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Inhibitory effects of selected plants on the growth of pathogenic fungi

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Abstract
Plants extracts and essential oils were evaluated for antifungal properties, against five pathogenic fungal species (*Trichophyton rubrum, Aspergillus niger, Candida albicans, Fusarium solani* and *Cryptococcus neoformans)*. Inhibitory effects on the production of fungal biomass and minimum inhibitory concentration were highly active even at lower concentration (2 µl/ml). On the basis of ability to test fungi *Mentha piperita* and *Cymbopogon citratus* was most inhibitory resulting at 95% reduction in the biomass was recorded against *Fusarium solani, Rhizopus microsporus* and *Cryptococcus neoformans.* The results confirmed that the highest antifungal activity of *Mentha piperita* and *Cymbopogon citratus* essential oil recorded here could be correlated to the presence of high level of oxygenated monoterpenes. *Mentha piperita* and *Cymbopogon citratus* essential oil (2-16µl /ml) significantly inhibited the growth of *Fusarium solani, Rhizopus microsporus* and *Cryptococcus neoformans* suggesting the potential value of *Cymbopogon citratus* essential oil for the treatment of various fungal diseases.

Keywords: essential oils, broth microdilution, minimum inhibitory concentration

Introduction
Fungi commonly cause superficial infections of the skin and mucous membranes. When they penetrate the tissues of an immunocompromised host, however, they can cause IFIs, which are associated with much greater morbidity and mortality. The fungal species most often associated with fatal diseases belong to the genera *Candida albicans, Aspergillus niger*, and *Cryptococcus neoformans*. *Candida* species are the fourth most common cause of nosocomial bloodstream infections (Karthaus *et al*., 2007) [5]. In the United States, *Candida albicans* causes most candidemias, followed by *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and, in fifth place, *Candida krusei* (Pfaller *et al*., 2007) [6].
C. albicans can cause serious infections of the oral mucosa, as well as disseminated infection in debilitated patients. Severe oropharyngeal candidiasis (OPC) afflicts many AIDS patients (Baccaglini *et al*., 2007) [1] and is a significant infection in cancer patients being treated with chemotherapy and/or radiotherapy (Ship *et al*., 2007) [7]. OPC is frequently the first clinical symptom recognized in human immunodeficiency virus-positive patients prior to the onset of overt AIDS (Ship *et al*., 2007) [7]. In cancer patients, the increased incidence of OPC results both from the debilitating effects of the cancer itself and from the immunosuppressive treatment for the cancer. Administration of broad-spectrum antibiotics for the management of bacterial infections in these patients may further predispose them to OPC (Soysa *et al*., 2008) [8]. Radiotherapy for oral cancer results in permanent damage to the salivary glands and frequently to lifelong xerostomia (Goldstein *et al.,* 2008) [4], another predisposing factor for OPC.
Mucormycosis are emerging infections that have high rates of morbidity and mortality. They show high resistance to antifungal agents, and there is a limited therapeutic arsenal currently available, therefore, there is a great need to give priority to testing therapeutic agents for the treatment of mucormycosis. Along this line, the use of essential oils and phytoconstituents has been emphasized as a new therapeutic approach.

Material and Methods
Collection of plant material
Fresh preparations of selected plants free from disease were collected from Mandla district (Madhya Pradesh.) in the month of September to March 2015. The plants were identified at Department of Post Graduate Studies and Research in Biological Sciences, Rani Durgavati University Jabalpur (M.P.). The leaves were washed thoroughly 2-3 times with running water and once with sterile distilled water, then air-dried on sterile blotter under shade.

Preparation of extracts
The plant material (500gm) was shade dried and ground to powder. Three portions (about 10 g each) of the same were taken separately in 100 mL each of methanol, hexane. After filtration, the solvents hexane and methanol extracts were removed in a rotary evaporator. The water extract was filtered through Whatman filter paper No. 1, concentrated and freeze-dried in a lyophilizer. Plants which showed activity were further extracted by soxhlet apparatus.

Soxhlet Extraction
Thoroughly washed dried leaves of the selected plants were dried in shade for 7 to 10 days and then powdered with the help of waring blender. 100 gram of shade-dried powder was filled in the thimble and extracted successively with hexane

and methanol, solvents in soxhlet extractor for 48h. The solvent extracts were concentrated under reduced pressure and preserved at 5^0C in air tight bottle until further use.

Extraction of essential oil

Air dried leaves of selected plants were chopped and ground into small pieces. The known amounts of processed samples were placed in a round bottom flask (1L) together with distilled water and hydro distillation was performed using Clevenger apparatus. Essential oil was collected, dehydrated using sodium sulphate and dispensed into dark bottles. Oil was stored at 4ºC until used.

Collection of Microorganisms

Fungi such as, *Trichophyton rubrum*, *Aspergillus niger*, *Candida albicans*, *Fusarium solani* and *Cryptococcus neoformans* were obtained from Microbial type culture collection IMTECH Chandigarh and *Rhizopus microsporus* collected from society for research diagnostics and treatment of fungal diseases SIHAM center Jabalpur and incubated at Sabouraud dextrose broth (SDB) 25 – 30^0C for 7 days.

Preparation of Inoculum

The fungal inoculums of *T rubrum, C neoformans, R. microsporus, A. niger* and *C.albicans* were prepared from 5 to 10 day old culture grown on Sabouraud Dextrose Agar medium. The petri dishes were flooded with 8 to 10 ml of distilled water and conidia were scraped using sterile spatula. The spore density of each fungus was adjusted with spectrophotometer (A_{595}nm) to obtain a final concentration of approximately 10^5 spores / ml.

Antifungal Activity

The antifungal activity was tested by disc diffusion method. The filter paper discs (5 mm in diameter) impregnated with 1mg/ml concentrations of the extracts were placed on test organism-seeded plates. Hexane and methanol were used to dissolve the extract and were completely evaporated before application on test organism-seeded plates. Blank disc impregnated with solvent methanol and hexane followed by drying was used as negative control. The activity was determined after 72 h incubation at 28°C. The diameters of the inhibition zones were measured.

Preparation of Sabouraud dextrose agar (SDA)

Peptone – 10g, Dextrose – 20g, Agar 20g, Distilled water–1000ml

Preparation of Sabouraud dextrose broth (SDB) –

Peptone – 10g, Dextrose – 20g, Distilled water – 1000ml

Assays for determination of antifungal activity

Effect of essential oils and plants extracts against test fungi was determined in terms of inhibition of biomass in liquid medium and Minimum Inhibitory Concentration (MIC) on solid medium as described below-

Disc diffusion assay

All the tests were performed according to Esteban *et al*., 2009. The inoculum was evenly spread on the surface of 10cm Petri dishes containing Sabouraud dextrose agar medium and exposed to air dry. Then, the antifungal disks were applied to the plates, after which the plates were incubated at 25°C for 5-10 days. After the colonies grew, the zones of inhibition around the disks were measured and recorded. Criteria of susceptibility and resistance of antifungal disks were measured. All tests were performed in duplicate.

Determination of MIC by agar dilution method

Minimum Inhibitory Concentration (MIC) of essential oils and plants extracts was determined by agar dilution assay. The agar plates were prepared using Potato Dextrose agar (PDA) (15 ml per petri dish) amended with various concentrations of plant essential oils (i.e. 2-16 µl/ml).These plates were inoculated with one ml cell suspension (106 cfu/ml) of *Fusarium solani, Rhizopus microsporus* and *Cryptococcus neoformans*. All the plates were incubated in triplicate for each concentration at 30°C for 48 h. Observation of the plates (fungal growth) was done at a time interval of 12 h up to 48 h of incubation. The MIC values were determined as the lowest concentration of essential oil preventing visible growth of *Fusarium solani, Rhizopus microsporus* and *Cryptococcus neoformans.*

Inhibition of biomass production

Method with slight modification was adapted. Briefly, 50 ml Sabouraud dextrose broth (SDB) Containing different concentrations of oils (2-32 µl\ml). was inoculated with 500 of freshly prepared spore suspension of test fungi. In the corresponding control an equal amount of distilled was added. Fluconazole in the concentration range of 2-32 µl\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 What man filter paper no.1. Mycelial yield was determined after drying the mycelial mat at 80°C for 24h and present loss in mycelial dry weight was calculated over control.

Broth microdilution method

Minimum fungicidal concentration (MFC) of essential oil was determined according to Broth Macro Dilution Assay. A range of essential oil concentrations (2-16 µl\ml) was prepared in Potato Dextrose broth (PDB) medium. Each flask was inoculated with 106 cfu/ml of the *Candida* strain. The flasks were incubated at 30°C, in an orbital shaking incubator (180 rpm) for 48 h. One ml of culture was taken from each flask (where growth was not observed) for serial dilution to make the inoculum of 106 cfu/ml and inoculated on PDA plates and incubated at 30°C for 48 h. The plates were observed and MFCs were determined.

Results

Plants extracts and essential oils were screened for antifungal susceptibility testing, presented in table (1to 9) and fig (1 to 3). All essential oils tested and their components exerted concentration dependent inhibitory effects on the production of fungal biomass and minimum inhibitory concentration and were highly active even at lower concentration (2 µl/ml). On the basis of ability to test fungi *Mentha piperita* and *Cymbopogon citratus* was most inhibitory resulting in 95%

reduction in the biomass was recorded against *Fusarium solani*, *Rhizopus microsporus* and *Cryptococcus neoformans.*

The present study shows that the plants extracts of *Cassia fistula* and *Annona squamosa* were quite effective and showed highest antifungal activity against *Fusarium solani*. The Plants extracts of *Jatropha curcas, Cuscuta reflexa* showed inhibitory effect against *Rhizopus microsporus* and *Cryptococcus neoformans.* It induced a significant growth inhibition zone in *Mentha piperita* at the lower concentration (2µl /ml) is 22 mm, 18 mm and 13 mm in *Fusarium solani*, *Rhizopus microsporus* and *Cryptococcus neoformans* and high at concentrations (8µl /ml) is 33 mm, 33 mm, and 22 mm.

The essential oil of *Cymbopogon nardus* showed no activity at low concentration (2µl /ml) and less effective at concentration (8µl/ml) is 15 mm, 20 mm and 11mm against *Fusarium solani*, *Rhizopus microsporus* and *Cryptococcus neoformans*.

The study showed that the plants extract of *Jatropha curcas* show highest antifungal activity 31 mm, 17 mm and 21 mm at concentration (16µl /ml) against *Rhizopus microsporus, Fusarium solani* and *Cryptococcus neoformans. Cuscuta reflexa* show highest antifungalactivity 24 mm, 16 mm and 8 mm at concentration (16µl /ml) against *Rhizopus microsporus, Fusarium solani* and *Cryptococcus neoformans. Cassia fistula* show highest antifungal activity 15 mm, 16 mm and 15 mm at concentration (16µl /ml) against *Rhizopus microsporus, Fusarium solani* and *Cryptococcus neoformans. Plumbago zeylanica* show highest antifungal activity 14 mm, 13 mm and 14 mm at concentration (16µl /ml) against *Rhizopus microsporus Fusarium solani* and *Cryptococcus neoformans. Annona squamosa* show highest antifungal activity 21 mm, 21 mm and 22 mm at concentration (16µl /ml) against *Rhizopus microsporus, Fusarium solani* and *Cryptococcus neoformans.*

MICs, MFCs of the essential oil of *Cymbopogon citratus* and *Mentha piperita*, it may be concluded that the antifungal activity of essential oils and plants extracts could be preliminarily investigated by the agar-well diffusion test for rapid screening. In the present study, *Cymbopogon citratus* and *Mentha piperita* essential oil showed better performance both in liquid as well as solid media.

The results confirmed that the highest antifungal activity of *Mentha piperita* and *Cymbopogen citratus* essential oil recorded here could be correlated to the presence of high level of oxygenated monoterpenes. Reported that the *Mentha piperita* and *Cymbopogon citratus* essential oil (2-16µl /ml) significantly inhibited the growth of *Fusarium solani* ,*Rhizopus microsporus* and *Cryptococcus neoformans* suggesting the potential value of *Cymbopogon citratus* essential oil for the treatment of various fungal diseases.

Table 1: Activity of methanolic plant extracts tested against *Fusariusm solani*-

Plant Extracts (Botanical name)	Common name	Inhibition Zone in Diameter (mm) at different Concentration			
		2µg/ml	4µg/ml	8µg/ml	16µg/ml
Jatropha curcas	Chandrajote	-	4	9	17
Cuscuta reflexa	Amar bel	4	7	12	16
Cassia fistula	Amaltash	5	8	15	24
Plumbago zeylanica	Chittavar	-	4	9	13
Annona squamosa	Sitaphal	3	9	16	21
Fluconazole (antifungal)		8	12	18	28

Table 2: Activity of methanolic plant extracts tested against *Rhizopus microsporus*

Plant Extracts (Botanical name)	Common name	Inhibition Zone in Diameter (mm) at different Concentration			
		2µg/ml	4µg/ml	8µg/ml	16µg/ml
Jatropha curcas	Chandrajote	-	13	19	31
Cuscuta reflexa	Amar bel	-	-	18	24
Cassia fistula	Amaltash	3	7	11	15
Plumbago zeylanica	Chittavar	4	6	10	14
Annona squamosa	Sitaphal	-	8	16	21
Fluconazole (antifungal)		7	15	22	34

Table 3: Activity of methanolic plant extracts tested against *Cryptococcus neoformans*

Plants Extracts	Common Name	Inhibition Zone in Diameter (mm) at different Concentration			
(Botanical name)		2µg/ml	4µg/ml	8µg/ml	16µg/ml
Jatropha curcas	Chandrajote	6	11	16	21
Cuscuta reflexa	Amar bel	-	-	-	8
Cassia fistula	Amaltash	2	5	7	15
Plumbago zeylanica	Chittavar	-	6	10	14
Annona squamosa	Sitaphal	-	-	14	22
Fluconazole (antifungal)		12	21	28	39

Table 4: Activity of essential oils against *Fusarium solani* at different concentration

Essential Oils	Inhibition Zone in Diameter (mm) at different Concentration			
	2µl/ml	4µl/ml	8µl/ml	16µl/ml
Mentha piperita	22	16	33	21
Cymbopogon citratus	18	24	26	19
Cymbopogon nardus	-	8	15	11
Fluconazole (antifungal)	24	28	36	37

Table 5: Activity of essential oils against *Rhizopus microsporus* at different concentration

Essential Oils	Inhibition Zone in Diameter (mm) at different Concentration			
	2µl/ml	4µl/ml	8µl/ml	16µl/ml
Mentha piperita	18	22	33	26
Cymbopogon citratus	4	7	11	13
Cymbopogon nardus	-	16	20	22
Fluconazole (antifungal)	21	27	37	31

Table 6: Activity of essential oils against*Cryptococcus neoformans* at different concentration

Essential Oils	Inhibition Zone in Diameter (mm) at different Concentration			
	2µl/ml	4µl/ml	8µl/ml	16µl/ml
Mentha piperita	13	19	22	24
Cymbopogon citratus	8	24	31	27
Cymbopogon nardus	-	5	11	9
Fluconazole	21	30	35	39

Table 7: Concentration dependent inhibition of *Mentha piperita* oil on biomass production

Concentration (µl/ml)	Inhibition in biomass production		
	Fusarium solani	***Rhizopus microsporus***	***Cryptococcus neoformans***
2	2.5	0.7	0.2
4	2.8	1.3	0.9
8	3.4	1.7	1.5
16	2.8	2.4	2.8
32	3.0	2.9	3.4

Table 8: Concentration dependent inhibition of biomass production of *Cymbopogon citratus*

Concentration (µl/ml)	Inhibition in biomass production		
	Fusarium solani	***Rhizopus microsporus***	***Cryptococcus neoformans***
2	0.6	0.4	0.1
4	1.0	1.2	0.6
8	1.6	1.9	1.2
16	2.4	2.2	1.9
32	2.9	3.1	2.6

Table 9: Concentration dependent inhibition of biomass production of *Cymbopogon nardus* oil

Different Concentration (µl/ml)	Inhibition in biomass production		
	Fusarium solani	***Rhizopus microsporus***	***Cryptococcus neoformans***
2	0.3	NI	0.1
4	0.8	0.2	0.5
8	1.2	0.9	1.1
16	1.8	1.4	1.6
32	2.0	1.7	2.0

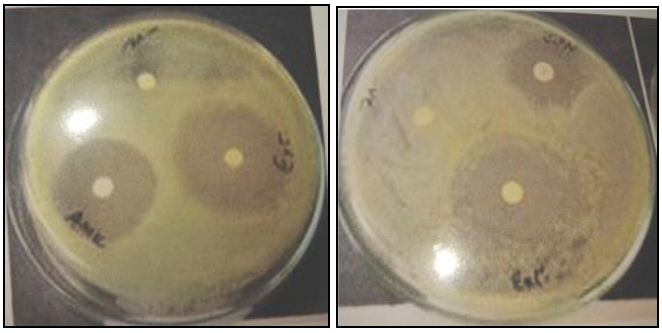

Fig 1: Activity of plant extracts against *Rhizopus Microsporus*

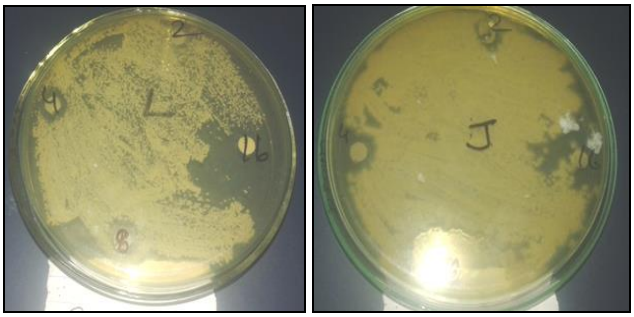

Fig 2: Activity of plant extracts against *Fusarium solani*

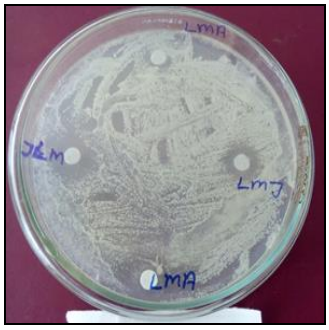

Fig 3: Activity of *Mentha piperita* against *Cryptococcus neoformans*

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