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Fungitoxic activity of Indian borage (*Plectranthus amboinicus*) volatiles

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ABSTRACT

Indian borage (*Plectranthus amboinicus*) was investigated for antifungal activity through agar well diffusion assay. Indian borage oil (IBO) was found to be effective against various fungi tested, as it inhibited the radial growth of mycelia and exhibited broad fungitoxic properties against *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus ochraceus* CFR 221, *Aspergillus oryzae*, *Candida versatilis*, *Fusarium* sp. GF-1019, *Penicillium* sp., and *Saccharomyces cerevisiae*. The effective concentration of IBO on the growth of *A. ochraceus* in yeast extract sucrose medium was determined. IBO completely inhibited ochratoxin (OTA) production by the toxigenic strain *A. ochraceus* at 500 ppm. Also, the application of IBO at 100 mg/g in food samples resulted in inhibition of the growth of *A. ochraceus* in food systems such as groundnut, maize and poultry feed and no detectable amount of OTA was found at a high moisture level of 30%, even after seven days. IBO has the potential for use as a botanical fungitoxicant against fungal attack in stored food commodities.

1. Introduction

Microorganisms play a central role in deterioration of stored foods. Fungi are significant destroyers of stored foodstuffs, rendering them unfit for human consumption and affecting their nutritive value. Quite often the growth of toxigenic fungi and mycotoxins produced in food and grains stored for long period of time present a potential hazard to human and animal health (Guo, Russin, Brown, Cleveland, & Widstrom, 1996; Soliman & Badeaa, 2002). During recent years, substantial interest has developed in the preservation of grains by the use of essential oils to effectively retard growth and mycotoxin production. Different extracts and essential oils of spices, herbs and other plant materials are becoming increasingly important in the food industry because of their antifungal, antitoxigenic and antioxidant activities. These plant isolates may improve the shelf life, quality and nutritional value of stored food commodities (Arfa, Combes, Preziosi-Belloy, Gontard, & Chalier, 2006; Mishra & Dubey, 1994).

Plant extracts, especially volatile essential oils from medicinal plants, have been reported to possess antimicrobial activity against a variety of food-borne, human and plant pathogens and pests (Burt, 2004; Soylu, Yigitbas, Soylu, & Kurt, 2007). Plants synthesise secondary metabolites with complex molecular structures and some of them have antimicrobial properties. These secondary metabolites include alkaloids, flavonoids, isoflavonoids, tannins, coumarins, glycosides, terpenes and phenolic compounds. Plant products with antimicrobial properties have possible application in food protection, preventing bacterial and fungal growth (Lanciotti et al., 2004).

Plectranthus amboinicus, also known as *Coleus amboinicus* Lour. and *Coleus aromaticus*, *Plectranthus aromaticus* (Benth.) Roxb, belongs to the Lamiaceae (Labiatae) family and has the following names: country borage, Cuban oregano, Indian borage, French thyme, Spanish thyme, Mexican mint and soup mint. *Plectranthus* has more than 300 species that are traditionally used to treat skin, digestive and respiratory diseases (Alsaabhi, Safiyeva, & Craker, 1999).

P. amboinicus (syn *P. aromaticus*) is one of the species of the genus *Plectranthus* that grows naturally in India and is distributed on the plains of Rajasthan and Uttar Kannada districts of the Western Ghats of India (Lukhoba, Simmonds, & Paton, 2006). Few studies have been conducted on the chemotype and activity of the essential oils from different species of *Plectranthus*. The phytochemical analyses of extracts of *Plectranthus* spp. have revealed the presence of abietene diterpenoids and sesquiterpenes (Orabi et al. 2000). The essential oil of *Plectranthus* spp. contains carvacrol, thymol, α-terpinolene, β-caryophyllene, 1,8-cineole, *p*-cymene and patchoulane (Marwah, Fatope, Deadman, Ochei, & Al-Saidi, 2007). Uncontrolled use of chemical antimicrobial preservatives have caused the appearance of microbial strains with more resistance to classic antimicrobial agents in stored food commodities. Many investigators have demonstrated the efficacy of the use of essential oils in preventing *Aspergillus flavus* growth and toxin production. But, a sufficient study has not been carried out on *Aspergillus*

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ochraceus, which is one of the main toxin sources. Hence, in the present study, the essential oil of Indian borage (*P. amboinicus*) has been investigated against fungal organisms, with emphasis on its effect on ochratoxin strain, *A. ochraceus*, and to identify its chemical constituents, which could be used as a substitute for fungicide to partially or completely inhibit the growth of fungi and mycotoxins.

2. Materials and methods

2.1. Microorganisms

Fungal strains *A. flavus*, *Aspergillus niger*, *A. ochraceus* CFR 221, *Aspergillus oryzae*, *Candida versatilis*, *Fusarium* sp. GF-1019, *Penicillium* sp. and *Saccharomyces cerevisiae* were obtained from the culture collection maintained at CFTRI, Mysore, India. Fungal isolates were maintained on potato dextrose agar and stored at 4 °C.

2.2. Raw materials and chemicals

2.2.1. Plant material

Fresh leaves of *P. amboinicus* were collected from local gardens of Mysore, India during the month of February 2008. The chairman, Department of Studies in Botany, University of Mysore, India, performed the botanical identification. A voucher specimen has been deposited in the herbarium of the University under the number Bot. 0002–2008.

Maize and poultry feed were procured from a local market in Mysore. 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA) and ochratoxin (OTA) standards were procured from Sigma–Aldrich Chemical Co., (St. Louis, MO). All chemicals used in the experiments were of analytical grade and purchased from standard chemical companies.

2.3. Indian borage essential oil (IBO)

The essential oil (volatile fractions) was isolated by hydrodistillation of Indian borage leaves using a Clevenger apparatus (Tiwari, Chansouria, & Dubey, 2003). The essential oil was stored in clean glass vials after removing water with anhydrous sodium sulfate.

2.4. Preparation of spore suspension

All the fungal cultures were grown on potato dextrose agar (PDA) slants at 25 °C until the spores became ramified (7–10 days). Spores were suspended by adding sterilised Tween 20 solution (0.1% v/v) in distilled water and inoculated at approximately 10^6 viable spores per ml.

2.5. Inhibitory activity of IBO

The antifungal activity of the volatile oil was studied against various fungi: *A. flavus*, *A. niger*, *A. ochraceus*, *A. oryzae*, *C. versatilis*, *Fusarium moniliforme*, *Penicillium* sp., and *S. cerevisiae* by agar well diffusion susceptibility test (Singh, Maurya, Catalan, & Lampasoma, 2004). Petri dishes with a diameter of 15 cm were prepared with potato dextrose agar. The wells from agar (6 mm in diameter) were then bored and 10 μl of essential oil were delivered to them. Each fungal suspension of 0.1 ml, containing spores at 10^{-6} cfu/ml, was inoculated onto the surface of the agar. The plates were incubated at room temperature for 5–7 days. They were observed for antifungal activity, and the mean growth values were converted into the inhibition percentage of mycelial growth (MGI) in relation to the control treatment, by using formula

$$\text{MGI}\ (\%) = [(d_c - d_t)/d_c] \times 100$$

where d_c and d_t represent mycelial growth diameter in control and treated petri plate, respectively.

2.6. Effect of IBO on fungal growth and OTA production

Yeast extract sucrose medium (YES) was used as a basal medium for growth and OTA production in stationary cultures (Abarca, Bragulat, & Castella, 1997). *A. ochraceus* spores suspension, prepared in Tween 20, was inoculated into sterile YES broth. IBO was incorporated into YES medium at zero hour to give concentrations of 100, 250 and 500 ppm. The cultures were incubated at 30 °C for 7 days. The fungal biomass was determined as dry weight (drying at 95 °C to constant weight). Ochratoxin was extracted according to the method of Stormer, Sandven, Huitfeldt, Eduard, and Skogstad (1998). The spores and the filtrate were acidified with 1 N HCl and extracted three times with chloroform, evaporated under vacuum to dryness and cleaned up with dichloromethane and phosphate buffer (pH 7). The toxin was quantified using HPLC analysis, using a Shimadzu LC-6A liquid chromatograph HPLC system (Shimadzu, Kyoto), equipped with a ODS Hypersil C18 column. The mobile phase used was water: acetonitrile: acetic acid (49:49:2). The Shimadzu florescence detector RF-10 AXL was set at 333 nm as excitation wavelength and 460 nm as emission wavelength. Injection volume was 5 μl. The concentrations of these compounds were calculated by comparing their retention time (11.02 min) and peak area with OTA standard.

2.7. Effect of IBO on fungal growth and toxin inhibition in food substrates

The food samples were aseptically ground to pass through a 20 BSM (British standard mesh) sieve and stored at −20 °C. The antifungal preparation was tested for inhibition of growth of *A. ochraceus* in food substrates, namely maize, groundnut and poultry feed. Food substrates were ground to grits and known quantity (100 g) was dispersed into 500 ml Erlenmeyer flasks and sterilised with 30% moisture at 121 °C and 15 psi pressure for 40 min in an autoclave. The samples were inoculated with a spore suspension of *A. ochraceus* CFR 221 (10^6 spores). The samples were treated with IBO at concentrations of 50 mg/g and 100 mg/g. The maize, groundnut and poultry feed samples were analysed for fungal biomass and ochratoxin content. The maize, groundnut and poultry feed samples without IBO served as controls. The samples were incubated at 28 ± 2 °C for 7 days.

2.8. Determination of antioxidant activity

Free radical-scavenging activity was measured by the 1,1-diphenyl 2-picrylhydrazyl (DDPH) method (Oktay, Culcin, & Kufrevioglu, 2003). Different concentrations (50, 100 and 200 ppm) of IBO extracts along with butylated hydroxyanisole (BHA) standards were taken in different test tubes. The volume was adjusted to 100 μl by adding MeOH. Four millilitres of a 0.1 mM methanol solution of DPPH were added to these test tubes and shaken vigorously. The test tubes were incubated in the dark at room temperature for 20 min. A control sample was prepared as above without extract, and methanol was used for the baseline correction. Changes in the absorbance of the samples were measured at 517 nm. Radical-scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

$$\text{radical-scavenging activity}\ (\%) = (\text{control OD} - \text{sample OD}/\text{control OD}) \times 100,$$

where OD is optical density.