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Screening of essential oils for the inhibition of *Aspergillus flavus* by Microtiter plate

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Abstract
Aspergillus flavus produces aflatoxin and contaminates cereals, oil seeds and nuts, due to the climatic change the aflatoxin infestation is wide spread to various crops Viz: corn, soya, wheat, rice and cotton seeds and nuts. Aflatoxin diet exposure has been observed through the consumption of food stuffs, the contamination of maize, peanuts, and oilseeds. Aflatoxin causes deleterious health effects Viz: human and animal health such as causing cancer, liver damage, diarrhea, dizziness and fever are the common symptoms of aflatoxin contaminated food consumption. The aflatoxin contamination is observed Pre and post-harvest, factors such as temperature, moisture, oxygen, and carbon dioxide, insect and rodent's infestation, incidence of broken grains or nuts, the cleaning of the product, will increase the incidence of toxigenic fungal load. Transport, waiting time for drying, frying system and storage condition affect. Some countries established maximum concentration for aflatoxin in specific products ranging from ppb to µg levels based on products, consumption pattern and country specific. We have screened essential oils (Eugenol and Rose oil) to test for the inhibition of the *Aspergillus flavus* growth. These essential oils Eugenol and Rose oil showed the maximum inhibitory activity and further can be used to control *Aspergillus flavus*.

Keywords: aflatoxin, essential oil, *Aspergillus*, antifungal activity

1. Introduction
Aspergillus flavus is responsible for spoilage of many foods and feeds, and causes decay on stored fruits damaged by insects, animals, early splits, and mechanical harvesting. Furthermore, *A. flavus* is able to produce aflatoxins in foods and feedstuffs (Rojas *et al.*, 2005) [20]. Post-harvest pathogens are controlled by a combination of storage technologies, physical methods and synthetic chemical fungicides (Eckert and Ogawa, 1988) [6]. But, alternative control methods are needed because of negative public perceptions about the use of pesticides, development of resistance to fungicides, and high cost for development of new chemical preservatives.
Numerous studies have documented the antifungal effects of plant essential oils against post-harvest fungal diseases (Shahi *et al.*, 2003; Guynot *et al.*, 2005; Mercier and Smilanick, 2005; Neri *et al.*, 2006; Irkin and Korukluoglu, 2007; Kumar *et al.*, 2007; Omidbeygi *et al.*, 2007) [21, 7, 12, 14, 8, 9, 15]. Antifungal property of the extracts or essential oils obtained from some plants against *A. flavus* has been evaluated (Montes-Belmont and Carvajal, 1998; Mahmoud, 1999; Paranagama *et al.*, 2003; Kumar *et al.*, 2007) [13, 10, 17, 9]. These authors reported that some plant oils and/or extracts could effectively inhibit the growth of this pathogen fungus.
The current study was to evaluate the antifungal property of essential oils in for controlling *A. flavus* in microtiter well plates in liquid medium to facilitate screening of possible essential oils and compounds by simple absorbance scanning at 595nm. In this study we are presenting data related to 1) Eugenol, 2) Rose oil screened by using this methodology.

2. Review of Literature
Adjou *et al.*, 2012 [1] studied antifungal activity of *Ocimum canum* essential oil against toxicogenic fungi. They evaluated the inhibition of *Aspergillus flavus* and *A. parasiticus* isolated from peanut and their aflatoxin production exposed to the essential oils extracted from fresh leaves of *Ocimum canum*. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of the oil were determined. The essential oil was found to be strongly fungicidal and inhibitory to aflatoxin production. Through GC/MS analysis, an amount of 30 components were identified, representing almost 95.2% of the oil. Essential oil of *O. canum* was characterized by major components such as terpinene-4-ol (41.18%), linalol

(14.7%) and terpene (6.9%). This plant offers novel approach to the management of storage fungi.

Paranagama *et al*, 2003 [17] developed a natural fungicide against aflatoxigenic fungi, using the essential oil of lemongrass. They isolated *Aspergillus flavus* from stored rice and identified as aflatoxigenic grain. Lemongrass was tested against *A. flavus* and the test oil was fungi static and fungicidal against the test pathogen at 0.6 and 1.0 mg/ml, respectively. The results obtained from the thin layer and gas chromatography indicated citral A & B as fungicidal constituents in lemon grass oil. During the fumigant toxicity assay of lemongrass oil, the sporulation and the mycelia growth of the test pathogen were in habited at the concentrations of 2.80 and 3.46mg/ml respectively. Thus they concluded that lemongrass oil could be used as anti-fungal agent to manage aflatoxin formation and growth of *Aspergillus flavus* in store.

Evaluated the effects of some essential oils i.e., eucalyptus, lemon grass and thyme on linear growth and spore. The highest reduction was obtained with eucalyptus and thyme at concentrations of 0.6% and lemon grass at 0.8% which reduced both diseases incidence and rotted part tissue more than 74 and 75% respectively. Meanwhile, other treatments showed moderate effect. It could be suggested that essential oils could make them an excellent treatment for controlling postharvest diseases of apple fruits. Antifungal activity of different concentrations of the EO was evaluated using disc diffusion method. The most abundant compounds identified in the EO were 1, 8-cineole (16.2%), α-pinene (15.6%), α-phellandrene (10.0%), and p-cymene (8.1%). The EO produced complete mycelial growth inhibition in all the test pathogens at a concentration of 7-8 μL/mL after five days of incubation. The minimum inhibitory concentration and minimum fungicidal concentration of the EO on the test fungi were in the range of 7-8 μL/mL and 8–10 μL/mL, respectively. These findings confirm the fungicidal properties of *E. camaldulensis* essential oils and their potential use in the management of economically important *Fusarium spp.* and as possible alternatives to synthetic fungicides. (Martin Muthee Gakuubi *et al.*, 2017) [11].

Determination of the Antifungal Activity by using the poisoned food technique as described by (Adjou *et al*., 2012) [1]. Various initial concentrations of plant extracts and essential oils will be prepared, containing 0.5% (v/v) of Tween-80 to cooled molten PDA (45°C) followed by manual rotation in a sterile Erlenmeyer flask to disperse the oil in the medium. 20 ml of the medium will be dispensed into sterile Petri dishes (9 cm in diameter) with enough care to avoid trapping air bubbles. The medium will be allowed to solidify at room temperature (23 ± 2°C) for about one hour. Agar discs with mycelia (6 mm in diameter) will be cut from the periphery of actively growing regions of the 7-day-old pure cultures using a sterile cork borer and aseptically inoculated at the center of the petri plates. Control plates without the essential oil and plant extract will be inoculated following the same procedure. Three replicates will maintain for each treatment and the plates will be incubated at 28ºC. The fungal colony diameter readings will be taken after three and five days of incubation. The percentage inhibition of the mycelial growth of the test fungus by the essential oil and plant extract will be calculated using the formula by Philippe *et al.,* 2012 [18].

3. Material and Methods

3.1 Pathogen fungus

The identified pathogen fungus *A. flavus* was collected from Department of Plant Pathology, College of Agriculture, UAS, Raichur for this study. Fungus culture was maintained on potato dextrose agar (PDA) at 4 °C for using further studies. The old cultures were transferred to fresh slant every two months in order to avoid a decline in strain viability.

3.2 Collection of the Essential Oils

Essential oils (Peppermint oil, Orange oil, Citronella oil, Methyl eugenol, Eugenol, Cumin seed oil, Rose oil, Lavender oil and Eucalyptus oil) were obtained from Sisco Research Laboratories Pvt. Ltd., Maharashtra.

Each essential oil was tested at various concentrations in the range of 0.05%, 0.1%, 0.25%, 0.5%, 1% & 2% in 10ml PDB and 10µl of Tween-20 (Sigma-Aldrich). Suspension of *A. flavus* with Tween-20 and *A. flavus* suspension without essential oils were used as controls. All tests were performed in triplicate with three repeats.

3.3 Media composition: (PDA)

Composition	1000ml
Peeled potato	200g
Dextrose	20g
Yeast extract	0.1g
Agar	15g

3.4 Media composition: (PDB)

Composition	1000ml
Peeled potato	200g
Dextrose	20g
Yeast extract	0.1g

3.5 Antifungal activity screening

The efficacy of the essential oils to inhibit *A. flavus* fungal growth was tested using 96-well microtiter plates. 300µl of distilled water was taken in separate wells. Controls (290µl of PDB + 10µl of *A. flavus* culture) were maintained without essential oils in a separate wells. Test samples (290µl of PDB with essential oils + 10µl of *A. flavus* culture) were maintained and performed in triplicates. All the plates were sealed with Para film and incubated at 27 ºC. Microplate readings were taken in Micro plate reader (Bio-Rad) for every 24 hours for 7 days at 595nm (Willem. F. *et al.,* 1989) [24].

4. Results and Discussions

4.1 Effect of essential oils on the growth of *A. flavus* in PDB medium

Eugenol and Rose oil were tested for the effect on the growth of *A. flavus* in PDB for 7 days. The results show that eugenol and rose oil significantly inhibited the growth of *A. flavus* Rose oil was the most effective oil followed by Eugenol.

The effects of eugenol and rose oil at various concentrations (0.05%, 0.1%, 0.25%, 0.5%, 1% & 2%) on the growth of *A. flavus* in PDB for 7 days were shown in Tables 1 & 2 and Fig 1 & 2.

Table 1: Absorbance OD at 595nm for essential oil Eugenol.

Time	Distilled water	Control	0.05%	0.10%	0.25%	0.50%	Only Media
0 hrs	0.059333	0.201667	0.188	0.185667	0.471667	0.395667	0.200333
24 hrs	0.034667	0.794667	0.275333	0.191	0.352333	0.338	0.226333
48 hrs	0.038667	1.956667	0.621333	0.242	0.323667	0.334333	0.337333
72 hrs	0.039333	1.969	1.209667	0.299333	0.304667	0.327667	0.432
96 hrs	0.046333	2.027	2.363667	0.304333	0.296	0.323	0.441333
120 hrs	0.044	1.465	2.604667	0.334667	0.284667	0.323	0.425333
144 hrs	0.054	1.474	2.527333	0.363667	0.283667	0.318667	0.396333
168 hrs	0.069333	1.4985	2.381	0.447667	0.287667	0.321333	0.374333

The essential oil eugenol inhibited the *A. flavus* at 0.25% is around 50% growth inhibition has been recorded and as the concentration of oil increases the inhibition is At 2% of Eugenol oil the wells appeared to be clumpsy. Hence affected the spectral readings. But the observations under microscope confirmed that the Eugenol has inhibited the *A. flavus*.

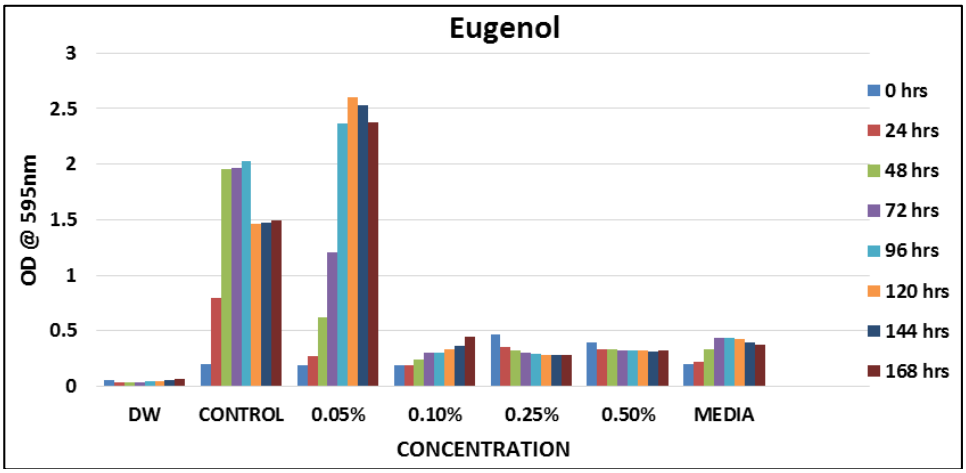

Fig 1: Eugenol inhibition time course

Table 2: Absorbance OD at 595nm for essential oil Rose oil.

Rose oil	DW	CONTROL	0.05%	0.10%	0.25%	0.50%	1%	2%	MEDIA
0 hrs	0.051667	0.228667	0.252	0.21	0.459	0.54	0.478	0.654	0.206667
24 hrs	0.042	0.867333	0.274	0.206667	0.255333	0.505667	0.627667	0.819	0.207
48 hrs	0.039333	2.340333	0.391333	0.319333	0.252333	0.449667	0.300333	0.719	0.265
72 hrs	0.043667	1.9625	0.76	0.375667	0.237	0.521667	0.245	0.619	0.277333
96 hrs	0.039667	1.9465	1.782667	0.489667	0.273333	0.462	0.244	0.518667	0.286
120 hrs	0.040333	1.9435	2.645333	0.882	0.406667	0.444	0.253667	0.414667	0.262333
144 hrs	0.040667	1.917	2.770667	1.655333	0.558667	0.433	0.257667	0.365	0.257333
168 hrs	0.040667	1.8555	2.769667	2.502667	0.842333	0.417667	0.259667	0.349333	0.317333

The essential oil rose inhibited the *A. flavus* growth by 50% at 1% and above and recorded concentration dependent inhibition.

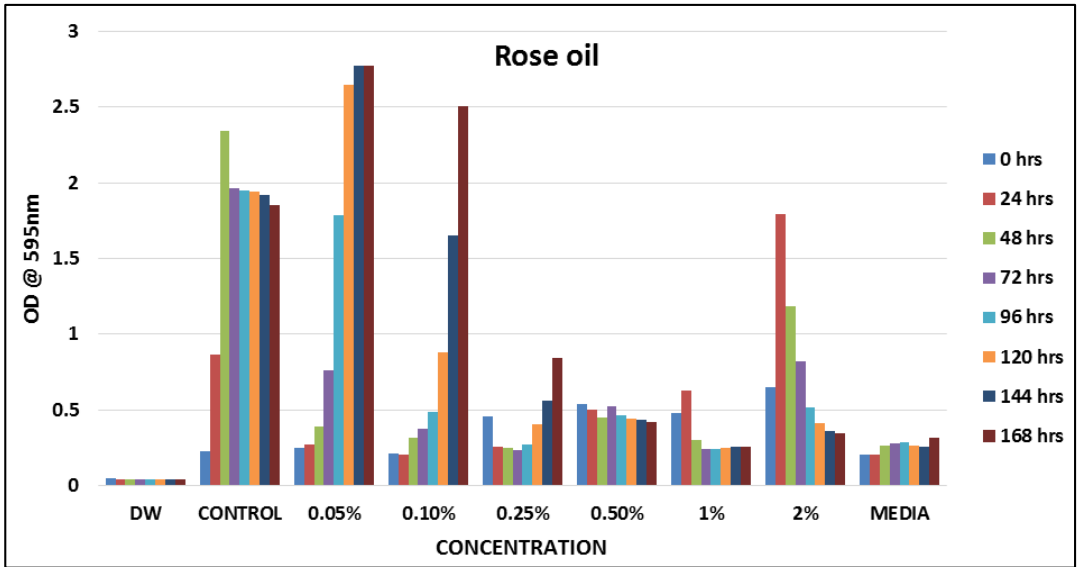

Fig 2: Rose oil inhibition time course

Essential oils, aromatic volatile products of the secondary metabolism of plants, have been in the applications for food flavouring and preservation as the demand by consumer for the safer natural products to control food spoilage without chemical residues and environmental pollution. There is a requirement of safer controlling agents and same time can be consumed without side effects in this direction essential oils fits in these categories. As these oils are being used for culinary purposes, flavouring agents and preservatives over the years. Our study to establish utility in controlling the *A. flavus* supports the safe consumer usage practices and without harming the nature.

In this study, Eugenol and Rose oil showed a pronounced antifungal efficacy against the tested fungi. The mycelium growth was recorded to change with increasing concentrations of the oil. As the concentration of the essential oil increased, a pronounced reduction in the percentage of spore germination was observed on *A. flavus*. Some studies focused on the effects of the compounds on fungal spore germination. Yenjit *et al*., 2010 [25], found that fernenol, arundoin, and the mixture of stigmasterol and b-sitosterol greatly inhibit spore germination and germ tube elongation in *Colletotrichum gloeosporioides* with EC50 values of 45.8, 62.3, and 86.9 mg/l. However, the spore germination was greatly inhibited at the concentration of 1 and 2%. Similar types of results were also reported by Bajpai *et al*., (2008) [2].

Potential utility of essential oil applications in biological control of aflatoxin producing fungi and insect pests has been established by various researchers. Present study confirms that the micro well based method can be used for screening aflatoxigenic fungi inhibition and our study clearly establishes that the essential oils can be used to control *A. flavus* and its utility needs to be established by testing by surface treating of infected samples. (refff)

A number of compounds and substances have been found to be effectively inhibit fungal growth and aflatoxin production, while others have stimulatory properties (Zaika *et al.*, 1987) [26]. In many instances low concentrations of test compounds stimulated fungal growth and/or toxin production, while higher concentrations completely inhibited them. Clove oil at 50 and 100 µg/ml and cinnamon oil at 50µg/ml stimulated the growth of *A. flavus* in liquid media whereas higher concentrations reduced the mycelial growth (Sinha *et al*., 1993) [22].

In the present study, Eugenol and Rose oil exhibited the most antimicrobial activity against *A. flavus*. There has been speculation on the contribution of the terpene fraction of the oils to their antimicrobial activity (Conner., *et al* 1993) [5]. The antimicrobial activity varies widely, depending on the type of spice or herb, test medium and microorganism (Snyder *et al*., 1997) [23]. Contents of essential oils in different species is influenced by genetic material, culture conditions, environment and by crop and post-crop processing (Charles *et al*., 1990 and Paakkonen *et al*., 1990) [4, 16]

5. Conclusions

Our study clearly establishes that the microtiter based screening can be used for testing antifungal activity *invitro* and optical density based method is suitable for screening antifungal activity. Eugenol has shown inhibition of 50% at 0.25% and rose oil at 1%. Hence further testing is necessary to be utilized as food preservant.

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