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Bioactivity of *Eugenia Earyophllata* Essential Oil against *Aspergillus flavus* and *Aspergillus parasiticus*

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Abstract

The aim of this study is to evaluate the inhibition of *Aspergillus flavus* (*A. flavus*) and *Aspergillus parasiticus* (*A. parasiticus*) isolated from wheat and broad bean seeds exposed to the essential oils obtained from clove *Eugenia earyophllata* (*E. earyophllata*). Different concentrations of the essential oil on spores germination, mycelium dry weight and aflatoxin production were determined *in-vitro*. Essential oils applied in 8 levels, included 0 (as control), 0.001, 0.005, 0.01, 0.05, 0.5, 1 and 2 mg/L. The results showed that the essential oil in some levels decreased and in others inhibited the growth of both fungi under *in-vitro* conditions. Spore germination was reduced by 25% and 30.7% at 0.01 mg/L in *A. flavus* and *A. parasiticus* compared with 100% in control treatment. Mycelium dry weight was also affected by the oil tested. Clove oil gave complete inhibition at its higher concentration (1.0 mg/L). Furthermore, aflatoxin production was highly affected at all concentrations of the essential oil tested. This plant offers novel approach to the management of storage fungi and this study suggests that the essential oil can be used as preservatives in foods.

Keywords: Bioactivity, essential oil, *Eugenia earyophllata*, *Aspergillus flavus*, *Aspergillus parasiticus*, aflatoxin

1. Introduction

Fungi are significant destroyers of foodstuffs during storage, rendering them unfit for human consumption by retarding their nutritive value and sometimes by producing mycotoxins drugs, they are limited in number. The increase of fungal resistance to classical drugs, the treatment costs, and the fact that most available antifungal drugs have only fungistatic activity, justify the search for new strategies [1]. The use of plants and their products for the treatment of a variety of fungal infections is an old practice. This is because many plants contain a variety of compounds that have promising potentials for antimicrobial activity. According to Naqui *et al*. [2] and Kalemba *et al.* [3], some plants contain compounds that are able to inhibit microbial growth. Aromatic plants have been widely used in folk medicine. It is known that most of their properties are due to their volatile oils. They have been empirically used as antimicrobial agents. Some essential oils show an important antifungal activity against yeasts, dermatophyte fungi and *Aspergillus* strains, which could predict therapeutic benefits, mainly for diseases with mucosal, cutaneous and respiratory tract involvement [4-6].

Essential oils were considered to be more effective than plant extracts [7]. Essential oils are rich source of biologically active compounds and they are potential sources of novel antimicrobial compounds. It was demonstrated that essential oils have been shown to possess antibacterial, antifungal, antiviral insecticidal and antioxidant properties [8]. Aflatoxins are natural poisons produced by two common fungi, *Aspergillus flavus* (*A. flavus*) and *Aspergillus parasiticus* (*A. parasiticus*). Aflatoxin contamination in a variety of field crops and agricultural commodities worldwide has a serious impact on the ec-

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onomies and food safety of these products. Aflatoxins were found to cause many other health hazards in both human beings and animals [9]. Many studies had been done to control aflatoxin poisoning and to prevent growth of the aflatoxin producing fungi. The most important of which was the use of essential oils and plant extracts. Their effects depend on the concentration of essential oils used [5, 7, 10-12].

The objective of the present study is to test the effect of clove essential oil on the growth of *A. flavus* and *A. parasiticus* and their aflatoxin production.

2. Materials and Methods

2.1. Source of Isolations

Associated fungi were isolated from stored wheat and broad bean seeds. The seeds were surface-sterilized in NaOCl for 2 min and rinsed in two changes of sterile distilled water. The seeds were blotted dry in between sterile Whatman No. 1 filter papers and plated on sterile potato sucrose agar (PSA) at the rate of 10 seeds per plate. Thirty seeds were plated per crop and incubated at room temperature of 25 ± 1°C. Sub-cultures were made from emerging colonies and pure cultures obtained for subsequent studies. Pure cultures of fungi were examined macroscopically and microscopically, and their identification was carried out by using a taxonomic schemes primarily based on morphological characters using the methods given by Singh *et al*. [13], Filtenborg *et al*. [14] and Tabuc [15]. Isolates of *A. flavus* and *A. parasiticus* were obtained from wheat and broad bean, respectively

2.2. Inoculum Preparation

The test organism *A. flavus* and *A. parasiticus* were sub cultured for 4 days and the spore mass from the slant was suspended in 3 mL of distilled water. This was used as the inoculum for the spore germination, mycelia dry weight and fermentative production of aflatoxin.

2.3. Essential Oils and Preparation of SMKY Media

Sucrose, magnesium sulfate, potassium nitrate and yeast extracts (SMKY) described by Davis *et al.* [16] was used in this study for testing the antifungal activity of essential oil on mycelia dry weight and detection of aflatoxin production. On spore germination, SMKY was prepared by modification of the method of Davis *et al*. as reported by Abdel-Rahim and Arbab [17]. A 1.0 L of media was distributed in 100 mL conical flask. The media was then sterilized at 121°C for 15 min. 5 mL of different concentrations of essential clove oils (0, 0.001, 0.005, 0.01, 0.05, 0.5, 1 and 2 mg/L) were added to 45 mL SMKY media after cooling.

2.4. Antifungal Assay

2.4.1. Spores Germination Test

The spore germination test was conducted by using the oil concentration with the synthetic SMKY broth [17]. A drop of about 0.5 mL was transferred from each solution onto a sterile glass slide and inoculated with 0.1 mL spore suspension (1×10^7 spore/mL). The inoculated slides were placed in the sterile petri dishes lined with moistened sterile filter paper to attain the relative humidity 90-100% approximately at 28°C for 20 h. 100 spores were examined and the extent of spore germination assessed by looking for germ tube emergence. The number of spores germinated was scored and reported as a percentage of spore germination.

2.4.2. Mycelia Dry Weight and Aflatoxin Production

A 50 mL of production medium (synthetic SMKY) whose pH adjusted to 6.0-6.4 was inoculated with 1.0 mL of the spore suspension and thoroughly mixed. These flasks with the inoculum were incubated at room temperature as stationary culture. After 8 days of incubation, the cultures of birth fungi were sprayed with chloroform to kill the spores, the mycelia mats were collected and weigh immediately and reweighed after drying at 80°C. The filtrate was then kept for the aflatoxin analysis.

2.5. Toxin Extraction

A 200 mL of chloroform was added to the filtrate of the culture and was shaken thoroughly for 1 h in a mechanical shaker. The solution was transfer quantitatively into a 250 mL separating funnel and allowed to separate. The chloroform phase was collected and drained into a conical flask (250 mL) containing 10 g of sodium sulfate (anhydrous) to absorb any water. By using quick fit distillation set up, the aflatoxin was extracted from the chloroform and stored in amber-colored

vials under refrigeration for analysis [18].

2.6. Detection of Toxin

For the detection of aflatoxin, the extract was subjected to thin layer chromatographic (TLC) as described by Jones [19]. The clean extract was done on percolated silica gel plates (20×20 mm) at a baseline drawn on the plate at a distance of 2 cm from the lower edge and allowed for one minute. The chromatoplates had previously been activated at a temperature of 105˚C for 90 min. The spots were left to dry for 1 min. The solvent system used was chloroform: methanol; 95:5. The chromate-plates were removed after 90 min, or when the solvent reached a mark 2 cm from the upper edge of the plates. They were dried mechanically and examined under the UV/Vis lamp at short and long wave, and the specks were circled with a pencil. The toxins were identified as florescent spots at the same height (R_f value). The retention factors (R_f) of the compounds/toxins detected in the media were calculated. R_f = distance traveled by the compound/distance traveled by the solvent. The R_f values were compared with the standard mycotoxins (Sigma®).

2.7. Statistical Analysis

All experiments were made in three replicates per treatment. One-way analysis variance (ANOVA) was performed on all the data and least significant differences ($P = 0.05$) between the control and treated samples were determined according to the Duncan's multiple range tests.

3. Results and Discussions

3.1. Cultural and Morphological Characteristics of the Fungal Isolates

On PSA, the fungus formed radial colonies of white airy mycelia, and when the growing fungus is about 1 inch in diameter, yellowish green spores were produced (Figure 1A). Conidiophore stipes were hyaline and coarsely roughened, often more noticeable near the vesicle. Conidia are globose to subglobose (3-6 µm in diameter), pale green and conspicuously echinulate. The vesicle, phialides, metulae and conidia form the conidial head (Figure 1B). The isolate was confirmed by morphological and cultural characters which were Colony: Colour, shape, margins and pigmentation; Mycelium: Colour, shape, septation; and Conidia: Colour, shape, size and septation. Based on the cultural and morphological description the fungus was identified as *A. flavus* [13-15]. *A. parasiticus* has deeper green colony (Figure 1C), only phialides, spherical head and conidia which tend to be smaller and more delicately spiny (Figure 1D) according to Singh *et al.* [13], Filtenborg *et al*. [14] and Tabuc [15].

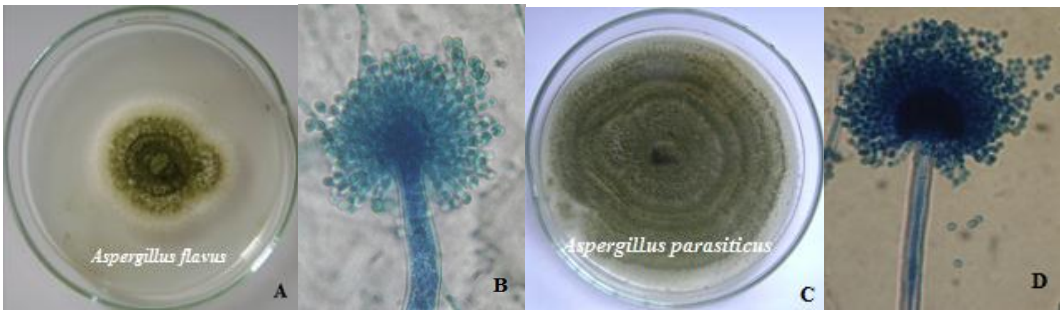

Figure 1. Colonies of *Aspergillus* on PSA Plate and their Conidial Heads (A & B for *A. flavus* and C & D for *A. parasiticus*)

3.2. Effect of Essential Oil on Spore Germination, Mycelia Growth and Aflatoxin Production

The present study investigated the effect of different clove oil concentration on the growth of fungi *A. flavus* and *A. parasiticus* as well as on their aflatoxin production. The results showed that the antifungal clove oil at its different concentrations was highly effective in reducing spore germination and had an inhibitory effect on *A. flavus* and *A. parasiticus* growth. A concentration of 0.5 mgL of clove oil was able to inhibit both spore germination completely (Figure 2, 3 and 4). However, it allowed 74.33 and 79.33 g of dry weight of mycelia growth in both fungi compared to 300 g in the control treatment.

The growth was completely inhibited at 1.0 mg/L in synthetic media for both fungi (Figures 3 and 4). Similar results were obtained by Bullerman *et al.* [20], they mentioned that clove oil at its different concentrations had an inhibitory effect on *A. flavus* growth. Bullerman [21] suggested the use of 1- 2% cinnmon oil to inhibit aflatoxin production by *A. parasiticus* in bread. According to Chatterjee [22], both the clove and cumin oils were effective because they both contain euge-

nol, the active ingredient against fungi, although clove oil contains far higher amounts of eugenol (95%) than cumin oil.

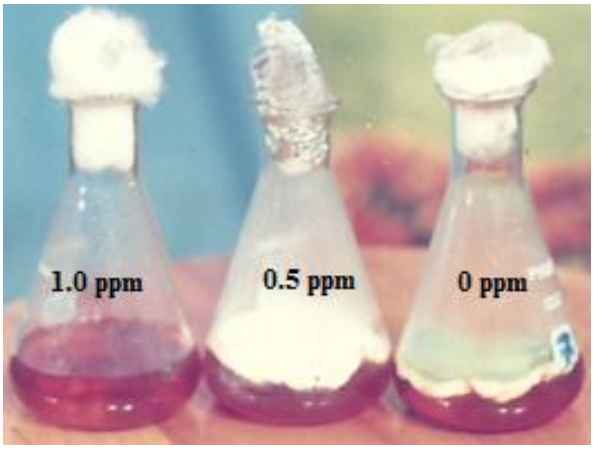

Figure 2. Effect of Clove Oil Concentrations on *A. flavus* Growth in Liquid Medium

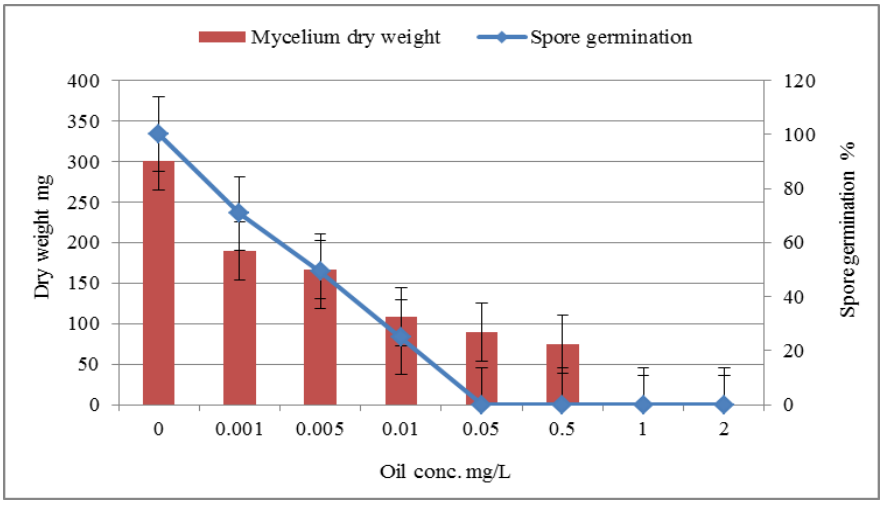

Figure 3. Average Dry Weight (mg) and Spore Germination Rate ± SD of *A. flavus* after Treatment with Different Essential Oil Concentrations

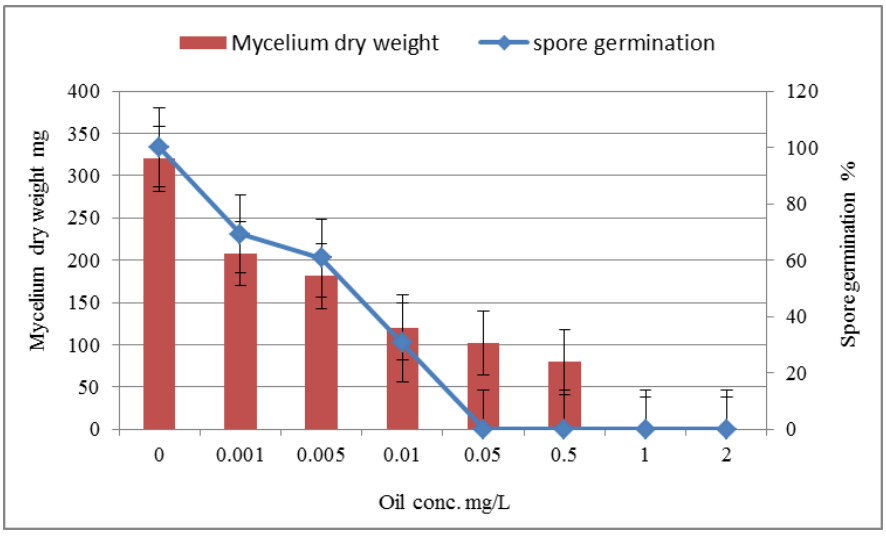

Figure 4. Average Dry Weight (mg) and Spore Germination rate ± SD of *A. parasiticus* after Treatment with Different Essential Oil Concentrations

Results in Tables 1 and 2 represent the effects of different concentrations of clove oil on aflatoxin production. Studying the effect of clove oil on aflatoxin fluorescence showed that *A. parasiticus* gave moderate fluorescence **(++)** while *A. flavus* gave slight fluorescence **(+)** at 0.01 mg/L compared with control treatment (0 mg/L). As to aflatoxin production, all concentrations gave less amounts of aflatoxin production than the control treatment (0 mg/L). Aflatoxin reduction was directly proportional with the increase of concentration from 0.001-1.0 mg/L. Significant effects of clove oil treatment were shown at higher concentrations rather than concentrations below 0.01 mg/L. Bullerman *et al*. [20] showed that lower concentrations of clove oil had an inhibitory effect on aflatoxin production. Moreover, Abdel-Rahim *et al*. [23] reported that at concentrations 0.03-0.05 mg/L, the aflatoxin was completely inhibited in liquid medium.

Table 1. Anti-aflatoxinogenic Assay with Essential Oil of *E. earyophllata*

EO con. (mg/L)	**Fluorescence intensity (Essential oil of E. earyophllata)**	
	A. flavus	*A. parasiticus*
00.00	+++	+++
0.001	++	++
0.005	+	++
0.01	+	++
0.05	-	-
0.5	-	-
1.0	-	-
2.0	-	-

Bright fluorescence (+++); moderate fluorescence (++); weak fluorescence (+); No fluorescence (-)

Table 2. Effect of *E. earyophllata* Essential Oils on Aflatoxin Production

EO con. (mg/L)	**Aflatoxin concentration (mg/L)**	
	A. flavus	*A. parasiticus*
00.00	100	150
0.001	0.046	0.083
0.005	0.0016	0.0056
0.01	0.00036	0.00076
0.05	0.0	0.0
0.5	0.0	0.0
1.0	0.0	0.0
2.0	0.0	0.0
LSD at 0.05	0.012	0.003

Values are mean 3 replicates

4. Conclusions

This study describes the antifungal activity of essential oil of clove (*E. earyophllata*) against mycotoxigenic fungi. Its antifungal activity has high effect on spore germination and mycelia dry weight of *A. flavus* and *A. parasiticus* isolated from wheat and broad bean seeds in Libya. Moreover, aflatoxin reduction was directly proportional to increase in the concentration of *E. earyophllata* essential oil. Results showed that clove essential oil could be used as natural fungicides with suitable potential for biological control of different pathogens, including the seed born fungi in storage. Higher concentrations of plant essential oil have created deterrence potential to plant pathogens and their toxins.

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