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RESEARCH ARTICLE

SCREENING OF *IN VITRO* ANTIFUNGAL ACTIVITY OF SOME INDIAN MEDICINAL PLANTS AGAINST *CANDIDA ALBICANS* AND *CRYPTOCOCCUS NEOFORMANS*

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ABSTRACT

The present investigation evaluates the anti-*Candida albicans* and anti-*Cryptococcus neoformans* activity of different extracts of thirty plants belonging to 16 families. Standard agar disc diffusion method was employed for determination of antifungal activity and standard two fold broth dilution methods was used for determination of the minimal inhibitory concentration (MIC) and the minimal fungicidal concentration (MFC). The test strain *Cryptococcus neoformans* used in the present study exhibited complete resistance to antifungal drugs clotrimazole and itriconozole. The result of the present study revealed that extracts of *Acacia catechu, Acacia ferruginea, Adenanthera pavonia, Albizia amara, Caesalpinia coriaria, Decalepis hamiltonii, Holoptelea integrifolia, Prosopis juliflora, Samanea saman* and *Solanum indicum* showed promising antifungal activity. The best antifungal activity was observed in toluene extract of *A. pavonia* and *C. coriaria,* chloroform extract of *A. amara* and methanol extract of *P. juliflora* and *S. saman* with zone of inhibition ranging from 9.2mm to 18.9mm against *C. albicans* and 10.5mm to 24.5mm against *C. neoformans* at 1mg/disc. The MIC and MFC values ranging from 0.125mg/ml to 0.5mg/ml and 1mg/ml to >2mg/ml against *C. albicans* and 0.0312mg/ml to 0.25mg/ml and 0.5mg/ml to 2mg/ml against *C. neoformans* respectively. The subsequent fractionation and phytochemical analysis confirmed that alkaloid fraction of *A. amara, P. juliflora* and *S. saman* and steroid fraction of *A. pavonia* and *C. coriaria* responsible for antifungal activity. Further investigations are required to identify active compounds responsible for activity and toxicological study using animal models before clinical application.

INTRODUCTION

During the last two decades, fungal infections have emerged as a major cause of morbidity and mortality in immunocompromised persons, in particular patients with acquired immunodeficiency syndrome (AIDS), patients undergoing cancer therapy, patients treated with immunosuppressive therapy for organ transplantation, patients in intensive care unit and extremely aged persons (Bajpai *et al.,* 2010; Jafari *et al.,* 2011; Saad *et al.,* 2010; Sortino *et al.,* 2011). *Candida albicans* (*C. albicans*) and *Cryptococcus neoformans* (*C. neoformans*) are opportunistic fungi, which are responsible for a variety of life threatening human diseases (Lee and Lee 2002). *C. albicans* and *C. neoformans* infections have been increased dramatically over the last few years, due to the explosion of AIDS and cancer epidemics as well as increasing number of solid organ transplant recipients around the world (Basha *et al.,* 2010; Jafari *et al.,* 2011). The current drugs available against *C. albicans* and *C. neoformans* are based on polyenes (amphotericin B), triazoles (itraconazole, fluconazole, voriconazole, ketoconazole and posaconazole) or echinocandins (caspofungin, micafungin, terbinafine and anidulafungin) (Abad *et al.,* 2007; Panacek *et al.,* 2009). However, administration of these antifungals are limited by a number of factors such as low potency, poor solubility, nephrotoxicity, hepatotoxicity, neurotoxicity and most importantly emergence and continuously increasing resistant strains of *C. albicans* and *C. neoformans* (Chee 2002; Jafari *et al.,* 2011; Khan and Ahmad 2011). The development of resistance in known fungal pathogens and emergence of new fungal pathogens intrinsically resistant to the currently available classical antifungal drugs demonstrate the urgent need for new, alternative, safe and more effective antifungal agents (Naeini *et al.,* 2009; Panacek *et al.,* 2009; Sati and Joshi 2011). The use of natural products in disease prevention and control as well as drug development and discovery has received increased attention in recent years (Jazari *et al.,* 2011; Sati and Joshi 2011). Plants which are traditionally used in the treatment of fungal infections or ailments could be a good source for new, safe and biodegradable antifungal drug discovery (Basha *et al.,* 2010). The recent reports from different countries have been proved such efficacy of botanicals in various drug discovery programmes (Amber *et al.,* 2010; Ouattara *et al.,* 2008; Thenmozhi and Rajeshwari 2010). But only 5-15% of the higher plants have been

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systematically investigated for the presence of bioactive compounds worldwide (Newman *et al.,* 2000). Many plants used in Indian traditional herbal medicine, in particularly ayurveda, yoga, unani, siddha, homeopathy and naturopathy have the potential to provide pharmacologically active natural products as a source for new antifungal drug discovery. However only a small percentage (<1%) of plants used in Indian traditional medicine have been scientifically and systematically evaluated for their clinical application for management of human pathogenic fungi (Sati and Joshi 2011; Verma and Dubey 1999). In view of these, as a part of our ongoing projects on the detection of antifungal and antibacterial compounds from higher plants, the authors *in-vitro* screened a large number of traditional medicinal plants for antimicrobial activity against some human pathogenic bacteria and fungi, with the ultimate aim of developing herbal formulation for disease management. The part of our ongoing projects results on antifungal activity of some plants extracts against *C. albicans* and *C. neoformans* are presented in this paper.

MATERIALS AND METHODS

Plant materials

Thirty fresh plant materials (Table-1), free from disease were collected from southern part of Karnataka (India), washed thoroughly 2-3 times with sterile distilled water, shade dried at room temperature (25±2 ^{0}C) for 15 days in the dark chamber, powdered and used for extraction. Voucher specimens were identified and deposited at the Herbarium of the Department of Studies in Microbiology, Bangalore University, Bangalore under No. BUB001 to BUB031.

Preparation of aqueous extract

Fifty grams of each, plant material was macerated separately with 250 ml sterile distilled water and centrifuged at 4000 g for 30 min. The supernatant was filtered through Whatman No. 1 filter paper and concentrated separately under reduced pressure using rotary flash evaporator. After complete evaporation of the water each of these extracts were kept in air-tight containers at 4 ^{0}C until tested. The dried crude plant extracts were re-suspended in DMSO (Dimethylsulphoxide) to a final concentration of 100 mg/ml and filtered through 0.45µm membrane filter for sterilization and subjected to antifungal activity assay at 2 mg/disc (Thippeswamy *et al.,* 2011).

Preparation of Solvent extract

Fifty grams of each, shade dried powder of *Adenanthera pavonia*, *Albizia amara, Caesalpinia coriaria, Prosopis juliflora* and *Samanea saman* were filled in the thimble separately and extracted successively with 200 ml of petroleum ether, toluene, chloroform, methanol and ethanol using a soxhlet extractor until colourless extract obtained on the top of the extractor. Each of the solvents extracts were concentrated separately under reduced pressure using rotary flash evaporator (Mohana *et al.,* 2009). The concentrated extracts were subsequently dried at room temperature under a steam of cold air and kept in air-tight containers at 4 ^{0}C until tested. The dried organic plant extracts were re-suspended in DMSO to a final concentration of 50 mg/ml and filtered through 0.45µm membrane filter for sterilization and then serial two-fold dilution were made in a concentration range from 50mg/ml to 0.09mg/ml and subjected to antifungal activity assay at 1mg/disc, 0.5mg/disc, 0.25mg/disc, 0.125mg/disc, 0.0625mg/disc, 0.0312mg/disc, 0.0156mg/disc, 0.0078mg/disc, 0.0039mg/disc and 0.0019mg/disc.

Separation of the active fraction

The toluene extract of *A. pavonia* and *C. coriaria,* chloroform extract of *A. amara* and methanol extract of *P. juliflora* and *S. saman* which are showed highest anti-Candidal and anti-Cryptococcal activity were further fractionated into different fractions following the procedure of Harborne (1998). All the fractions were dried under reduced pressure, dissolved in DMSO and subjected to antifungal activity assay at 50mcg.

Organisms used

The human pathogenic fungi *Candida albicans* (NCIM 3471) and *Cryptococcus neoformans* (NCIM 3541) were purchased from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India and these cultures were grown and maintained on MGYP medium (Malt extract-0.3 gm, Glucose-1.0 gm, Yeast extract-0.3gm, Peptone-0.5gm and distilled water-100 ml), 48 h old culture served as test fungi for antifungal activity assay.

Antifungal activity assay

Standard agar disc diffusion method was employed for the determination of antifungal activity (Bajpai *et al.,* 2010). 100µl of standardized inoculum containing 10^7 CFU/ml of fungal suspension was uniformly spread on MGYP agar plate and allowed to dry for 10 min. Four layer six mm sterilized paper discs (Whatman No. 1) impregnated separately with desired different concentrations of each extracts and fractions were placed on the surface of pre-inoculated MGYP test plates (Chee 2002). The plates were kept at 4 ^{0}C for 1 h. for diffusion of extract, thereafter the plates were incubated at 30 ^{0}C for 48 h. The disc devoid of extract and the presence of same amount of DMSO served as negative control. The antifungal disc contains Amphotericin-B (100mcg), Clotrimazole (10mcg), Itriconozole (10mcg) and Nystatin (100mcg) obtained from Hi-media Pvt. Ltd. Mumbai, India, were used as positive control. After incubation, zone of inhibition if any around the disc was measured in mm (millimetre). Each assay in this experiment was replicated four times.

Determination of MIC and MFC by broth dilution method

A standard two-fold broth dilution technique was employed to determine the MIC and MFC value (Unlu *et al.,* 2010). Twenty milligram of toluene extract of *A. pavonia* and *C. coriaria,* chloroform extract of *A. amara* and methanol extract of *P. juliflora* and *S. saman* which are showed highest activity against *C. albicans* and *C. neoformans* were incorporated separately into sterile test tubes containing 10 ml MGYP broth to obtain a concentration of 2mg/ml. Then serial two fold dilutions were made in concentration range from 2mg/ml to 0.0039mg/ml. A 100µl of standardized suspension of each test organism (10^7 CFU/ml) was transferred to each tube. MGYP

broth, devoid of extract served as control. The tubes were incubated at 30 ^{0}C for 48 h. Afterwards 50µl of incubated broth was taken from all the tubes were sub-cultured on treatment free MGYP agar plates and incubate for 72 h at 30 ^{0}C. The MIC is defined as the lowest concentration of the extract to inhibit fungal growth; whereas MFC was defined as the lowest concentration of the extract completely prevents fungal growth on extract free MGYP agar medium.

RESULTS AND DISCUSSION

The present study was conducted to investigate the anti-*Candida albicans* and anti-*Cryptococcus neoformans* activity of different extracts of 30 plants belonging to 16 families. The medicinal plants chosen based on Indian herbal traditional usage. *In vitro* antifungal effects are reported as zone of inhibition. The susceptibility of *C. albicans* and *C. neoformans* to aqueous extract of 30 plants were evaluated by disc diffusion method and presented in (Table 1). Tukey HSD statistical data analysis revealed that aqueous extract of *A. catechu, A. ferruginea, A. pavonia, A. amara, C. coriaria, D. hamiltonii, H. integrifolia*, *P. juliflora, S. saman* and *S. indicum* showed promising antifungal activity against *C. albicans* and *C. neoformans* at 2mg/disc. Among these the best antifungal activity was observed in the extract of *A. pavonia, A. amara, C. coriaria, , P. juliflora* and *S. saman* with zone of inhibition ranging from 9.1mm to 15.5mm and 10.0mm to 19.6mm against *C. albicans* and *C. neoformans* respectively at 2mg/disc. The modest antifungal activity was observed in extract of *A. catechu, D. hamiltonii* and *S. indicum* with zone of inhibition ranging from 7.9mm to 9.1mm against *C. albicans* and 8.5mm to 9.5mm against *C. neoformans* at same concentration. The extracts *viz., A. pavonia, A. amara, C. coriaria, P. juliflora* and *S. saman* showed strong activity in aqueous system were subjected to successive solvent extraction.

The inhibitory activity of the five different successive solvent extracts of *A. pavonia, A. amara, C. coriaria, P. juliflora and S. saman* against *C. albicans* and *C. neoformans* is presented in (Table 2). Among the five different extracts tested in each plants, the best antifungal activity was observed in methanol extract of *P. juliflora* (zone of inhibition (z.i.) 18.9mm & 24.5mm (Fig. 1.)) and *S. saman* (z.i. 15.6mm (Fig. 2.) & 22.3mm), chloroform extract of *A. amara* (z.i 16.6mm & 22.4mm), toluene extract of *C. coriaria* (z.i 9.6mm & 11.5mm) and *A. pavonia* (z.i 9.2mm & 10.5mm) against *C. albicans* and *C. neoformans* respectively at 1mg/disc. The present study clearly indicates that methanol, chloroform and toluene are the most effective solvent for extracting antifungal compounds from *P. juliflora* and *S. saman, A. amara*, *C. coriaria* and *A. pavonia* respectively. As the concentration of the extract loaded on the disc increased, the diameter of the zone of inhibition around the paper disc also increased (Table-3). Further phytochemical analysis and antifungal activity guided assay revealed that alkaloid fraction of *A. amara, P. juliflora* and *S. saman* and steroid fraction of *A. pavonia* and *C. coriaria* showed antifungal activity with zone of inhibition ranging from 10.3mm to 19.6mm and 11.6mm to 21.1mm against *C. albicans* and *C. neoformans* respectively at 50mcg concentration (Table 3). Comparative efficacy of the extracts, with synthetic fungicides viz., Amphotericin-B (100mcg), Clotrimazole (10mcg), Itriconozole (10mcg) and Nystatin

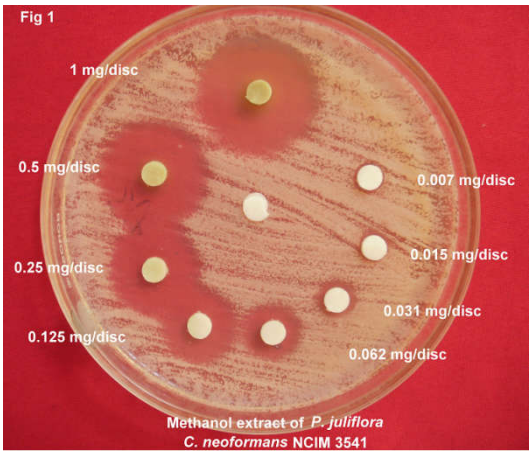

Fig. 1: Antifungal activity of methanol extract of *P. juliflora* against *C. neoformans* at different concentrations

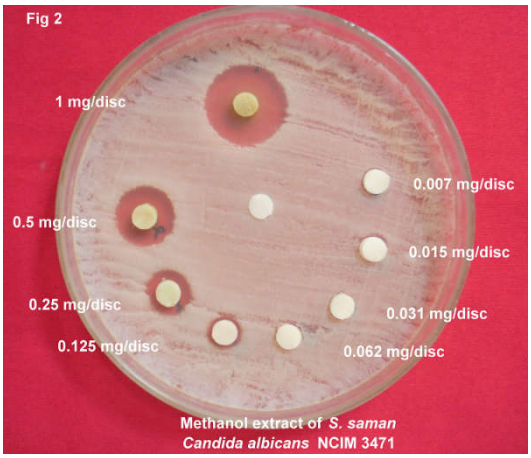

Fig. 2: Antifungal activity of methanol extract of *S. saman* against *C. albicans* at different concentrations

(100mcg) against *C. albicans and C. neoformans* revealed that Nystatin shows highest antifungal activity with zone of inhibition 27.0mm against *C. albicans* and 17.0mm against *C. neoformans.* The fungicide Clotrimazole and Itriconozole were not effective against *C. neoformans* (Table-3). The results of the present investigation demonstrate that *C. neoformans* is resistant to Clotrimazole and Itriconozole, but this fungus is strongly inhibited by extracts of *A. pavonia, A. amara, C. coriaria, P. juliflora and S. saman.* The inhibition zone in some case reached more than the twice the value of the standard fungicides used as positive control.

The inhibitory activity and lethal activities, as defined by MIC and MFC values of extracts of *A. pavonia, A. amara, C. coriaria, P. juliflora and S. saman* against *C. albicans and C. neoformans* were presented in the (Table-4). The MIC value of the extracts ranged from 0.125mg/ml to 0.5mg/ml against *C. albicans* and 0.0312mg/ml to 0.25mg/ml against *C. neoformans*, while the MFC value ranged from 1mg/ml to >2mg/ml against *C. albicans* and 0.5mg/ml to 2mg/ml against *C. neoformans*. The lowest MIC value was observed in methanol extract of *P. juliflora* (0.125mg/ml and 0.0312mg/ml), followed by chloroform extract of *A. amara* (0.125mg/ml and 0.0625mg/ml) and methanol extract of *S. saman* (0.5mg/ml and 0.125mg/ml) against *C. albicans and C. neoformans* respectively. However in determining the MFC, some of the extracts with good MIC values did not have a

Table 1: Screening of *in-vitro* antifungal activity of aqueous extract of some Indian medicinal plants against *C. albicans* and *C. neoformans*

Plant Name	Family	Antifungal activity	
		C. albicans	*C. neoformans*
Acacia catechu	Mimosaceae	+	++
Acacia chundra	Fabaceae	-	-
Acacia ferruginea	Mimosaceae	+	+
Adenanthera pavonia	Mimosaceae	++	+++
Albizia amara	Fabaceae	++++	++++
Anageissus lotifolia	Combretaceae	-	-
Asperagus racemosus	Liliaceae	-	-
Bauhinia acuminata	Caesalpiniaceae	-	-
Caesalpinia coriaria	Caesalpiniaceae	+++	++++
Calatropis gigantea	Apocyanceae	-	-
Carissa carandas	Apocyanceae	-	-
Cassia alata	Fabaceae	-	-
Cassia siamea	Fabaceae	-	-
Cassia tora	Fabaceae	-	-
Coleus amboinicus	Lamiaceae	-	-
Decalepis hamiltonii	Apocyanceae	+	++
Dodonaea viscosa	Sapindaceae	-	-
Ficus bengalensis	Moraceae	-	-
Ficus religiosa	Moraceae	-	-
Gliricidia sepium	Fabaceae	-	-
Holoptelea integrifolia	Ulmaceae	+	+
Prosopis juliflora	Fabaceae	++++	++++
Ricinus communis	Euphorbiaceae	-	-
Saccharum spontaneum	Poaceae	-	-
Samanea saman	Fabaceae	++++	++++
Sesbania grandiflora	Fabaceae	-	-
Solanum indicum	Solanaceae	++	++
Spilanthes paniculata	Asteraceae	-	-
Thespesia populnea	Malvaceae	-	-
Vitex nigundo	Lamiaceae	-	-

Data given are mean of four replicates; Leaves are used as test material; No activity was observed around control (DMSO impregnated) disc; The notations were used to estimate the zone of inhibition (ZI) with *C. albicans* and *C. neoformans* as follows: **-**, no antifungal activity ZI **≤7.5 mm**; **+**, scantly antifungal activity ZI **7.6mm to 8.5mm**; **++**, modest antifungal activity ZI **8.6mm to 9.5mm**; **+++**, strong antifungal activity ZI **9.6mm to 10.5 mm**; **++++**, very strong antifungal activity ZI **≥10.6 mm**.

Table 2: Antifungal activity of different solvent extracts of some selected plants against *C. albicans* and *C. neoformans*

Plant samples	Organisms	Zone of Inhibition in mm				
		Petroleum ether extract	Toluene extract	Chloroform extract	Methanol extract	Ethanol extract
A. pavonia	*C. albicans*	7.2±0.5	9.2±0.5	-	-	8.1±0.8
	C. neoformans	-	10.5±0.7	-	-	9.0±0.5
A. amara	*C. albicans*	-	11.2±0.6	16.6±0.5	14.0±0.3	12.5±0.5
	C. neoformans	-	15.2±0.5	22.4±0.7	19.2±0.4	18.0±0.4
C. coriaria	*C. albicans*	8.0±0.4	9.6±0.4	8.0±0.5	-	-
	C. neoformans	-	11.5±0.8	9.2±0.6	-	8.1±0.5
P. juliflora	*C. albicans*	-	-	12.0±0.5	18.9±0.5	-
	C. neoformans	-	-	16.0±0.4	24.5±0.5	-
S. saman	*C. albicans*	-	-	9.0±0.5	15.6±0.6	10.0±0.6
	C. neoformans	-	6.0±0.5	15.0±0.2	22.3±0.8	17.0±0.5

Data given are the mean of four replicates ± standard error, $p < 0.001$, No activity was observed around control (DMSO impregnated) disc.

corresponding good MFC suggesting such extracts have strong fungistatic activity than fungicidal activity. In the recent years there are several reports are available on the antifungal activity plant extract against *C. albicans and C. neoformans* (Amber *et al.,* 2010; Augustine *et al.,* 2005; Sati and Joshi 2011). None of the earlier reports have demonstrated the antifungal potency of *A. catechu, A. ferruginea, A. pavonia, A. amara, C. coriaria, P. juliflora, H. integrifolia*, *S. saman* and *S. indicum* against *C. neoformans*. In the present investigation the anti-cryptococcal activity of these plants has been demonstrated for the first time. Even though reports are available on the anti-candidal activity of *S. saman* (Ferdous *et al.,* 2010), but they were not evaluated MIC and MFC value. In the present investigation, for the first time demonstrate the anti-candidal activity of *A. pavonia, A. amara, C. coriaria, P. juliflora and S. saman* along with MIC and MFC value. Further investigations such as isolation and characterization of the active compounds responsible for antifungal activity and toxicological experiments using animal models are required before clinical application.

In the past 20 years, the frequency of the systemic fungal infections such as candidiasis and cryptococcosis has increased dramatically, due to the explosion of AIDS and cancer epidemic around the world (Ouattara *et al.,* 2008). Candidiasis is the fourth most common infection in immunocompromised persons (Augustine *et al.,* 2005). Among the different types of drugs prevailing in the market, antifungal antibiotics are very small (Sortino *et al.,* 2011). At present there are four classes of systemic antifungal compounds such as polyene antibiotics, azole derivatives, allylamines/thiocarbamates and fluopyrimidines are available

Table 3: Comparative efficacy of standard antifungal drugs, organic extract and active fraction of some medicinal plants against *C. albicans* and *C. neoformans*

Classical antifungal drugs and plants	Plants extract and fraction	Concentration	Zone of Inhibition in mm	
			C. albicans	*C. neoformans*
Amphotericin-B	-	100mcg	10.0±0.2	10.0±0.1
Clotrimazole	-	10mcg	17.0±0.4	-
Itriconozole	-	10mcg	12.0±0.2	-
Nystatin	-	100mcg	27.0±0.3	20.0±0.2
A. pavonia	Toluene	1mg	9.2±0.5	10.5±0.7
		0.5mg	7.7±0.6	8.4±0.7
		0.250mg	-	7.6±0.6
		0.125mg	-	-
	Steroid	50mcg	10.3±0.8	11.6±0.5
A. amara	Chloroform	1mg	16.6±0.5	22.4±0.7
		0.5mg	11.9±0.5	14.6±0.5
		0.250mg	9.5±0.8	11.0±0.7
		0.125mg	7.8±0.7	9.5±0.5
	Alkaloid	50mcg	15.3±0.5	16.73±0.4
C. coriaria	Toluene	1mg	9.6±0.4	11.5±0.8
		0.5mg	7.9±0.4	9.6±0.7
		0.250mg	-	8.1±0.3
		0.125mg	-	-
	Steroid	50mcg	11.3±0.6	12.6±0.4
P. juliflora	Methanol	1mg	18.9±0.5	24.5±0.5
		0.5mg	15.2±0.6	22.0±0.8
		0.250mg	11.0±0.2	19.0±0.5
		0.125mg	8.8±0.6	13.9±0.4
	Alkaloid	50mcg	19.6±0.6	21.1±0.5
S. saman	Methanol	1mg	15.6±0.6	22.3±0.8
		0.5mg	11.6±0.5	15.0±0.5
		0.250mg	9.4±0.6	12.2±0.3
		0.125mg	7.6±0.6	9.2±0.4
	Alkaloid	50mcg	15.6±0.4	17.0±0.5

Data given are the mean of four replicates ± standard error, $p < 0.001$, No activity was observed around control (DMSO impregnated) disc.

Table 4: Determination of MIC and MFC value of some plants extract against *C. albicans* and *C. neoformans*

Plant samples	Extract used	Organisms	MIC mg/ml	MFC mg/ml
Adenanthera pavonia	Toluene extract	*C. albicans*	0.5	>2
		C. neoformans	0.250	2
Albizia amara	Chloroform extract	*C. albicans*	0.125	2
		C. neoformans	0.062	1
Caesalpinia coriaria	Toluene extract	*C. albicans*	0.5	>2
		C. neoformans	0.125	2
Prosopis juliflora	Methanol extract	*C. albicans*	0.125	1
		C. neoformans	0.031	0.5
Samanea saman	Methanol extract	*C. albicans*	0.5	1
		C. neoformans	0.125	0.5

Data given are the mean of four replicates, $p < 0.001$. No growth inhibition was observed in control tube containing DMSO.

in clinical use (Sati and Joshi 2011). But, none of these existing systemic antifungals satisfies the medical needs completely, because of weaknesses in spectrum, potency, pharmacokinetic properties. Thus, the discovery of new, safe and more effective antifungal drugs is a major challenge to the pharmaceutical industry (Khan and Ahmad 2011). The use of natural products in disease prevention and control as well as in drug development has received increased attention in recent time (Verma and Dubey 1999). Approximately 25% of globally prescribed drugs are obtained from plants (Newman *et al.,* 2000). In addition, plants can also serve as important sources of new drugs, new drug leads and new chemical entities. Even-though, WHO (World Health Organization) is encouraging, promoting, and facilitating the effective use of herbal medicine for health programs, only 1% of the total known medicinal plants species is acknowledged to therapeutic value for human health benefits (Newman *et al.,* 2000; Verma and Dubey 1999). However a large percentage of the estimated 3,50,000 plant species on earth is yet to be investigated for their pharmacological and phytochemical potential (Lee and Lee 2010). Considering these the present investigation is an important step in developing plant based drugs which are eco-friendly for the management of the pathogenic fungi.

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