms [17, 18] have reported thyme, clove, cinnamon, bay, oregano, garlic and lemongrass to be some of the best broad spectrum candidates for inhibition of food-borne pathogens and spoilage organisms.

The objective of this study was to evaluate the inhibition of growth and aflatoxin production of *Aspergillus flavus* strain (ATCC 16872) by various essential oils in culture medium.

Material and Methods

Plant materials

Six herbs namely, fennel (*Foeniculum vulgare* L.); coriander (*Coriandrum sativum* L.); caraway (*Carum carvi* L.); rosemary (*Rosmarinus officinalis* L.); basil (*Ocimum basilicum* L.) and peppermint (*Mentha x piperita* L.) were purchased from local markets and authenticated in the herbarium of Faculty of Science, Cairo University and National Research Center, Egypt. One kg of each plant seeds (for fennel, coriander, caraway) or leaves (for rosemary, basil, peppermint) were subjected to hydrodistillation. The volatile oil then collected and dried in desiccators over anhydrous Ca SO₄. Each volatile oil sample was kept in dark bottle till used.

Preparation of Test microorganism and culture

Aspergillus flavus strain (ATCC 16872), were kept on potato-dextrose-agar (PDA) slant at 25°C for 10 days. Periodic transfers were done to keep the

microorganism viable. Spores were obtained and harvested by washing off the surface of the slant with 10 ml of sterile 0.1% Tween 80 solution (Merck, Germany) to obtain a concentration of “10⁶” spore/mL and was utilized the same day.

Determination of mycelial weight

Flasks containing mycelia were filtered through pre weighed Whatman filter no. 1 and were then washed with distilled water. The mycelia were 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 net dry weight of mycelia was then determined.

Inhibition of A.flavus growth and aflatoxin production in the presence of essential oils

Fifteen ml of YES medium, was put in a 250 ml-flasks and then autoclaved at 120°C for 15 min. Inoculation was carried out by adding 1 ml of a suspension of spores (“10⁵” spores) of a toxigenic *A. flavus* strains without (control) or with 50 µl, 100 µl and 150 µl of one of the tested essential oils. The flasks were incubated in the dark for 14 days at 25°C. After the incubation period, the growth of the mycotoxigenic fungi *A. flavus* in all flasks was visually examined.

Extraction of aflatoxin B1 from A. flavus cultures

Extraction of mycotoxins produced in the YES culture was carried out according to the method of Munimbazi and Bullerman [19]. Where, the mycelium of each flask contained YES medium was harvested by filtration through Whatman paper (No.4), then extracted by 100 ml chloroform. Chloroform extract was dried by addition of anhydrous sodium sulfate. The residue was transferred to vial and evaporated off using a stream of nitrogen at temperature below 60°C. The dry film was used for the detection of aflatoxins by high performance liquid chromatography (HPLC).

The percentage of inhibition of fungal growth and aflatoxins were calculated using equation:

$$\% \text{ inhibition} = (\text{control} - \text{treatment} / \text{control} \times 100).$$

Determination of aflatoxins by HPLC

Derivatization: The derivatives of tested samples and standards (control) were done as follow: Two hundred

µl hexane were added to the clean up dry film of standard and tested samples followed by 50 µl Trifluoroacetic acid (TFA) and mixed by vortex vigorously for 30 s. The mixture was let to stand for 5 min. To the mixture 450 ml water- acetonitrile (9 +1 v/v) by pipet were added and mixed well by vortex for 30 seconds, and the mixture was left to stand for 10 min. to form two separate layers. The lower aqueous layer was used for HPLC analysis [20].

Apparatus: The HPLC system consisted of Waters Binary Pump Model 1525, a Model Waters 1500 Rheodyne manual injector, a Waters 2475 Multi-Wavelength Fluorescence Detector, and a data workstation with software Breeze 2. A phenomenex C18 (250 x 4.6 mm i.d.), 5 µm from Waters corporation (USA). An isocratic system with water: methanol: acetonitrile 240:120:40. The separation was performed at ambient temperature at a flow rate of 1.0 mL/min. The injection volume was 20 µL for both standard solutions and sample extracts. The fluorescence detector was operated at wavelength of 360 nm for excitation and 440 nm for emission.

Quantitation: The mixed solutions of standard as well as sample extract after derivatisation were filtered through a 0.22 µm membrane filter and loaded (20 µL) into a 20 µL injection loop. The elution order of the four aflatoxins was G2, B2, G2a (G1 derivative), B2a (B1 derivative). AFs contents in samples were calculated from chromatographic peak areas using the standard curve.

Statistical analysis

All data from three independent replicate trials were subjected to statistical analysis using statistical software (SPSS, 10.0; Chicago, USA). Data were reported as means ± standard deviations. The significant differences between mean values were determined by Duncan's Multiple Range test ($p < 0.05$), following one-way ANOVA.

Results

Antifungal activities of essential oils on mycelial growth

Each essential oil showed notable antifungal activities against *A. flavus*.

Statistical results showed that kind and amount of essential oils have a significant influence on the antifungal activity $p < 0.05$ (Table 1).

Table 1: Effect of different concentrations of essential oils on the mycelia dry weight inhibition % in YES medium.

Treatment	Concentration (ppm)	Mycelial dry weight inhibition % ^d
Peppermint	500	45.107 ± 4.875 ^{a,c}
	750	53.541 ± .595 ^b
	1000	68.412 ± 1.637
Basil	500	46.093 ± 2.086 ^{a,c}
	750	64.217 ± 2.554 ^b
	1000	71.666 ± 1.527
Coriander	500	51.610 ± 5.485 ^{a,c}
	750	66.553 ± 2.050 ^b
	1000	71.666 ± 1.527
Fennel	500	45.133 ± 3.208 ^{a,c}
	750	54.447 ± 2.724 ^b
	1000	64.694 ± 1.795
Caraway	500	53.071 ± 2.024 ^{a,c}
	750	60.957 ± 2.420 ^b
	1000	71.666 ± 1.527
Rosemary	500	61.433 ± 1.769 ^{a,c}
	750	66.983 ± 3.774 ^b
	1000	71.666 ± 1.527

^a significant differences between concentration 500 & 750; ^b significant differences between concentration 750 & 1000; ^c significant differences between concentration 500 & 1000 in the same column; ^d Data are means of triplicates (± standard deviation). % inhibition = (control-treatment / control x100).

As can be seen, essential oil concentration of 1000 ppm has the highest antifungal activity for all tested essential oils. Complete inhibition of growth of *A. flavus* was observed for basil, caraway, coriander and rosemary at 1000 ppm (Fig. 1).

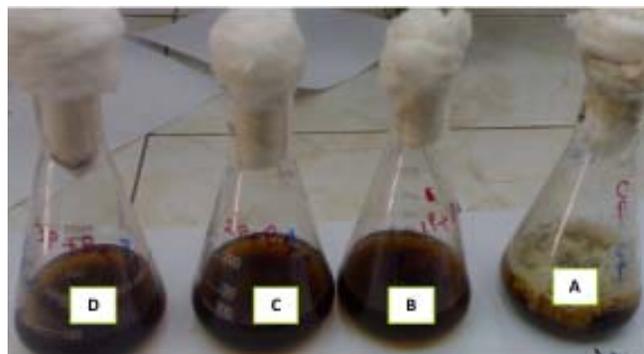


Figure 1: Flask A, control flask showing growth of *Aspergillus flavus* in YES medium; Flask B, C and D, containing basil, coriander and caraway essential oil at concentration of 1000 ppm respectively showing no growth of *Aspergillus flavus* in YES medium.

Effect of essential oils on Inhibition of aflatoxin B1 production

Each essential oil showed notable inhibition of aflatoxin B1 production by *A. flavus*. Statistical results showed that kind and amount of essential oils have a significant influence on the aflatoxin inhibition with $p < 0.05$ (Table 2).

Table 2: Effect of different concentrations of essential oils on aflatoxin B1 inhibition % in YES medium.

Treatment	Concentration (ppm)	Aflatoxin B1 inhibition % ^d
Peppermint	500	9.639 ± 655 ^{a,c}
	750	20.683 ± .723 ^b
	1000	21.643 ± .938
Basil	500	23.282 ± .594 ^{a,c}
	750	23.804 ± .702 ^b
	1000	23.883 ± .635
Coriander	500	24.907 ± .627 ^{a,c}
	750	25.133 ± .621 ^b
	1000	25.382 ± .576
Fennel	500	22.964 ± .663 ^{a,c}
	750	23.201 ± .663 ^b
	1000	23.985 ± .674
Caraway	500	21.066 ± .709 ^{a,c}
	750	22.390 ± .683 ^b
	1000	24.980 ± .663
Rosemary	500	16.464 ± .849 ^{a,c}
	750	17.271 ± .795 ^b
	1000	19.551 ± .755

^a significant differences between concentration 500 & 750; ^b significant differences between concentration 750 & 1000; ^c significant differences between concentration 500 & 1000 in the same column; ^d Data are means of triplicates (± standard deviation); % inhibition = (control-treatment /control x100).

Essential oils of basil and coriander showed marked inhibition of aflatoxin production by *A.flavus* at all concentrations tested 500,750 and 1000 ppm.

Discussion

The inhibition of *Aspergillus flavus* growth by essential oils has already been previously reported [21, 22]. In our study, results indicated antifungal efficacy of all tested essential oils. The extent of inhibition of fungal growth and aflatoxin B1 production was dependent on the concentration of essential oils used. The total inhibition of *Aspergillus flavus* growth was observed at 1000 ppm concentrations of essential oils of basil, coriander, caraway and rosemary. Rasooli et al. [11] obtained the same result using 450 µg/mL of *Rosamarinus officinalis* essential oil. Soliman and Badea [23] also, reported complete inhibition of *Aspergillus flavus*, *A. parasiticus*, and *A. ochraceus* by the oils of thyme and cinnamon (<500 ppm), marigold (<2000 ppm), spearmint, basil, (3000 ppm). However, they did not specify chemical composition of their oils as well as in our study.

Although they used Czapek-Dox Agar as nutrient medium [24]. Our results conducted in YES medium, which is more nutritious medium than Czapek-Dox Agar, indicated complete inhibition of *A. flavus* at 1000 ppm of basil, coriander, caraway and rosemary oils. This

indicated that highly nutritious medium such as YES could not support fungal cells resistance against the tested oils.

The antifungal effect of the tested oils could be related to several components known to have biological activities, such as methyl chavicol and 1-linalool for basil, d-linalool for coriander, carvone and limonene for caraway, 1,8-cineole and limonene for rosemary [25].

It may be deduced that fungal growth inhibition and subsequent aflatoxin B1 production were related mostly to linalool and 1,8-cineole contents of the oils. It should be noted that there was a gradual increase in inhibition due to the increased concentration of tested essential oils.

Sometimes, fungal growth inhibition was reported to be associated with the degeneration of fungal hyphae as after treatment with *Thymus vulgaris* L., *Lavandula R.C.*, and *Mentha piperita* L. [26]. Other studies showed that the main target of the oils were cell wall and cell membrane as in the presence of thyme essential oils at 250 ppm, the plasma membrane of *A. parasiticus* was seen to be irregular, dissociated from the cell wall, invaginated and associated with the formation of lomasomes [27]. Or ultra-structural changes depending on essential oil concentration as in *Ageratum. Conyzoides* [28].

Changes in plasma membranes and mitochondria were also reported by Rasooli et al. [29] who investigated the action of the essential oil of two species of *Thymus* on *A. niger*. TEM observations by de Billerbeck et al. [30], carried out to determine the ultrastructural modifications of *A. niger* hyphae after treatment with *Cymbopogon nardus* (L.) essential oil, revealed reduced diameter and thinning of the hyphal walls.

Considering the large number of different groups of chemical compounds present in the essential oil, it is most likely that its antimicrobial activity is not attributable to a specific mechanism alone but to several targets in the cell [31, 32].

Some studies have concluded that whole essential oils have greater antibacterial activity than the major components mixed, which suggests that the minor components are critical to the activity and may have a synergistic effect or potentiating influence [33]. This is the case of *Salvia officinalis* [34] and certain species of *Thymus* and *Origanum vulgare* [35].

Our results revealed that all tested essential oils showed notable inhibition of aflatoxin B1 production by *A. flavus* at high concentrations, but basil and coriander oils showed marked inhibition of aflatoxin B1 production at all concentrations tested (500,750 and 1000 ppm).

Recently, the natural products such as plant extracts have been identified as potential candidates against AFB1. A study showed that essential oils reduce DNA binding of aflatoxin. Essential oils from common spices such as nutmeg, ginger, cardamom, celery, xanthoxylum, black pepper, cumin and coriander were tested for their ability to suppress the formation of DNA adducts by AFB1 *in vitro* in a microsomal enzyme-mediated reaction. All oils were found to inhibit adduct formation very significantly and in a dose-dependent manner. The adduct formation appeared to be modulated through the action on microsomal enzymes, because an effective inhibition on the formation of activated metabolite was observed with each oil. The enzymatic modulation is perhaps due to the chemical constituents of the oils and this could form a basis for their potential anticarcinogenic roles [36].

In another research, the effects of garlic oil, such as diallyl disulfide (DADS) and diallyl sulfide (DAS) on AFB1-induced DNA damage in cultured primary rat hepatocytes were shown. About 0.5 and 2 mM DAS or 0.5 and 1 mM DADS significantly decreased the DNA damage induced by AFB1 as compared with the AFB1 control, according to the unscheduled DNA synthesis test [37].

Our results showed that, both fungal growth and aflatoxin B1 biosynthesis of *A. flavus* were suppressed by all the tested oils. The inhibitory effect of the oils varied according to type of oil and increased in proportional to their concentrations.

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