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Chemical composition, antifungal and antiaflatoxigenic activities of *Ocimum sanctum* L. essential oil and its safety assessment as plant based antimicrobial

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ABSTRACT

The study deals with the efficacy of *Ocimum sanctum* essential oil (EO) and its major component, eugenol against the fungi causing biodeterioration of food stuffs during storage. *O. sanctum* EO and eugenol were found efficacious in checking growth of *Aspergillus flavus* NKDHV8; and, their minimum inhibitory concentrations (MICs) were recorded as 0.3 and 0.2 μl ml^{-1}, respectively. The *O. sanctum* EO and eugenol also inhibited the aflatoxin B_1 production completely at 0.2 and 0.1 μl ml^{-1}, respectively. Both of these were found superior over some prevalent synthetic antifungals and exhibited broad fungitoxic spectrum against 12 commonly occurring fungi. The LD_{50} value of *O. sanctum* EO on mice was found to be 4571.43 μl kg^{-1} suggesting its non-mammalian toxic nature. The findings of present study reveals the possible exploitation of *O. sanctum* EO and eugenol as plant based safe preservatives against fungal spoilage of food stuffs during storage.

1. Introduction

Food safety is an increasingly important public health issue. Nearly, 30% people in the world suffer from food borne diseases every year caused by microbes (Burt, 2004; Shephard, 2008). Moulds are opportunistic biological agents of ubiquitous nature which firmly colonize the foods because of their powerful arsenal of hydrolytic enzymes which can cause a high degree of deterioration of edibles and responsible for considerable economic losses (De Souza et al., 2005). Among moulds, *Aspergillus flavus* Link ex. Fries is a potent food spoiling fungus decreasing shelf life of food products (Bandyopadhyay et al., 2007; Bluma and Etcheverry, 2008). The toxigenic strains of *A. flavus* produce aflatoxins, a group of highly toxic secondary metabolites. Aflatoxins are mutagenic, carcinogenic, teratogenic, hepatotoxic, immunosuppressive as well as inhibitor of several metabolic systems and also recognized as 6th of the 10 most important health risks by WHO for developing countries where short life span is prevalent (Joseph et al., 2005). Thus, food contamination with these fungi and presence of aflatoxin is a major concern, which has received world wide attention due to their deleterious effects on human and animal health as well as their importance in international food trade (Mishra and Das, 2003; Soubra et al., 2009).

The undesirable effects of chemical preservatives and restrictions forced by the food industries on their application as food additives have renewed interest to search for alternative antimicrobial agents to be used in food processing and post harvest storage technology. Over the last few decades, there has been an increasing consumer's demand for foods devoid of synthetic preservatives. The attraction of modern society towards 'green consumerism' (Smid and Gorris, 1999) desiring fewer synthetic ingredients in foods. The recommendation of herbal products as 'generally recognized as safe' (GRAS) as food additive in different developed countries may lead interest in plant based food preservative to protect the food commodities and other processed food without rendering any mammalian and environmental toxicity.

Different crude extracts of spices, herbs and other plant materials are becoming increasingly important in food industries because of their antifungal and bioregulatory properties (Holley and Patel, 2005; Vagi et al., 2005; Kumar et al., 2007a; Lee et al., 2007). Volatile substances from different aromatic plants have proven to be efficient antifungal against food spoiling moulds (Kumar et al., 2007b, 2008; Rasooli et al., 2008).

EO of *O. sanctum* L. (holy basil) has been used in cure of many diseases in Indian system of medicine (Prakash and Gupta, 2005; Hannan et al., 2006; Muthuraman et al., 2008) but its application as antimicrobial ingredient in food preservation to control the fungal infestation and aflatoxin production is still unexplored. Therefore, the present study was performed to evaluate the sensitivity of *A. flavus* to some essential oils (EOs) with special reference to antifungal and aflatoxin B_1 (AFB_1) suppression by *O. sanctum* EO so as to recommend its possible application as a safe and plant based food additive.

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2. Materials and methods

2.1. Test fungus

The toxigenic isolate *A. flavus* NKDHV8 was isolated from seeds of *Hordeum vulgare* L. by serial dilution technique (Aziz et al., 1998) and identified on the basis of colony and morphological characteristics (Raper and Fennel, 1977). The toxigenicity of the isolate was determined (Sinha et al., 1993) and selected for detailed study. The culture of test fungus *A. flavus* NKDHV8 was maintained on Potato Dextrose Agar (PDA) medium (Potato, 200 g; Dextrose, 20 g; Agar, 18 g and distilled water 1000 ml, pH 5.6 ± 0.2) in the Laboratory of Herbal Pesticide, Department of Botany, Banaras Hindu University.

2.2. Isolation of essential oils

Leaves of some aromatic angiospermic taxa (Table 1) of the locality were collected for the extraction of EOs. The plants were identified with the help of Flora of BHU Campus (Dubey, 2004). Fresh leaves (500 g) of each plant species were thoroughly washed with distilled water and subjected to hydrodistillation in Clevenger's apparatus for 3 h. The volatile fraction (EO) was separated and stored in clean glass vial after removing water traces passing through anhydrous sodium sulphate (Tripathi et al., 2004).

2.3. Fungitoxicity of EOs

The fungitoxicity of isolated EOs was evaluated against toxigenic isolate *A. flavus* NKDHV8 following poisoned food technique (Kumar et al., 2008) using PDA as nutrient medium. Requisite amount of the EO was dissolved separately in 0.5 ml 5% tween-20 in presterilised Petri plates (90 mm diameter). Then after, 9.5 ml of PDA medium was pipetted aseptically to each Petri plate and mixed thoroughly so as to obtain the final concentration of 1.0 μl ml^{-1}. Disc of 5 mm diameter of test fungus *A. flavus* NKDHV8 was cut with the help of cork borer from the periphery of 7 days old culture and inoculated aseptically to the centre of poured Petri plate of treatment and control sets and incubated at 27 ± 2 °C for 7 days. Control sets were kept parallel to the treatment sets without EO. Diameter of fungal colony of treatment and control sets were measured and percent mycelial inhibition was calculated.

$$\text{Percentage of mycelial inhibition} = \frac{dc - dt}{dc} \times 100$$

where, dc, average diameter of fungal colony in control sets; dt, average diameter of fungal colony in treatment sets.

During antifungal screening, EO of *O. sanctum* exhibited more fungitoxic activity than the other tested EOs and chosen as test plant for detailed study.

Table 1
Antifungal screening of some higher plant essential oils against *Aspergillus flavus* NKDHV8.

Plants	Family	Plant part	Percent growth inhibition at (1.0 μl ml^{-1})
Acorus calamus L.	Araceae	Leaf	92.98 ± 6.34^{b}
Adenocalymma allicea Mart.	Bignoniaceae	Leaf	88.75 ± 4.06^{b}
Aegle marmelos (L.) Correa.	Rutaceae	Leaf	74.89 ± 7.03^{de}
Ageratum conyzoides L.	Asteraceae	Leaf	85.85 ± 2.87^{c}
Amomum subulatum Roxb.	Zingiberaceae	Leaf	84.79 ± 4.04^{c}
Citrus sinensis (L.) Osbeck	Rutaceae	Peel	70.85 ± 6.89^{defg}
Curcuma longa L.	Zingiberaceae	Leaf	72.13 ± 8.44^{def}
Cymbopogon martini (Roxb.) Wats	Poaceae	Leaf	72.87 ± 7.92^{def}
Eucalyptus citriodora Hook.	Myrtaceae	Leaf	67.98 ± 9.85^{efg}
Eugenia jambolana Lam.	Myrtaceae	Leaf	67.02 ± 11.82^{fg}
Helianthus annus L.	Asteraceae	Leaf	75.43 ± 3.40^{d}
Lantana camara L.	Verbenaceae	Leaf	87.34 ± 3.74^{bc}
Melaleuca leucadendron L.	Myrtaceae	Leaf	63.62 ± 13.87^{g}
Murraya koenigii (L.) Sprenge.	Rutaceae	Leaf	42.13 ± 11.42^{i}
Ocimum basilicum L.	Lamiaceae	Leaf	72.54 ± 9.02^{def}
Ocimum sanctum L.	Lamiaceae	Leaf	100^{a}
Salvia officinalis L.	Lamiaceae	Leaf	92.48 ± 2.79^{b}
Tagetes erecta L.	Asteraceae	Leaf	54.78 ± 10.73^{h}
Vetiveria zizanoides (L.) Nash.	Poaceae	Leaf	40.63 ± 11.32^{i}
Zingiber officinale Rosc.	Zingiberaceae	Leaf	89.57 ± 5.66^{bc}

Values are mean (n = 3) ± SD; $P < 0.05$.
The means followed by same letter in the column are not significantly different according to ANOVA and Tukey's multiple comparison tests.

2.4. GC/GC–MS analysis

The EO of *O. sanctum* was analyzed through gas chromatography (Perkin Elmer Auto XL GC) equipped with a flame ionization detector. The GC conditions were as follows: column, EQUITY–5 (60 m × 0.32 mm × 0.25 μm); H_2 was the carrier gas; column Head pressure 10 psi; oven temperature program isotherm 2 min at 70 °C, 3 °C/min gradient to 250 °C, isotherm 10 min; injection temperature, 250 °C; detector temperature 280 °C. GC–MS analysis was also performed using Perkin Elmer Turbomass GC–MS. The effluent of the GC column was introduced directly into the source of MS. Spectra were obtained in the EI mode with 70 eV ionization energy. The compounds were identified by comparison of their relative retention times and the mass spectra with those of authentic reference compounds shown in the literature (Adams, 2007). Eugenol was found to be the major component of *O. sanctum* EO.

2.5. Antifungal and AFB_1 inhibition assay

Minimum inhibitory concentration (MIC) and antiaflatoxigenic efficacy of *O. sanctum* EO and its major component eugenol (Ozone international, Mumbai) was determined on the toxigenic isolate *A. flavus* NKDHV8 using SMKY (sucrose, 200.0 g; magnesium sulphate, 0.5 g; potassium nitrate, 0.3 g; yeast extract, 7.0 g; distilled water, 1000 ml; pH, 5.6 ± 0.2) broth as nutrient medium (Sinha et al., 1993). Different concentrations of the EO and eugenol *viz.* 0.1, 0.2, 0.3, 0.4 and 0.5 μl ml^{-1} were prepared separately by dissolving their requisite amount in 0.5 ml 5% tween-20 and then mixing it with 49.5 ml of SMKY medium in 150 ml Erlenmeyer flask. The control sets were kept parallel to the treatment sets without EO and eugenol. The flasks were inoculated aseptically with 1 ml spore suspension (≈10^{6} spores ml^{-1}) of *A. flavus* NKDHV8 in 0.1% tween-80 and incubated at 27 ± 2 °C for 10 days. After incubation, content of each flask was filtered (Whatman No. 1) and biomass of filtered mycelium was determined by oven drying at 100 °C till their weight remains constant. The filtrates were separately extracted with 40 ml chloroform in a separating funnel (250 ml) to determine the AFB_1 production. The chloroform extract was separated and dehydrated with anhydrous sodium sulphate and evaporated till dryness on water bath at 70 °C. The residue left after evaporation was re-dissolved in 1 ml chloroform and 100 μl of it was spotted on TLC plate (20 × 20 cm^{2} of silica gel). The plate was then developed in toluene:isoamyl alcohol:methanol (90:32:2;v/v/v) solvent system proposed by Reddy et al. (1970). The intensity of AFB_1 was observed in ultraviolet fluorescence analysis cabinet at an excitation wavelength of 360 nm (AOAC, 1984). For quantitative estimation, blue spots of AFB_1 on TLC were scraped out and dissolved in 5 ml cold methanol and centrifuged at 3000g for 5 min. Optical density of supernatant was recorded at 360 nm and the amount of AFB_1 was calculated following Sinha et al. (1993).

$$\text{Aflatoxin}B_1\text{content}(\mu g\ L^{-1}) = \frac{D \times M}{E \times l} \times 1000$$

where, D, absorbance; M, molecular weight of AFB_1 (312); E, molar extinction coefficient of AFB_1 (21,800) and l, path length (1 cm cell was used).

2.6. Fungitoxic spectrum

The spectrum of fungitoxicity of the *O. sanctum* EO and eugenol was determined at 0.3 and 0.2 μl ml^{-1}, respectively, by the usual poisoned food technique using PDA against 12 fungal species *viz. Alternaria alternata* (Fr.) Keissel., *Aspergillus candidus* Link., *Aspergillus fumigatus* Fresen., *Aspergillus niger* Van Tiegh., *Aspergillus paradoxus* Fennell & Raper, *Aspergillus terreus* Thom., *Aspergillus versicolor* (Vuillemin) Tiraboschi, *Cladosporium cladosporoides* Fresen., *Curvularia lunata* (Wakker) Boedijn, *Fusarium nivale* (Fr.) Ces., *Fusarium oxysporum* L. and *Penicillium* sp. isolated from selected food commodities (*Oryza sativa* L., *Triticum aestivum* L., *Zea mays* L., *H. vulgare* L., *Cicer arietinum* L., *Pisum sativum* L. and *Cajanus cajan* L.).

2.7. Comparison with some synthetic antimicrobials

The fungitoxic efficacy of the EO oil of *O. sanctum* and eugenol against *A. flavus* was compared with some earlier reported synthetic fungicides *viz.* benzimidazole (Benomyl), diphenylamine (DPA), phenyl mercuric acetate (Ceresan), zinc dimethyl dithiocarbamate (Ziram), carbendazim 12% + mancozeb 63% (SAAF), carbendazim 50% WP (Bavistin), sulphur 80% WP (Wettasul-80), organo mercurial dust (Agrosan GN), copper oxychloride (Blitox-50) and mencozeb (Dithane M-45) following poisoned food technique (Kumar et al., 2008).

2.8. Determination of safety limit of *O. sanctum* oil

The safety limit of *O. sanctum* essential oil was determined by acute oral toxicity recording LD_{50} value on mice (*Mus musculus* L.) with an average weight 35 g and age 3 months. Requisite amount of EO was mixed properly with tween-80 and distilled water (2:1) to prepare different solutions containing desired dose of oil *viz.* 0, 25, 50, 75, 100, 125, 150, 175 and 200 μl for each set of mice (12 mice per set). 0.5 ml each solution of EO was orally administered through a syringe with catherator to each