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Research Article

In vitro Synergistic effect of *Cassia fistula* leaves extracts with Fluconazole against clinical isolates of *Candida albicans*

Yogesh Bansal, Charulata, *Devendra Singh Negi

Department of Chemistry, HNB Garhwal University, Srinagar (Garhwal), Uttarakhand, India

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ABSTRACT

The aim of the study was to evaluate the synergistic antifungal action of crude DMSO (Di methyl sulphoxide) & Petroleum ether leaves extract of *Cassia fistula* in conjunction with fluconazole against candidial species isolated from *Candidasis* patients. Correlation was observed between the leaves & fluconazole, therefore it was also found that the antifungal action of *Cassia fistula* leaves extract was enhanced (up to 3 times) in combination with fluconazole on fluconazole resistance *Candida albicans* strains isolated from patients with Candidiasis. The plant appears to play an important role in overcoming the azole resistant of Candidial strains. IC_{50} was determined by agar well diffusion and broth dilution method in the presence of fluconazole and leaves extracts of *Cassia fistula* in different solvents. Synergistic behavior of leaves extracts in Pet. Ether and DMSO extract of *Cassia fistula* with fluconazole was checked by broth dilution method.

Key words: *Candida albicans, Cassia fistula*, Fluconazole, Synergism.

INTRODUCTION

Cassia fistula Linn. (Hindi-Amaltas; English-Golden Shower or Indian Laburnum), a medium sized tree belonging to the family - Caesalpiniaceae, is widely cultivated throughout India as an ornamental tree. It has been used extensively in the folklore medicine for the treatment of a variety of diseases. Pharmacologically, the plant has been investigated for its antibacterial, laxative[1], hepatoprotective[2], hypocholesterolaemic[3], anti-diabetic[4], antitumor and antioxidant effects[5]. The plant is rich in phenolic antioxidants such as anthraquinones, flavonoids and flavan-3-ol derivatives[6]. In the recent years relatively more emphasis is being laid on the examination of natural products including substances of plant origin for their anti fungal activity. The mechanism of action of these substances is not known clearly. Although *Candida* species are often present as benign commensal organisms in the digestive tract of healthy individuals, produce a broad range of serious illnesses in compromised hosts[7]. Therapy for serious *Candida* infections has been difficult because of the limited number of available antifungal agents and their drug resistance[8]. Alteration in sterol biosynthesis that results in the substitution of other sterols for ergo sterol, Over expression of the target protein so that sufficient enzyme activity remains even in the presence of the drug, Over expression of various membrane efflux pumps that reduce intracellular drug concentration, and Alteration in the amino acid sequence of the target protein that reduces its binding affinity for azoles[9-10].This paper suggests a way of overcoming or preventing, drug resistance antifungal by combination therapy[11]. Combination therapy sounds attractive, it has been reported that anti microbial combination can be selected against the development of resistance that's why in the present study we analyzed the phenomenon of the interaction between Azoles and *Cassia fistula* leaves.

MATERIALS AND METHODS

IC_{50} was determined by agar well diffusion and broth dilution method in the presence of fluconazole and leaves extracts of *Cassia fistula* in different solvents. Synergistic behavior of leaves extracts in pet ether and DMSO extract of *Cassia fistula* with fluconazole was checked by broth dilution method.

The study group consisted of Candidasis subjects selected from Himachal Institute of Dental Sciences, Poanta Sahib living in Poanta Sahib (urban area study group) and outskirt areas of Poanta Sahib (sub urban area study group) during Oct.2010-Mar.2011.

Exclusion criteria

- Subjects on any antibiotic or antifungal therapy, 15 days prior to sampling-drugs interfere with the candidal CFU ´s (Colony forming units) in the oral cavity.
- Patients who had received chemotherapy or radiotherapy or any other immunosuppressive therapy one year prior to the sampling-immunosuppressive therapies are known to increase fungal CFU´s in oral cavity.
- Patients with any intra oral prosthesis- denture wearing causes increased fungal CFU´s in oral cavities.
- Patients who had undergone any surgical procedure one year prior to sampling –invasive procedure cause immunosuppression which further causes increased CFU´s in oral cavity.

*Author for correspondence: E-mail: devendra_negi@yahoo.com

Table 1: Distribution of subjects according to sex in various age groups.

	Sex	Age group (in yrs) 8-21	Age group (in yrs) 21-40	Age group (in yrs) 41-60	Total	%age	Mean & S.D
Sub urban area Study group	Male	7	8	18	33	66	Mean-45.3
	Female	5	7	5	17	34	& S.D-8.01
	Total	12	15	23	50	100	
Urban area Study Group	Male	11	7	0	18	60	Mean -39.07
	Female	7	5	0	12	40	& S.D-4.03
	Total	18	12	0	30	100	

Table 2: Number of isolates from each method:-

	Method	Positive cultures Number	%age	Negative cultures Number	%age	Total
Sub urban area study group	Oral rinse	37	74	13	26	50
	Scraping	24	48	26	52	50
Urban area study group	Oral rinse	21	70	9	30	30
	Scraping	13	43.33	17	56.66	30

On statistical analysis of comparison of two methods, it was found that Z= 2.67(p >0.01) which is significant.

Table 3: Effect of *Cassia fistula* leaves extract (in different solvents) on isolated *Candida* species with their IC_{50} valves

	C.albicans(29*)	*C.glabrata*(3*)	*C.kruseii(2*)*	*C.pseudotropicalis*	*C.tropicalis*
1)DMSO leaves extract	0.8±0.06mg/ml	2.8±0.65 mg/ml	4±0.23 mg/ml	0.6±0.034 mg/ml	0.1±0.024 mg/ml
2)Pet. Ether leaves extract	1±0.021 mg/ml	3.1±0.39 mg/ml	4.8±0.14 mg/ml	0.8±0.039 mg/ml	0.1±0.017 mg/ml
3)Methanolic leaves extract	1.8±0.083 mg/ml	3.8±0.58 mg/ml	5±0.48 mg/ml	1.7±0.19 mg/ml	0.6±0.049 mg/ml
4)Ethanolic leaves extract	2±0.15 mg/ml	3.9±0.46 mg/ml	5±0.17 mg/ml	1.9±0.28 mg/ml	0.8±0.068 mg/ml
5)Fluconazole	0.1±0.024 µg/ml	1.8±0.26 µg/ml	14±0.34 µg/ml	0.4±0.054 µg/ml	0.4±0.15 µg/ml

Note -1) Values are mean of 8 replicates (IC_{50}± SD valves).
*2) * means the number of isolates used for IC_{50} calculation.*
3) IC_{50}- 50% inhibitory concentration of leaves extract & fluconazole.

Table 4: Species wise distribution of various fungal species

S.No.	Name of fungal species	No.of colonies	%age	Confirmatory test
1	*Candida albicans*	35	77.77	Germ tube.Carbohydrate fermentation, Corn meal
2	*Candida* glabrata	3	5.88	Carbohydrate fermentation
3	*Candida krusei*	2	3.92	Carbohydrate fermentation, Corn meal
4	*Candida* pseudotropicalis	2	3.92	Carbohydrate fermentation, Corn meal
5	*Candida* tropicalis	1	1.96	Carbohydrate fermentation, Corn meal
6	*Penicillium marneffei*	1	1.96	Colony morphology,pigment production & LCB preparation
7	*Chaetomium*	1	1.96	Colony morphology & LCB preparation

After complete evaluation 50 subjects were included in the sub urban area study group and 30 in the urban area study group.

Sample collection:-

Sample from oral cavity: Two methods were used for obtaining the sample from oral cavity from the Urban area study group and Suburban area study group:- Scraping method and Oral rinse method (Table No.2).

Collection of plant material: The plant material used is the dried leaves of herbs *Cassia fistula* (BSD112753) were collected from HNB Garhwal University, Srinagar (Garhwal) in Jan 2009. Plant was identified by Botanical Survey of India, Dehradun. Leaves of selected plants were dried (room temperature) and powdered with a mortar.

Extraction of plant material: The plant material taken for the study was stored under refrigerated conditions till use.

The sample was prepared by grinding one gram of sample in 1 ml of different solvents like Methanol, Ethanol, Dimethyl sulphoxide and Petroleum ether individually, in pre-chilled mortar and pestle and the extract was centrifuged at 10,000 rpm at 4^0C for 10 minutes. The supernatant, thus obtained was used within four hours for anti fugal activity.

Methods of identification & isolation of Candida species*:* The samples were observed everyday, for the appearance of growth. Smear was prepared from the growth from mucosal scraping and lesions scraping which appeared usually 48-72hrs after incubation. Preliminary identification was done by Gram staining and the Gram positive fungal colonies were sub cultured on SDA plate and Gram negative colonies were not further used in the study. After preliminary identification the colonies were subjected to various confirmatory test as per standard procedure like Lacto phenol cotton blue preparation, Germ tube test, Corn meal Tween 80,Agar culture test agar, carbohydrate fermentation test& slide culture [12-13].

Growth and maintenance of culture: *Candida* species was isolated from patients with candidiasis & was maintained on Sabaroud's agar slant (Glucose: 40g/lt, Peptone: 10g/lt, Agar agar:20g/lt)[14].

Preparation of inoculums: Two to three colonies of test culture were inoculated in nutrient Broth and were incubated at 37^0C.The broth was incubated for 4 hr. in a shaker incubator until the turbidity of broth (~10^6cfu/ml) matches with that of $BaSO_4$ 0.5 McFarland standards.

RESULTS AND DISCUSSION

The sub urban area group consists of 66% male patients & the maximum cases were in the age group of 4th decade to 6th decade with a mean of 45.3 years & S.D is 8.01 in table 1. The urban area group consists of 60%males with maximum subjects in the age of 2nd decade to 4th in table 1.Our study showed that 70% of the patients in the suburban group had oral lesions,in table 2 & isolated fungal species were identified preliminary by SDA plate & Gram staining, *Candida* species were further identified and confirmed by methods given in the table 3.

The antifungal activity of DMSO. Pet.ether, Methanolic, Ethanolic leaves extract of *Cassia fistula* & fluconazole on various *Candida* species isolated from candidiasis patients are shown in table 4. Growth of *Candida albicans, Candida glabrata, Candida krusei, Candida pseuditropicalis & Candida tropicalis* were inhibited by four leaves extracts of *Cassia fistula* prepared in different concentration. Crude DMSO leaves extract was most active plant extract against *Candida krusei* with an IC_{50}

Table 5: Candidiasis patients with Fluconazole resistant *Candida albicans* strains.

S. no.	Age/ sex/ Area	OPD card number	Oral manifesta-tion	Scrap ing	Oral rinse	CFU / ml	Type of colonie s	Morpho-logy	Gram Stain-ing	Gram tube	Confirmator y test	Organism	Fluco-nazole IC_{50}
1	32/F/ sub urban	50429	Candidiasis	-ve	+ve	1674	one	Whitish round opaque colonies	+ve	+ve	Carbohydrat e fermentation , corn meal	*Candida albicans*	52 µg/ml
2	44/m/ sub urban	53725	Candidiasis	-ve	+ve	5920	two	Whitish round opaque colonies	+ve for both	+ve for one & -ve for other	Carbohydrat e fermentation , corn meal	*Candida albicans Candida glabr ata*	35µg/ml
3	49/m/ sub urban	62157	Ulcer, Candidiasis	+ve	+ve	5388	one	Whitish round opaque colonies	+ve	+ve	Carbohydrat e fermentation , corn meal	*Candida albicans*	41µg/ml
4	37/F /sub urban	48467	Ulcer, Candidiasis	+ve	+ve	5448	one	Whitish round opaque colonies	+ve	+ve	Carbohydrat e fermentation , corn meal	*Candida albicans*	48µg/ml
5	39/F/ sub urban	47013	Ulcer, Xero-stomia, Can-didiasis	-ve	+ve	Conf l-uent	Three	Whitish round opaque colonies	-ve for one	+ve for one	Carbohydrat e fermentation , corn meal	*Candida albicans*	58µg/ml
6	51/m/ sub urban	51488	Ulcers, Xerostomia Candidiasis	+ve	+ve	Conf l-uent	two	Whitish round	+ve f or both	+ve for one & -ve for other	Carbohydrat e fermentation , corn meal	*Candida albians & Candida Psuedotropica lis*	40µg/ml

Table 6: Synergistic effect of Fluconazole & *Cassia fistula* leaves extract on fluconazole resistance strains of *Candida albicans*.

S.No.	Age/Six/Area	Fluconazole(µg/ml)+ $C.F^{DMSO}$	%age inhibition	Fluconazole(µg/ml)+ $C.F^{Pet.ether}$	%age inhibition
1	32/F/Sub urban	0.0µg/ml+0.0mg/ml	0	0.0µg/ml+0.0mg/ml	0
		52µg/ml+0.8mg/ml	98	52µg/ml+0.9mg/ml	92
		26µg/ml+0.4mg/ml	90	26µg/ml+0.225mg/ml	82
		13µg/ml +0.2mg/ml	70	13µg/ml +0.45mg/ml	74
2	44/M/Sub urban	35µg/ml +0.9mg/ml	95	35µg/ml +1.1mg/ml	90
		17.5µg/ml+0.45mg/ml	92	17.5µg/ml +0.5mg/ml	85
		8.75µg/ml+0.22mg/ml	80	8.75µg/ml+0.27mg/ml	80
3	49/M/Sub urban	41µg/ml +1.2mg/ml	98	41µg/ml +1.3mg/ml	98
		20.5µg/ml+0.6mg/ml	92	20.5µg/ml+0.65mg/ml	85
		10.2µg/ml+0.3mg/ml	81	10.2µg/ml+0.37mg/ml	75
4	37/M/Sub urban	48µg/ml +0.8mg/ml	95	48µg/ml +0.9mg/ml	90
		24µg/ml + 0.4mg/ml	90	24µg/ml + 0.45mg/ml	85
		12µg/ml+0.2mg/ml	80	12µg/ml+0.225mg/ml	78
5	34/F/Sub urban	58µg/ml + 0.8mg/ml	99	58µg/ml + 1mg/ml	99
		29µg/ml + 0.4mg/ml	92	29µg/ml + 0.5mg/ml	87
		14µg/ml+ 0.2mg/ml	79	14µg/ml+ 0.25mg/ml	75
6	52/M/Sub urban	40µg/ml + 1.2mg/ml	98	40µg/ml + 1mg/ml	90
		20µg/ml + 0.6mg/ml	95	20µg/ml + 0.5mg/ml	85
		10µg/ml+ 0.3mg/ml	87	10µg/ml+ 0.25mg/ml	77

Note –$C.F^{Pet\ ether}$- Petroleum ether leaves extract Cassia fistula,
$C.F^{DMSO}$ - DMSO leaves extract Cassia fistula.

valve of 4 ±0.23 mg/ml. For *Candida albicans, Candida glabrata, Candida pseuditropicalis & Candida tropicalis,* the DMSO leaves extract & Pet. Ether leaves extract were potent inhibitors with an IC_{50} of 0.8 ±0.06 mg/ml, 2.8 ±0.65 mg/ml, 0.6 ±0.34 mg/ml, 0.1 ±0.24 mg/ml & 1 ±0.021 mg/ml, 3.1 ±0.39 mg/ml, 4.8 ±0.14 mg/ml, 0.1 ±0.17 mg/ml respectively. Fluconazole which was used as positive control exhibited strong antifungal activity with the IC_{50} of 0.1 ±0.024 µg/ml, 1.8 ±0.26µg/ml, 14 ±0.34 µg/ml, 0.4 ±0.54µg/ml, 0.4 ±0.15µg/ml against *Candida albicans, Candida glabrata, Candida krusei, Candida pseuditropicalis & Candida tropicalis.*

Alternation of In vitro susceptibility of Isolated Candida albicans to fluconazole

In table 5, we noted that incidence of oral *Candida* carriage significantly increased with age of the patient. The *Candida* positive subjects were older than the *Candida* negative subjects. Table 5 also shows the incidence of fluconazole resistance cultures of *Candida albicans* from six patients with Chronic Candidiasis, Ulcers and Xerostomia. These fluconazole resistance cultures of *Candida albicans* have shown a very high IC_{50} value ranges from 35 to 58µg/ml & referred as "Drug tolerance" in table 5. To further define the para meters of both *Cassia fistula* leaves extract (both in DMSO & Pet. Ether solvent) with Fluconazole were varied from their IC_{50} to ½ IC_{50} and then ¼ IC_{50} and excellent results were observed, depicted in table 4.

In this work, Fluconazole was found to be synergic in interaction with Crude DMSO leaves extract of *Cassia fistula* or Crude Pet. leaves extract of *Cassia fistula* against six resistant strains isolated from 80 patients, in table 6. The IC_{50} of Fluconazole & Crude DMSO leaves extract of *Cassia fistula* or Crude Pet. leaves extract were found to be with in a range of 35-58µg/ml and 0.8-1.2 mg/ml or 0.9-1.3 mg/ml respectively. When isolated, Fluconazole resistant *Candida albicans* strains were grown under combination of Fluconazole +*Cassia fistula*^{DMSO} and Fluconazole +*Cassia fistula* $^{Pet.\ Ether}$ at their IC_{50},1/2 IC_{50} & 1/4 IC_{50} (in table 6), actively growing *Candida albicans* cells got killed rapidly & efficiently by 0.8 mg of *Cassia fistula* leaves extract in DMSO plus Fluconazole 52 µg/ml. No viable cells (among 5×10^5) were detected after 2 hr. of exposure to drugs (in combination). However cells that were exposed to fluconazole (52µg/ml) were not killed even during the overnight incubation (16hr) and moreover cells exposed to combination treatment, did not grow up to the same density as the control. Same experiment has been repeated with remaining isolated *Candida albicans* strains and similar results were observed as depicted in the table 6. The results reveal that at least 75-85% growth inhibition of *Candida albicans* was observed even at ¼ IC_{50} of their combination. It shows that both are acting synergistically.

Our findings suggested options for expanding the utility of existing antifungal drug classes. Current therapeutic agents, azole and amphotericin B are only partially effective for the treatment of candidiasis[15]. Therefore, more effective drugs and drug combination are needed. More recently it has been confirmed that this approach could add some microbiological benefits to the treatment of candidiasis in non neutropenia patients[16].The results presented here demonstrated that isolated resistant *Candida* species were killed by normal IC_{50} valve of leaves extract. Combination of anti fungal drug with *Cassia fistula* leaves extract, in comparison with single drugs, may confer the benefit of increased efficiency, sparing toxicity or both. We emphasize that in the present study we analyzed the phenomenon of interaction

between azole and *Cassia fistula* leaves extract and not the mechanism of their interaction. The bases of drug interaction are potentially multifactorial and complex.

CONCLUSION

The results made us conclude that fluconazole and *Cassia fistula* leaves extract exhibit synergistic fungicidal activity against *Candida albicans*, this finding serve as evidence of utility of combination treatment approach to treat fungal disease.

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