Int.J.Curr.Microbiol.App.Sci (2018) *7*(11): 3700-3711

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 11 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.711.426

Antifungal Activity of *Cassia* sps against Clinically Isolated Antibiotic Resistant *Cryptococcus neoformans*

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Keywords

Cryptococcosis, *Cryptococcus neoformans*, encapsulated basidiomycetous yeast

Article Info

Accepted:
04 October 2018
Available Online:
10 November 2018

A B S T R A C T

A total of 100 clinical samples were collected from microbiology laboratories in and around Chennai, Tamil Nadu, India for screening the *Cryptococcus neoformans* from patients. Among the 100 clinical samples 48 isolates showed *Cryptococcus neoformans* growth. The antibiotic sensitivity pattern of *Cryptococcus neoformans* isolates from the clinical specimens of patients showed highly resistant to antibiotics. The leaves of plant species, *Cassia angustifolia, Cassia albus, Cassia wislizenii* and *Cassia tora* were collected and subjected to ethanolic extraction. The ethanolic leaves extracts were tested alone against antibiotic resistant *Cryptococcus neoformans* isolated from the clinical specimens of patients. Voriconazole has been used as standard antibiotic reference. The antimicrobial activity of individual ethanolic plant extract was higher in *Cassia tora* than the other extracts tested against antibiotic resistant *Cryptococcus neoformans* isolated from the clinical samples of patients. *Cassia tora* extract exhibited inhibition zone of 2.0 and 2.1 cm against clinically isolated antibiotic resistant *Cryptococcus neoformans* and Standard strains of *Cryptococcus neoformans*. There is a scope to use ethanolic extracts of the leaves of *Cassia tora* against antibiotic resistant *Cryptococcus neoformans* isolated from clinical specimens of patients.

Introduction

Cryptococcosis is a worldwide opportunistic mycosis whose etiologic agent, *Cryptococcus neoformans*, is an encapsulated basidiomycetous yeast that has been classified into two varieties and five serotypes: *C. neoformans* var. *neoformans* (serotypes A, D and AD) and *C. neoformans* var. *gattii* (serotypes B and C) (Pinner *et al.*, 1995; Spitzer *et al.*, 1993). In 1999, Franzot *et al.*, (1999) based on molecular studies, proposed a separate variety for serotype A as *C. neoformans* var. *grubii* (Kwon-Chung and Bennett, 1992), however the new variety is not completely accepted. The two or three varieties of *C. neoformans* isolated from patients differ in certain aspects, among

which, geographic distribution is mentioned frequently (Williamson, 1994). Kwon-Chung (2002) showed that *C. neoformans* has two basidiomycetous teleomorphs, *Filobasidiella neoformans* var. *neoformans* and *F. neoformans* var. *bacillispora* (Chasakes and Tyndall, 1975). Alpha isolates are the most frequent mating types reported among clinical isolates (Staib, 1962).

Cryptococcal infections have increased dramatically over the last years. This high incidence can be due in large part to the explosion of acquired immune deficiency syndrome (AIDS) epidemic around the world and the use of more potent immunosuppressive agents by increasing numbers of solid organ transplant recipients (Mitchell and Perfect, 1995; De Bedout *et al.,* 1999).

Invasive fungal diseases are increasing and significant cause of mortality and morbidity in the immunocompromised patients. Many immune system defects may cause increased risk for these opportunistic fungal infections, but the major predisposing factors are neutropenia and AIDS (Emmanue I. Odongo-Aginya, 2000). Most important fungal pathogens in these settings are *Candida albicans* and some other candida species, *Cryptococcus neoformans* and some other rare agents (Ajello *et al.,* 1998).

C. neoformans has been divided into two varieties, *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* (Ikeda *et al.,* 1982; Bennett *et al.,* 1997). These two varieties of *C. neoformans* are easily differentiated by their biochemical properties, and have distinctive serotypes based on the antigenic composition of those capsular polysaccharides, which play an important role in pathogenicity (Bennet *et al.,* 1978; Baro *et al.,* 1998). *C. neoformans* var. *neoformans* corresponds to serotypes A, D, and AD, whereas *C. neoformans* var. *gattii* corresponds to serotypes B and C (Bennet *et al.,* 1977; Kwon-Chung *et al.,* 1982). Differences between the two varieties with regard to pathogenicity and geographical distribution have been documented (Kabasawa *et al.,* 1991; Baro *et al.,* 1998).

C. neoformans var. *neoformans* has a worldwide distribution and has been associated with a variety of environmental sources, in particular, bird excreta and decaying wood (Ruiz *et al.,* 1981). The most common isolate responsible for human infection is *C. neoformans* var. *neoformans* serotype A (Ellis and D.H., 1987).

Cryptococcus reported to be isolateed from AIDS patients has almost exclusively been of serotype A, even in areas where serotypes B or C are isolated more frequently in the general population. *C. neoformans* var. *gattii* has a more restricted global distribution occurring in tropical and subtropical areas (Kwon- Chung *et al.,* 1984), and plant debris associated with a number of *Eucalyptus species* (Ellis *et al.,* 1990; Pfeiffer *et al.,* 1992). These trees, native to Australia, are its natural habitat (Halliday *et al.,* 1999).

Despite the increasing number of studies on the epidemiology of *C. neoformans*, the role of these trees and the nature of infectious propagule are not well understood. However, identification of the two varieties of *C. neoformans* isolated from clinical and environmental sources provides useful information for epidmiological and ecological investigations.

Cryptococcus neoformans is a ubiquitous organism that can cause acute, subacute, or chronic systemic disease in humans. Clinical infection in humans usually involves the pulmonary and central venous systems. Osseous involvement occurs in 5-10% of patients with disseminated cryptococcosis.1

Extrapulmonary Cryptococcosis is typically chronic, with alternating periods of remission and exacerbation for as long as 16-20 years. 2 Hematogenous spread from pulmonary infection is the most likely route. Spread from a site of latent infection in a lymph node or direct spread from the skin is another possibility.1 Isolated cryptococcal osteomyelitis without involvement of organic systems other than osseous structures can occur but is rare.

However, most cases show pathological involvement of the other systemic organisms. 3 Most cases of cryptococcal osteomyelitis occur in immunocompromised patients. Cryptococcal osteomyelitis with or without other sites of infection in immunocompetent patients is less frequent.

The present study aims to identify the antibiotic resistant *Cryptococcus neoformans* from the clinical specimens and to find the new therapeutic compounds of medical importance from the higher plants. The main objectives of present study includes to find out the most predominant yeast in clinical specimens. To isolate and identify *Cryptococcus neoformans* from the clinical samples and check the antibiotic sensitivity pattern of *Cryptococcus neoformans* isolates from the clinical specimens of patients. To screening the antibiotic resistant *Cryptococcus neoformans* from clinical isolates and select the plant species with antimicrobial activity which are locally available in abundance.

Materials and Methods

This study was conducted at the different microbiology laboratories at Chennai, Tamil Nadu, India. The samples were collected from One hundred patients undergoing chemotherapy, surgery and diabetic mellitus treatment. Patients were examined for signs or symptoms of Cryptococcosis at baseline, independent of any signs or symptoms of Cryptococcosis. Males represented 60 of patients and females 40 were studied. The following clinical history was obtained for each patient: Age, sex, underlying diseases, prior surgical procedures, recent history of fungal infection and antimycotic treatment.

Only those patients who had not used antimycotic agents in the previous four weeks were included. The samples obtained from the CSF, blood, and urine of the study subjects were inoculated on several and selective agar media including Sabouraud dextrose agar with antibiotics (G-penicillin 20 U/ml, streptomycin 25 µg/ml).

Collection of Samples

Samples were taken sites by using a swabbing method (Hanan M. Al-Abeid *et al.*, 2004). A sterile cotton swab was immediately inoculated into a Brain hearth infusion broth containing 50µg/ml chloramphenical. Tubes were incubated at 37°C for up to 48 hrs when streaking in to Sabouraud dextrose agar (SDA) was performed to obtain isolated colonies. All plates were incubated at 35°C for 24 to 48 hours and yeast like colonies were isolated.

Identification of Isolates

Guizotia abyssinica and D-proline, selective and differentiating media for Cryptococcus spp., were used for isolation and identification, respectively (Danielle *et al.*, 1989). The yeast - like colonies were identified by classical methods using the following tests: Morphology, Formation of Capsules, Assimilation of Carbon and Nitrogen sources, Fermentation of Carbohydrates and Urea hydrolysis (Sullivan *et al.*, 1995). The methods described by Microbiology: A laboratory Manual by Cappuccino (1999) was followed for all the procedures.

Antibiotic Sensitivity Test

Antibiotic susceptibility testing of the clinical isolates along with the quality control strains were performed using BHI agar instead of Muller Hinton agar by disk diffusion method. Antibiotic susceptibility test was conducted by adopting Kirby-Bauer disc diffusion method. The cultures were streaked closely with swab on the medium in the form of lawn.

In the plate containing antibiotics such as: Amphotericin-B (15µg), Ketoconazole (10µg), Itraconazole (10µg), Clotrimazole (10µg), Miconazole (10µg), Fluconazole (10µg), Voriconazole (10µg), discs were placed and incubated at 37°C following overnight incubation the culture was examined for areas no growth around the disc (Zone of inhibition).

Antimicrobial Activity

Selection of plants

Based on the literature, the following plants were selected, on the presence of different groups of phytochemical compounds that they possess and based on the availability of the plants.

Collection of selected plants

The leaves of the plants above listed were collected largely in polythene bags and immediately transported to the laboratory for processing.

Preparation of crude extract

The leaves of the plants collected were individually washed with tap water, blotted with filter paper and spread over news paper for air drying under shade. After complete dryness, the leaves of individual plants was powdered using a mixer grinder.

A known quantity of leaf powder (50 g) of each plant was taken in a 250 ml conical flask and added with 100 ml of ethanol (85%). Ethanol was used for the extraction of phytochemicals because it has the ability to dissolve the phytochemical compounds like tannins, polyphenols, flavonols, terpenoids and alkaloids (Ivanovska *et al.,* 1996).

The ethanol-leaf powder mixtures were kept at room temperature for 48 hours and rapidly stirred using glass rod every 8 hours. After 48 hours, the extract of each plant was filtered through Whatmann No.1 filter paper to exclude the leaf powder.

Then each filtrate was kept in beaker on a water bath at 45ºC until the solvent gets evaporated. A greasy final material (Crude extract) obtained for each plant was transferred to screw cap bottles and stored under refrigerated condition till use.

Preparation of stock solution

By using digital electronic balance, 200 mg of each crude extract was carefully taken in a standard measuring flask and 5ml of ethanol was added to dissolve the extract and one or two drops of emulsifier (Triton-X100) was added to completely dissolve the extract.

Then it was made up to 200 ml by adding distilled water. This forms the stock solution of 1000 ppm (i.e., 1mg/ml). For the antimicrobial assay using individual plant extract, the stock solution of 1000 ppm was directly used.

Antimicrobial Assay

Standard Strains Used

Standard reference strains *Cryptococcus neoformans* obtained from the Microbial Type Culture Collection (MTTC) of Institute of

Microbial Technology (IMTECH), Chandigarh were used for the present study. This Standard reference strains were maintained by subculture method in Sabouraud dextrose agar slants.

Kirby Bauer Disc Diffusion Technique

This technique was used to test the sensitivity of selected test organisms to the ethanolic leaf extracts individually and in combination as described above (Bauer *et al.,* 1966).

Preparation of antimicrobial disc using crude extracts

Discs of 5 mm in diameter from a sheet of filter paper were punched out, placed in petridishes allowing a distance of 2-4 mm between each disc and sterilized in a hot air oven at 160ºC for 1 hour.

After allowing the disc to cool, 20 µl (0.02 ml) of each test solution was added on to each disc and then the discs were dried at 37ºC in an incubator for one hour (Cheesbrough, 1984). For control set, the discs were added with distilled water (200 ml) containing 5ml ethanol + 2 drops of emulsifier at 20µl/disc.

Preparation of plates

The Petri plates of 100mm diameter with nutrient agar were swabbed with broth culture of each test bacteria in separate plates by using sterile swab. Over this, prepared antimicrobial discs were placed under aseptic conditions.

Three discs of each extract were placed in triangles. Control sets with a standard antibiotic Voriconazole (10 µg/disc) were simultaneously maintained. Also the discs without plant extract(discs prepared using 200 ml distilled water + 5 ml ethanol + one or two drops of emulsifier) were also maintained as another set of control for each test organism. The plates were then incubated at 37ºC for 24 hours and the zone of inhibition (IZ) was measured and recorded.

Results and Discussion

A total of 100 clinical samples were collected for the study from different medical laboratories of Chennai, Tamil Nadu, India. The phenotypic characteristics of yeast isolates identified from clinical samples are indicated in Table 1.

A percentage of 68 showed for *Cryptococcus sps.* From this, 48 isolates showed Capsule staining positive activity and identified as *Cryptococcus neoformans* by using sugar fermentation and assimilation tests. Among the 48 isolates of *Cryptococcus neoformans* 21 isolates showed Fluconazole resistance (Table.3 and Chart.3).

The phenotypic characteristics of *Cryptococcus neoformans* isolates from clinical samples are indicated in Table 1. Preliminary identification of the isolates was performed by conventional tests (Capsule formation, urease positive, sugar fermentation and assimiliation test, Nitrate negative).

Table.2 showed *Cryptococcus neoformans* isolates from patients. Prevalence of Fluconazole resistant *Cryptococcus neoformans* isolated among two sex groups of sample donors is depicted in Table.4. A higher percentage of 46.15 isolates were obtained from Male, and was followed by Female patients. Antifungal susceptibility pattern and percentage of *Cryptococcus neoformans* isolates was showed in Table.4 and Table.5.

Ethanolic extracts of the plant leaves includes *Cassia angustifolia, Cassia albus, Cassia wislizenii and Cassia tora* showed antimicrobial activity against Fluconazole resistant *Cryptococcus neoformans* (Table.5).

Table.1 The lists of plants selected for the study are given here under (Figure1 to 3)

Plants selected	Family	Major class of phytochemical(s) present	References
Cassia angustifolia	Malvaceae	Flavonoids	**Duke (1985)**
Cassia albus	Malvaceae	Flavonoids	
Cassia wislizenii	Malvaceae	Flavonoids	
Cassia tora	Malvaceae	Flavonoids	

Table.2 Identification of *Cryptococcus neoformans*

S. No.	Tests	Results
1.	Colony morphology on SDA	**Large whitish cream colour.**
2.	Lacto phenol cotton blue staining	**Showing large budding cells**
3.	Capsule test	**Positive**
4.	Fermentation of Carbohydrates	
	Glucose	**Positive**
	Maltose	**Positive**
	Galactose	**Positive**
	Trehalose	**Positive**
	Fructose	**Positive**
	Sucrose	**Negative**
	Lactose	**Negative**
5.	Sugar Assimilation test	
	Glucose	**Positive**
	Maltose	**Positive**
	Sucrose	**Positive**
	Galactose	**Positive**
	Xylose	**Positive**
	Trehalose	**Positive**
	Lactose	**Negative**
	Melibiose	**Negative**
	Cellobiose	**Negative**
	Inositol	**Negative**
	Raffinose	**Negative**
	Dulcitol	**Negative**
6.	Urease test	**Positive**
7.	Nitrate test	**Negative**

Table.3 *Cryptococcus neoformans* isolates from patients

S. No.	Patients	No. of individual screened	Number of Cryptococcus isolates	*Cryptococcus neoformans*	
				No. of isolates	Percentage (%)
1.	Male	60	38	26	**68.4%**
2.	Female	40	30	22	**70%**
Total		**100**	**68**	**48**	**70.5%**

Table.4 Fluconazole resistant *Cryptococcus neoformans* and Fluconazole sensitive *Cryptococcus neoformans*

S. No	Patients	No. of individual screened	Others isolates	Number of Cryptococcus isolates	*Cryptococcus neoformans*		Fluconazole resistant *Cryptococcus neoformans*		Fluconazole sensitive *Cryptococcus neoformans*	
					No. of isolates	Percentage (%)	No. of isolates	Percentage (%)	No. of isolates	Percentage (%)
1.	Male	55	17	38	26	68.4	12	46.15	14	**53.84**
2.	Female	45	15	30	22	73.3	9	42.85	13	**59.0**
Total		**100**	**32**	**68**	**48**	**70.5**	**21**	**44.68**	**27**	**56.25**

Table.5 Antifungal susceptibility pattern and percentage of *Cryptococcus neoformans* isolates

S. No	Patients	No. of individual screened	No. of *Cryptococcus neoformans* isolates	Antibiotics used						
				AM-B	KZ	IZ	Cl	MZ	FZ	VZ
1.	Male	60	26	15.3 (4)	30.7 (8)	30.7 (8)	23.00 (6)	26.9 (7)	46.15 (12)	0.07 (2)
2.	Female	40	22	28.5 (6)	22.7 (5)	36.3 (8)	31.80 (7)	27.2 (6)	42.85 (9)	13.6 (3)
Total		100	48	20.8 (10)	27.0 (13)	33.3 (16)	27.0 (13)	27.0 (13)	43.7 (21)	10.4 (5)

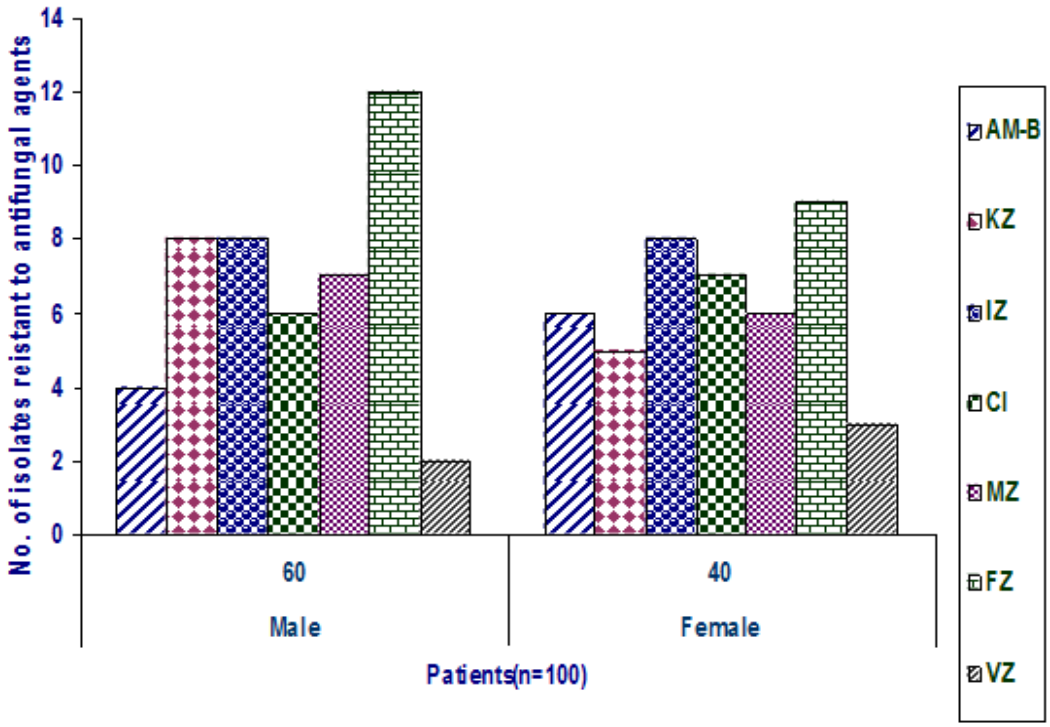
AM-B=Amphotericin-B; KZ=Ketoconazole; IZ=Itraconazole; CL=Clotrimazole; MZ=Miconazole; FZ=Fluconazole; VZ=Voriconazole.

Table.6 Effect of ethanolic extracts of selected plant leaves on Fluconazole resistant *Cryptococcus neoformans*

S. No.	Ethanolic extract of the plant leaves used	Zone of inhibition in cm	
		IFRCN	SFRCN
1	*Cassia angustifolia*	1.3	**1.4**
2	*Cassia albus*	1.7	**1.8**
3	*Cassia wislizenii*	1.9	**1.9**
4	***Cassia tora***	**2.1**	**2.0**

(Values are mean of three replicates)
IFRCN – Clinically isolated Fluconazole Resistant *Cryptococcus neoformans*
SFRCN – Standard Fluconazole Resistant *Cryptococcus neoformans*

Chart.1 Antifungal susceptibility pattern and percentage of *Cryptococcus neoformans* isolates



AM-B=Amphotericin-B; KZ=Ketoconazole; IZ=Itraconazole; CL=Clotrimazole; MZ=Miconazole; FZ=Fluconazole; VZ=Voriconazole.

Antifungal reference standard, Voriconazole had equal effect on Fluconazole resistant *Cryptococcus neoformans* tested and also tested for standard *Cryptococcus neoformans* strains (Table.6). The present study used phenotypic methods to determine the stability of oral yeast colonization in patients and to characterize oral yeasts. Additionally, an attempt was made to determine the antifungal susceptibility testing to oral cavity of patients *Cryptococcus neoformans* isolates. Diseases, particularly fungal diseases are increasing day by day. Diagnostic and treatment measures are also in increasing trend. Antibiotics are gaining more and more importance from time to time from the time of its first discovery for the treatment of bacterial and fungal diseases. Indiscriminate use of antibiotics leads to development of resistance to various antibiotics, particularly by mutation developed.

This study was carried with an aim to isolate *Cryptococcus neoformans* from Clinical samples from different medical laboratories and subjected to antibiotic sensitivity test by using Amphotericin-B, Ketoconazole, Itraconazole, Clotrimazole, Miconazole, Fluconazole, Voriconazole. But, the fluconazole antifungal agent showed highly resistant to *Cryptococcus neoformans.* Among the 48 isolates of *Cryptococcus neoformans* 43.7% (21 isolates) of isolates showed resistant to Fluconazole.

Antibiotic sensitivity test to the *Cryptococcus neoformans* isolates from the clinical specimens was studied for now the effective therapeutic agent as well as to know the resistant pattern, particularly to Fluconazole. The tremendous therapeutic advantage afforded by antibiotics is being threatened by the emergence of increasingly resistant strains

of microbes. Outside the hospital, Fluconazole resistant *Cryptococcus neoformans* is of greatest concern; these reports also indicate the appearance of fluconazole resistant *Cryptococcus neoformans* infections. Fluconazole resistant *Cryptococcus neoformans* is a significant problem in the hospital and patients.

The locally available medicinal plants were subjected ethanolic extraction and then tested against fluconazole resistant *Cryptococcus neoformans* isolates from clinical samples of patients. The plant species includes *Cassia angustifolia, Cassia albus, Cassia wislizenii and Cassia tora* were used in this present study. Results on the analysis of ethanolic extracts of the plants leaves include *Cassia angustifolia, Cassia albus, Cassia wislizenii and Cassia tora* showed antimicrobial activity against Fluconazole resistant *Cryptococcus neoformans.* Among the plant leaves tested *Cassia angustifolia* showed minimum activity against Fluconazole resistant *Cryptococcus neoformans*. Ethanolic extracts of *Cassia tora* showed high antimicrobial activity against Fluconazole resistant *Cryptococcus neoformans* and Standard *Cryptococcus neoformans* strains that include 2.0 and 2.1 respectively. (Table.5). Antifungal reference standard, Voriconazole had equal effect on Fluconazole resistant *Cryptococcus neoformans* tested and also tested for standard *Cryptococcus neoformans* strains (Table.6).

Nineteen flavnoids from *Cassia tora* showed antimicrobial activity against FRCN. It exhibits antimicrobial activity against strain of *Cryptococcus neoformans* with minimum inhibitory concentrations ranging from 3.13 to 12.5 µg/ml. (Karuppusamy *et al.,* 2002). The plant species include *Cassia tora* showed highest antimicrobial activity that 2.1, 2.0 cm against clinically isolated *Cryptococcus neoformans* and Standard strain of *Cryptococcus neoformans* respectively, in this present study.

Nowadays, the scheme therapeutic to treat *C. neoformans* has few alternatives, which are basically represented by amphotericin B and 5-fluorocytosine in association with azole drugs. However, whether the strains have resistance against these drugs the patients show an increase in risk of death. Moreover, the amphotericin B may cause important unwanted effect, such as impairment of glomerular filtration and hepatic function, hypokalaemia and hypomagnesaemia, anaemia, thrombocytopenia, anaphylactic reactions, and neurotoxicity. In attempt to decrease these unwanted effects, liposome-encapsulated and lipid-complexed preparations have been used, however they are significantly more expensive and less efficient than native drug (Gruszecki *et al.,* 2003; Blau and Fauser, 2000). Consequently, there is an increasing need for new compounds with antifungal activity.

Natural products, including plants, may be a source of compounds with antifungal effects and therefore possible candidates for the development of new antifungal agents.

Natural products have been widely studied as an alternative for treating yeasts such as *C. neoformans*. Cáceres *et al.,* (2012) tested the ethanol extract of Smilax domingensis against *C. neoformans*, showing a MIC of 500 g/mL. In the same year, Manoj and Muragan (2012) tested the methanol extract of *Plagiochila beddomei* against *C. neoformans* (MTCC 6333) also showing a MIC of 500 g/mL. In another recent article tested the fungicides activities of seven species of Lippia, the results showed an anti-*C. neoformans* only for the species *L. sidoides*, with the MIC of 625 g/mL (Fabri *et al.,* 2011).

The parallel results have also been reported by Iqbal-Ahmad (2001) while screening ethanolic

extracts of 25 medicinal plants of the family Asclepiadaceae and reported that out of 25 plant extracts screened, 13 extracts showed strong antimicrobial activity whereas rest of the plant extracts showed less activity or minimum antimicrobial activity against *Cryptococcus neoformans* strains.

The antimicrobial activity of individual ethanolic plant extract was higher in *Cassia tora* than the other extracts tested against antibiotic resistant *Cryptococcus neoformans* isolated from the clinical samples of patients. *Cassia tora* extract exhibited inhibition zone of 2.0 and 2.1 cm against clinically isolated antibiotic resistant *Cryptococcus neoformans* and Standard strains of *Cryptococcus neoformans*. There is a scope to use ethanolic extracts of the leaves of *Cassia tora* against antibiotic resistant *Cryptococcus neoformans* isolated from clinical specimens of patients.

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How to cite this article:

Vijayalakshmi, T. M. and Murali, R. 2018. Antifungal Activity of *Cassia* sps against Clinically Isolated Antibiotic Resistant *Cryptococcus neoformans*. *Int.J.Curr.Microbiol.App.Sci.* 7(11): 3700-3711. doi: https://doi.org/10.20546/ijcmas.2018.711.426