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Effect of essential oil of *Cymbopogan caseius* and *Cymbopogan nardus* against Aflatoxin producing *Aspergillus flavus*

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Abstract

Effect of individual and combined essential oils of *Cymbopogan caesius* and *C. nardus* on growth of aflatoxin producing fungus *Aspergillus flavus* and aflatoxin production was investigated *in vitro*. Different concentrations (50 ppm, 100 ppm, 200 ppm and 300 ppm) of essential oils significantly inhibited fungal growth and total aflatoxin content in Mycological broth as compared to that of control. Combinations of essential oils had synergistic inhibition of fungal growth and aflatoxin production as compared to their individual oil. Inhibition of fungal growth in combined essential oils ranged from 37.4% to 100% with an average of 75.7%, respectively. The results of the present study suggest that the synergistic effect of plant extracts can be used for control of fungal growth and aflatoxin production.

Keywords: Antifungal, Antiaflatoxigenic, C. caesius, C. nardus

Introduction

The aflatoxins (AFs) are a group of toxic and carcinogenic polyketide secondary metabolites, which are produced by strains of *Aspergillus flavus, A. parasiticus, A. nomius* and *A. pseudotamarii* (Payne, 1998). The International Agency for Research on Cancer (IARC) has classified aflatoxin B_1 (AFB_1) as a Group I carcinogen, primarily affecting the liver (IARC, 1993). AFs are found as contaminants in various agricultural commodities such as cereals, tree nuts, groundnut and cottonseed (Pittet, 1998). Aflatoxin B_1 (AFB1), the most potent of the four naturally occurring aflatoxins namely aflatoxin B_1, B_2, G_1 and G_2 is highly toxic and carcinogenic metabolite produced by *Aspergillus* species of agricultural commodities (Leontopoulos *et al*., 2003). Aflatoxins have been detected in various food commodities from many parts of the world (Smith and Moss, 1985) and are presently considered as one of the most dangerous contaminants of food and feed. Besides their effect on the health of human and animals, aflatoxin also has an impact on the agricultural economy through the loss of crop production and the time and costs involved in monitoring and decontaminating efforts as FAO and WHO have imposed regulatory guidelines of 20 ppb of total aflatoxins as the maximum allowable limit in food or feed substrate. In some European countries aflatoxin levels are regulated below 5 ppb (Jiujiang *et al*., 2002). However, the potential for such toxicity is highest in countries like Kenya, where consumption of contaminated maize, a staple food led to 317 cases of poisoning and 125 deaths in rural area (Lewis *et al*., 2005; Molyneux *et al*., 2007). The genus *Cymbopogon* (Poaceae) is known to include about 140 species. Among these, more than 52 have been reported to occur in Africa, 45 in India, six each in Australia and South America, four in Europe, two in North America and the remaining are distributed in South Asia (Jagadish Chandra, 1975). Most of these species produces characteristic aromatic essential oils that have commercial importance in perfumery, cosmetics and pharmaceutical applications. The *Cymbopogon* essential oils are characterized by monoterpene constituents like citral, Citronellol, citronella, linalool, elemol, 1,8-cineole, limonene, geraniol, β-caryophyllene, methyl heptenone, geranyl acetate and geranyl format (Ganjewala, 2009). Citral is one of the important components of the oil present in several species of *Cymbopogon* with wide industrial uses such as raw material for perfumery, confectionery and vitamin A. Lemon grass is an aromatic tropical grass with clumped, bulbous stems that ultimately

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becomes a leaf blade. It has lemon flavor. It has branched cluster of stalked flower grows up to 6 inches tall. The antifungal activity of *Cymbopogon* oils being attributed to its Geraniol content (Bard *et al.*, 1988). The oil valued highly in the perfuming industry as a source of high grade Geranoil (Mallavarapu *et al.*, 1998). It is fungistatic against the filamentous fungi *Aspergillus niger, Chaetomium globosum* etc. (Delespaul *et al.*, 2000) and is considered to provide protection against mosquitoes (*Anopheles culcifacies*) (Ansari & Razdan, 1999). *Cryptococcus neoformans*, a fungus that causes infection during the last stages of AIDS is inhibited by both Palmarosa oil and geranoil (Viollon and Chaumont, 1994).The objective of the present study was to investigate the efficacy of individual and combinations of essential oils of *C. caesius* and *C. nardus* for inhibition of growth of aflatoxin producing fungus *A. flavus* and aflatoxin production.

Materials and Method

Leaves of *C. caesius* (1 Kg fresh) was collected from southern India. *C. caesius* is a major problematic weed in orchards like mango in several states of Southern India, grows up to the height of 2 meters and flowering takes place during the months of Nov-Jan. The mature flower head is rich in oil (1.1-1.6%). They were a shade dried and then finely chopped with a sharp blade. *C. nardus* (Citronella) (1 Kg fresh) was collected from Biomass Research Station, Banthra of National Botanical Research Institute, Lucknow, India.

Extraction of Essential oil

Chopped leaves of *C. nardus* and inflorescence of *C. caesius* were collected and shade dried. The essential oil was extracted using Clevenger apparatus for about 5 h. *C. nardus* leaves yielded 0.8% and *C. caesius* inflorescence yielded 1.2% essential oil on dry weight basis. The obtained essential oils were dried over sodium sulphate (HiMedia) and stored at 4 ^{O}C until tested and analyzed (Mahdavi *et al.*, 2013).

Antifungal activity of Essential oil

For antifungal assay, PDA plates were prepared and 40 µL of spore suspension containing 18 x 10^4spores mL^{-1} was spread on the PDA plates using a sterilized glass spreader. Impregnated discs containing essential oil of *C. caesius* and *C. nardus* were placed on each inoculated Petriplate and incubated at 28^{0}C for 5 days. Paper discs containing 20% DMSO were used as control. The activity was expressed on the basis of measurement of inhibition zone as compared to control (Bauer et al., 1966).

Mycelial weight Determination

250 mL flasks consisting of Mycological Broth (HiMedia) and shaked at 200 rpm using Lab-Line incubator shaker at 28^{0}C for 96 hrs. Inoculum consisted of 40µL of 18 x 10^4 spore's mL^{-1} in each experimental flask respectively. The fungal biomass was estimated on the basis of fresh weight of combined mycelial and spores. Flask containing mycelia were filtered through Whatman filter paper no. 1 and washed with distilled water. The mycelia were placed on pre weighed Petriplate and were allowed to dry at 40 ^{o}Covernight. The Petriplate containing dry mycelia was weighed (Rasooli and Abyaneh, 2004). Percent growth inhibition on the basis of the dry weight is calculated as:

Control weight – Sample weight / Control weight x 100

Aflatoxin production assay

Different concentrations (50 ppm, 100 ppm, 200 ppm and 300 ppm) of essential oils individually and in combinations were added to each conical flask containing Mycological Broth (HiMedia Ltd). Control did not have plant extracts. All the flasks were inoculated with 1 mL of conidial suspension (1 X 10^7 mL^{-1}) of *A. flavus* and mixed thoroughly by vigorous shaking following the method of Oyebangi and Efiuvwevwere (1999). All treated conical flasks were incubated at 28 ± 2 ^{o}C for 8 days.

Samples preparation and separation with immunoaffinity column

Sample preparation and separation with aflatoxin column procedures were performed according to the instructions of the test kit manual (Rida Aflatoxin Column Art No.: R5001/5002) (R-Biopharm GmbH, 2005). 25 mL of methanol (70%) was added to 5 mL of Broth and the solution was extracted by mixing gently for 10 min at room temperature. Afterwards, the extract was filtered through a paper filter and 15 mL of distilled water were added to 5 ml of filtered solution. Then, 0.25

mL Tween 20 were added and stirred for 2 min. According to test kit manual, clean up procedure was performed as follows. The column was rinsed with 2 mL distilled water for equilibration. The column was filled with approximately 1 mL sample extract. A suitable adapter was attached on top of the column and a syringe was used as a sample reservoir. Syringe was filled with the rest of the sample extract. This was passed slowly and continuously through the column (flow rate: approximately 1 drop/s) and discarded. The column was rinsed with 10 mL distilled water and the passed solution was discarded. The column was dried by passing air through the column for approximately 10 s, in order to make sure that all the residual buffer would be removed from the column. The syringe was removed and a clean and closable vial directly placed below the column. 0.5 mL of methanol was passed slowly through the column (flow rate: approximately 1 drop/s). Toxin containing eluate was diluted 1:10 with the sample dilution buffer (Phosphate Buffer Solution (PBS), pH 7.2) and used 50 µl per well in the assay.

Aflatoxin determination by ELISA

According to Ridascreen Total Aflatoxin (Art no. R4701) test kit manual (R-Biopharm, GmbH, 2005). 50 µl aflatoxin standard solutions and 50 µl prepared test samples were added into separate wells of micro-titer plate, in duplicate. Then, 50 µl of the diluted enzyme conjugate was added to each well, mixed gently and incubated for 2 h at room temperature (20–25°C) in the dark. The liquid was then removed completely from the wells; the each well was washed with 250 µl washing buffer (PBS–Tween Buffer, pH 7.2). The washing procedure was repeated for three times. After the washing step, 50 µl enzyme substrate (urea peroxide) and 50 µl chromogen (tetramethyl-benzidine) were added to each well and incubated for 30 min at room temperature in the dark. Finally, 100 µl of the stop solution (1 N H_2SO_4) were added to each well and the absorbance was measured at 450 nm in ELISA plate reader (Bio-Rad Model 680.). Aflatoxin standard 0, 0.5, 1.5, 4.5, 13.5 and 40.5 ppb are used for the preparation of caliberation curve (Fig 1).

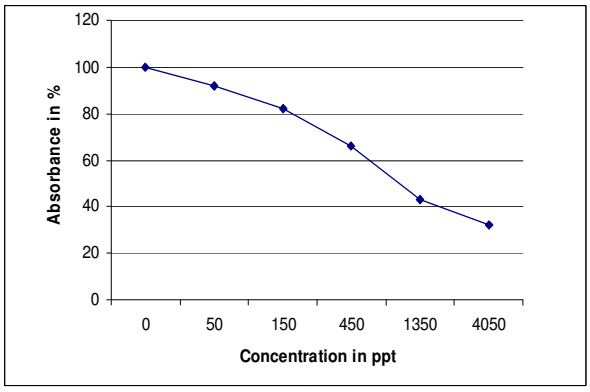

Figure. 1 Calibration curve for total aflatoxin using different concentrations of total aflatoxin standards.

Results and Discussion

Essential oils were isolated from *C. caesius* and *C. nardus* grasses by hydrodistillation using Clevenger apparatus. Antifungal activities of various different concentrations of essential oils were investigated against aflatoxin producing fungi *A. flavus* using the paper disc method. Four different concentrations (50 ppm, 150 ppm, 200 ppm and 300ppm) were made by dilution with Triton X-100. All the three concentrations of *C. caesius* significantly inhibited fungal growth as compared to that of control (Fig.2). Percent inhibition of fungal growth ranged from 7.8% to 12%, the lowest being in 50 ppm and highest in 300 ppm (Figure 2). The fungal growth inhibition by *C. nardus* was 7.9% and 11.9% in essential oil concentration of 150 ppm and 300 ppm, respectively. The lowest concentration (50 ppm) was not found to affect growth of *A. flavus* (Fig. 2).

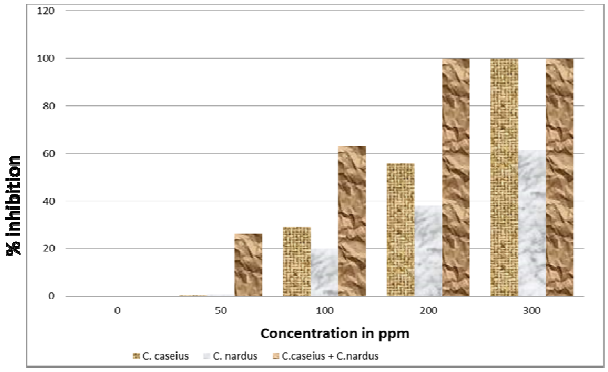

Figure 2. Inhibitory effect of *C. caesius* , *C. nardus* and combination of both essential oil against aflatoxin production.

The effect of different concentrations of essential oils of *C. caesius* and *C. nardus* on growth of *A. flavus* and total aflatoxin production were studied in mycological broth medium using incubator shaker at 28°C for 96 hrs. The fungal biomass in control and treated samples was calculates as the method described earlier (Rasooli and Abyaneh, 2004). Both the two essential oils individually and in combinations were found to inhibit fungal growth and aflatoxin production as compared to that of control (Table 1). All the three different concentrations of essential oil of *C. caesius* were significantly inhibited mycelia weight and aflatoxin production (Table 1). The lowest concentration of *C. nardus* showed lowest inhibition percentage of mycelia weight, whereas combined *C. caesius* and *C. nardus* oils inhibited 37.4% of fungal growth at the concentration of 50 ppm, respectively. The essential oils investigated individual and in combination were found to inhibit both the fungal growth and aflatoxin production as compared to that of control (Table 1).

Table 1. Effect of essential oil of *C. caseius* and *C. nardus* against Aflatoxigenic *A. flavus* MTCC 2799.

Plant	concentration (ppm)	Mycelial wt (mg)	% inhibition of Mycelial wt	TAF* (in ppb)	% inhibition TAF*
	Control	49.9±6.2	0	36.45	0
C. caseius	50	44.2±5.6	11.4	34.4	0.5
	100	31.6± 8.3	36.6	28.5	29
	200	11.8±2.8	76.3	16	56
	300	NG	100	ND	100
C. nardus	50	47.4±3.3	0.5	35.8	1.8
	100	39.6±4.2	20.6	29.2	20
	200	16.4±9.2	67	22.6	38
	300	2.3±1.2	95	14	61.5
C. caseius + *C. nardus*	50	31.2±1.8	37.4	26.8	26.4
	100	12.8±1.3	74.3	13.4	63.2
	200	4.3±0.7	91.2	ND	100
	300	NG	100	ND	100

*** TAF- Total Aflatoxin , NG- No growth, ND – not Detected**

Percent inhibition of fungal growth in between individual *C. caesius* and C. *nardus* essential oil ranged from 0.5% to 100%, the lowest being in *C. nardus* at the concentration of 50 ppm and highest in *C. caesius* at 300 ppm concentration, respectively (Table 1). Combined essential oils showed synergistic antifungal activity as compared to their individual oil. Percent inhibition of fungal growth in combined essential oils ranged from 37.4% to 100% with an average of 75.7%, respectively.Quantitative analysis of total aflatoxin was performed by combined ELISA following the method described earlier (Sidhu *et al*., 2009). Both the essential oils investigated individually and in combination significantly inhibited aflatoxin production as compared to that of control. Percent inhibition of total aflatoxin content in between individual essential oils ranged from 1.8% to 100% with an average of 25.6% (Fig. 2). Combinations of essential oils had synergistic inhibition of aflatoxin production as compared to their individual oils. Combined essential oils of C. *caesius* and *C. nardus* inhibited 26.4%, 63.2% and 100% at the concentration of 50, 100, 200 and 300 ppm as compared to that of control. Citronella oil (0.2%

v/v) have been reported to inhibit the growth of aflatoxin producing fungal strains of Aspergillus such as *A. flavus* IMI242684, *A. flavus* M113, *A. flavus* S 156 and *A. parasiticus* IMI 102566 (Thanaboripat *et al*., 2004). Essential oil of Cinnamon (*Cinnammomum zeylanicum*), peppermint (*Mentha piperita*), basil (*Ocimum basillicum*), Origanum (*Origanum vulgare*), the flavouring herb epazote (*Teloxys ambrosioides*), Clove (*Syzygium aromaticum*) and Thyme (*Thyme vulgaris*) have caused a total inhibition of *A. flavus* on maize kernel. One of the characteristics of aflatoxin deactivation processes is that it should destroy the mycelia and spores of the toxic fungi, which may proliferate under favorable conditions (Namazi *et al*., 2002).The results of this study showed that essential oils of *C. caesius* and *C. nardus* significant inhibited mycelia growth resulted in considerable inhibition of aflatoxin production.

Kumar et al. (2009) also reported that Essential oil of *C. flexuosus* and its components were efficient in checking fungal growth and aflatoxin production. *C. flexuosus* essential oil absolutely inhibited the growth of *A. flavus* and aflatoxin B_1 production at 1.3 µl ml^{-1} and 1.0 µl ml^{-1} respectively.Reports by earlier workers (Morozumi, 1978; Azzouz and Bullerman, 1982, Bahk and Marth, 1983; Bullerman *et al.*, 1977; Hitikoto *et al.*, 1980; Yin and Cheng, 1998) support the fact the extract of certain spices and herbs of medicinal importance exhibit antifungal property. These natural antifungal agents can be potentially exploited in controlling the growth of fungi and consequently inhibiting aflatoxin formation (Yin and Cheng, 1998). Most of the researcher and research are now focusing on the use of plant metabolites particularly essential oil which can be exploited as source of antimicrobial agent as food additive in the form of preservation to control or inhibit the growth of food borne pathogen which is responsible for gastrointestinal infection. Essential oil is active in very small concentration as well as it does not impart any change in taste and odour, it also increases the shelf life of food. Synthetic preservative used nowadays may have some health related issues. So, it is very necessary to search for new antimicrobial agent which is safe and equally effective in controlling food borne infection. The essential oil of both studied plant have great potential to control fungal pathogen particularly *A. flavus* as well as aflatoxin associated with it.

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