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In vitro antifungal, anti-elastase and anti-keratinase activity of essential oils of *Cinnamomum*-, *Syzygium*- and *Cymbopogon*-species against *Aspergillus fumigatus* and *Trichophyton rubrum*

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ABSTRACT

This study was aimed to evaluate effects of certain essential oils namely *Cinnamomum verum*, *Syzygium aromaticum*, *Cymbopogon citratus*, *Cymbopogon martini* and their major components cinnamaldehyde, eugenol, citral and geraniol respectively, on growth, hyphal ultrastructure and virulence factors of *Aspergillus fumigatus* and *Trichophyton rubrum*. The antifungal activity of essential oils and their major constituents was in the order of cinnamaldehyde > eugenol > geraniol = *C. verum* > citral > *S. aromaticum* > *C. citratus* > *C. martini*, both in liquid and solid media against *T. rubrum* and *A. fumigatus*. Based on promising antifungal activity of eugenol and cinnamaldehyde, these oils were further tested for their inhibitory activity against ungerminated and germinated conidia in test fungi. Cinnamaldehyde was found to be more active than eugenol. To assess the possible mode of action of cinnamaldehyde, electron microscopic studies were conducted. The observations revealed multiple sites of action of cinnamaldehyde mainly on cell membranes and endomembranous structures of the fungal cell. Further, test oils were also tested for their anti-virulence activity. More than 70% reduction in elastase activity was recorded in *A. fumigatus* by the oils of *C. verum*, *C. martini*, eugenol, cinnamaldehyde and geraniol. Similar reduction in keratinase activity in *A. niger* was recorded for the oils of *C. martini* and geraniol. Maximum reduction (96.56%) in elastase activity was produced by cinnamaldehyde whereas; geraniol caused maximum inhibition (97.31%) of keratinase activity. Our findings highlight anti-elastase and anti-keratinase activity of above mentioned essential oils as a novel property to be exploited in controlling invasive and superficial mycoses.

Introduction

Fungal infections caused by various pathogenic and opportunistic groups are on the rise in the different parts of the world. Invasive aspergillosis caused by *Aspergillus* is considered as a major cause of morbidity and mortality in immunocompromised hosts and mortality rates may range from 40 to 90% in high risk populations (Dagenais and Keller 2009). Other chronic infections associated with the immunocompromised patients are caused by dermatophytes mainly *Trichophyton* sp, and had shown increased incidence in recent years especially in the tropical countries (Venkatesan et al. 2007). With the increasing number of immunosuppressed patients at an unprecedented rate, the management of these fungal infections would be a definite challenge to mankind.

Current antifungal therapy for such fungal infections has been threatened by the development of drug resistant strains, host toxicity of available polyenes and fungistatic mode of action of azoles (Barker and Rogers 2006). Therefore, development of newer drugs with improved efficacy and safety or alternative mode of combating infections is needed. Recent developments in fungal genomics have provided unprecedented opportunities for identifying new antifungal drug targets and subsequently exploiting in disease control. Targeting virulence and pathogenicity are now considered as valuable anti-pathogenic approaches (Gauwerky et al. 2009). Establishment of infection by fungi depends on the host–cell interaction with complex interplay of secretion of virulence factors mainly proteinases including elastinases, keratinases, gelatinases, lipases and phospholipases. These extracellular enzymes are probably essential for these organisms to degrade structural barrier and to obtain nutrient and in establishing infections (Voltan et al. 2008). Plant products traditionally being used in ethnomedicine have been expected to deliver newer antifungal compounds. Antifungal activities of the essential oils or their major constituents against *Aspergillus* and *Trichophyton* spp. have been reported by

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several workers (Zacchino et al. 1999; Cavaleiro et al. 2006; Bajpai et al. 2009). The influence of essential oils on fungal virulence factors synthesis and activity are not yet explored or poorly known.

In lieu of this, the present study was aimed to determine *in vitro* growth inhibition of *Aspergillus fumigatus* and *Trichophyton rubrum* by four plant essential oils such as *Cinnamomum verum*, *Syzygium aromaticum*, *Cymbopogon citratus* and *Cymbopogon martini* and their respective major ingredients namely cinnamaldehyde, eugenol, citral and geraniol. These major compounds were chosen to assess their role in contributing activities alone and in their respective oils. Further, possible target sites of most active agent on fungal cell and inhibition of elastase and keratinase enzymes was examined.

Materials and methods

Plant essential oils and drugs

Essential oils and active compounds were obtained from Himalaya Drug Co. (*Cinnamomum verum*, cinnamon); Himedia Ltd. (eugenol and cinnamaldehyde, 98% purity each), Aroma Sales Corporation (oils of *Cymbopogon citratus*, lemongrass; *Cymbopogon martini*, palmrosa; and citral and geraniol) and Dabur Co. Ltd. (*Syzygium aromaticum*, clove). The drug powder of fluconazole was purchased from Himedia Laboratories, Mumbai, India. Stock solution of fluconazole was prepared in dimethyl sulphoxide (DMSO) at a concentration of 25 mg ml^{-1} and stored at −20 °C until used. The purity of oils and active compounds was determined by physicochemical analyses such as specific gravity, refractive index, optical rotation and solubility in alcohol (data not shown) at Fragrance and Flavour Development Centre, Kannauj, India. Chemical composition of oils was determined by gas chromatography–mass spectrometry at Sophisticated Analytical Instrument Facility of Indian Institute of Technology, Mumbai, India (Khan and Ahmad 2011) and Advanced Instrumentation Research Facility, Jawaharlal Nehru University, New Delhi, India. Essential oils were diluted 10 times in 1% DMSO and used in assays.

Gas chromatography and gas chromatography–mass spectrometry analysis

The percentage composition of oil *C. citratus* was determined by GC-FID and the compounds were identified by GC–MS. GC analysis was carried out on a Shimadzu 2010 Gas Chromatograph equipped with an FID and 25 m × 0.25 mm × 0.25 μm WCOT column coated with diethylene glycol (AB-Innowax, 7031428, Japan). Injector temperature was set at 270 °C and detector at 280 °C. Nitrogen was used as a carrier gas at a flow rate of 3.0 ml/min at a column pressure of 74.9 kPa. 0.2 μl of sample were injected into column with a split ratio of 90.0. The linear temperature program of 60–230 °C set at a rate of 3 °C min^{-1} with hold time at 230 °C for 10 min. The samples were then analyzed on the same Shimadzu instrument fitted with the same column and following the same temperature program as above. MS parameters used were: ionisation voltage (EI) 70 eV, peak width 2 s, mass range 40–600 amu and detector voltage 1.5 V. Results were based on GC-FID. Peak identification was carried out by comparison of the mass spectra with database of NIST05 and Wiley 8 libraries. Identification of compounds was confirmed by comparison of their relative retention indices with literature values (Davies 1990).

Fungal strains

Aspergillus flavus NRRL501 was kindly provided from the fungal culture collection of the Agricultural Research Service, USA; *Aspergillus fumigatus* MTCC2550, *Alternaria solani* MTCC2101 and *Trichophyton rubrum* MTCC296 were purchased from Microbial Type Culture Collection, India; *Aspergillus niger* IOA-3 and *Trichophyton rubrum* IOA-9 were collected from Jawaharlal Nehru Medical College and Hospital, AMU, Aligarh, India and are maintained at the departmental culture collection.

Assays for determination of antifungal activity

Effect of essential oils against test fungi was determined in terms of inhibition of biomass in liquid medium and mycelial radial growth on solid medium as described below.

Inhibition of biomass production

Method of Shafique et al. (2011) with slight modification was adapted. Briefly, 50 ml Sabouraud dextrose broth (SDB) containing different concentrations of oils (0.005–0.32% v/v) was inoculated with 500 μl of freshly prepared spore suspension (~1.5×10^6 cfu/ml) of test fungi. In the corresponding control an equal amount of distilled water was added. Fluconazole in the concentration range of 1.25–200 μg/ml was also tested as a positive control. The flasks were incubated at 28 ± 2 °C for 5 days. Thereafter, mycelial biomass from triplicate samples for each treatment was collected on pre-weighed Whatman filter paper no. 1. Mycelial yield was determined after drying the mycelial mat at 80 °C for 24 h and percent loss in mycelial dry weight was calculated over untreated control.

Inhibition of mycelial radial growth

Method of Quiroga et al. (2004) with little modification was adapted. Briefly, a 5 mm diameter disc of inoculum of the test fungi was cut from the periphery of an actively growing culture and placed onto the SDA petriplates amended with test oils (0.005–0.32% v/v). Fluconazole amended plates (1.25–200 μg/ml) were included as positive control. The SDA plates without oils and inoculated with corresponding fungi were served untreated controls. All the inoculated plates were incubated at 28 ± 2 °C for 5 days. Three replicates for each combination of test fungi and oils concentrations were used. The mean diameter of the radial growth of the fungi was recorded at the end of the incubation period and percent growth inhibition was calculated over untreated control.

Antifungal activities of cinnamaldehyde and eugenol against ungerminated and germinated conidia

The assay comprising the activities against ungerminated and germinated conidia of test fungi employed the modified method of De Lucca et al. (1997). For ungerminated conidia susceptibility assay, 90 μl of freshly obtained conidial suspension (~1.5×10^6 cfu/ml) was added to Eppendorf tubes containing SDB (final volume 1 ml) with a range of concentration of test agent (0.005–0.16% v/v). Control sample contained no test agent. Mixed samples were incubated at 30 °C for 30 min. Viable count was enumerated by plating 100 μl onto SDA plates and incubating at 28 ± 2 °C for 24 h. For germinated conidia susceptibility assay, freshly obtained conidial suspension was first allowed to incubate at 30 °C for 8 h (*A. fumigatus* MTCC2550) and for 10 h (*T. rubrum* IOA-9) at 120 rpm and then treated as for ungerminated conidia susceptibility assay. Each assay was carried out three times per isolate per agent concentrations and data are presented as mean ± SD.

Determination of effect of essential oils on hyphal morphology and ultrastructure

Light microscopy

The time (0, 2, 4, 8, 16, 24, 32, 48 h) and concentration (0.005–0.32% v/v) dependent toxicity of oils *C. verum* and