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SYNERGISM BETWEEN *CASSIA FISTULA* AND FLUCONAZOLE; "A NEW APPROACH AGAINST CANDIDIASIS"

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ABSTRACT: Natural products have great potential against microbes and can be used to overcome the problem of drug resistant pathogens. They are helpful in reducing the toxicity of existing antifungal compounds. This present *in vitro* study demonstrated the synergistic effectiveness of *Cassia fistula* (CF) leaves extracts and fluconazole (Flu) against isolated Flu resistant strains (FRSs) of *Candida albicans* (CA) as the growth of FRS was found to be sensitive to extracts of CF leaves. In the present study CF leaves' extracts were prepared in different solvents and their IC_{50} values were estimated by micro dilution method. It was found a statistical significant decrease in concentration of cellular lipids, phospholipid of FRS when treated against the CF leaves or combination of CF leaves and Flu. Further, H^3 uptake analysis revealed that both methods affected the DNA synthesis as well as decrease in DNA contents of FRS. Hence, the results of this study would be significant for the development of new anti candidal drugs from CF leaves' extracts in combination with known synthetic drugs like Flu to control drug resistant CA.

INTRODUCTION: The pace of drug discovery has increased with the introduction of new targets and new inhibitors of these targets being reported each month [1]. Several authors have several studies on anti-microbial properties of natural extracts and natural products [2, 3, 4]. Due to the difficulty in identifying drug targets unique to fungi that are not shared with the human host, only restricted number of antifungal agents has been widely used for treating candidial systemic infections [5].

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Fluconazole (Flu) is the most common azole drug that target lanosterol 14-α –demethylase (Erg 11) in the ergosterol biosynthesis pathway and used as antifungal agent for prevention and treatment of candidiasis [6]. Prolonged use of above drug in recent years has contributed to the development of Flu resistance in candida species [7].

For this reason it has now become very important to reduce the Flu concentration by different combination methods. On this study, we assess the synergism between Flu with CF (*Cassia fistula*). CF is called as 'An Indian Laburnum', also known as the golden shower tree and is a flowering plant belongs to the family Fabaceae [8]. The species is native to the Indian Subcontinent and adjacent regions of Southeast Asia and has antifungal

properties [9], hepato protective activity [10], hypo cholesteroleamic activity [11], hypo glucamic activity [12], anti plasmodial activity [13] and anti-tumour activity [14]. Therefore, in the present study, we proposed to study the antifungal effect of CF leaves extracts in different solvents and Flu against isolated fluconazole resistant strains (FRS) of *Candida albicans* (CA). We also evaluated the intracellular changes made by CF leaves extract on isolated resistant strains CA.

MATERIALS AND METHODS: The present study has been designed to evaluate the antifungal action of *Cassia fistula* (CF) leaves extracts and commercially available drug fluconazole (Flu) on clinical isolates of fluconazole resistant strains (FRS) of *Candida albicans* (CA). The study protocol was approved by local ethical committee. The written informed consent was obtained from patients before their enrolment in the study.

Collection of plant material: CF leaves were collected from HNB Garhwal University, Srinagar (Garhwal), Uttarakhand, INDIA. This plant was identified and confirmed by Botanical Survey of India, Dehradun (BSD 112753). The leaves of the same were obtained and stored under refrigerated conditions till use.

Preparation of leaf extract: The extract was prepared by grinding leaves in solvents like Methanol, Ethanol, Dimethyl sulphoxide and Petroleum ether (wt/vol). This mixture was than centrifuged at 10000 rpm at 4ºC for 10 min. The supernatant, thus obtained was used within four hours for estimation of anti-fungal activity.

Patient selection: For the isolation of flu resistant candidal species eighty samples were collected from eighty oral candidiasis patients during Aug 2012 to April 2013. Selected patients had not received any antibiotic or antifungal therapy (15 days prior to sampling) and any surgery, chemotherapy, radiotherapy or immune suppressive therapy (one year prior to sampling). Patients wearing intra oral prosthesis were also excluded from the research.

Sample collection: Required samples were obtained from the patients through oral rinse method [15]. Patients were asked to rinse the mouth with 10ml of normal saline for 60 sec. Then, they were asked to spit the oral rinse in a sterile container. The samples which could not be sent immediately to laboratory were refrigerated. 25 µl of oral rinse was inoculated on Sabaroud's agar plate (SDA) with in 8 hrs of collection and incubated at 37ºC for 48-72 hrs.

Isolation of identifications of fungal species: Preliminary identification was done by Gram staining [15] and Gram positive fungal colonies were sub cultured on SDA plate. The colonies were further subjected to various confirmatory tests as per standard procedure like Lacto phenol cotton blue preparation, Germ tube test, Corn meal Tween 80, Agar culture test, carbohydrate fermentation test and slide culture [16, 17, 18, 19].

Growth of isolated Candida species was maintained on Sabaroud's agar slant (Glucose 40 g/L, Peptone 10g/L , Agar – agar 20 g/L) and SDA agar slat was prepared by standardize procedure [19].

Assessment of the MIC and cellular changes: The minimum inhibitory concentration and 50% inhibitory concentration (IC_{50}) values of the fungal strains under study were determined by the micro dilution methods in accordance with the CLSI M7 – A2 protocol [20]. MIC was defined as the lowest concentration that did not result in any visible growth of the yeast compared with the growth in the control well. Further, intracellular changes in selected fluconazole resistant strain were studied via, total lipid [21], phospholipids, ergosterol, H^3 uptake studies [22].

Statistics analysis: All the data provided in the study represents means ± standard error of the mean (S.E.M.). For all other analyses ANOVA was used ($p<0.05$).

RESULTS AND DISCUSSION:

Isolation of Candida species: The study samples (n=80) were collected from 80 subjects with a mean age of 45.3±8.01. In our study, Candidial species were preliminary identified by Gram staining and then confirmed by Germ tube, Carbohydrate fermentation and colony formation. Our study observed that 77.77% of samples from patients (suffering from Candidiasis, Xerostomia and Mouth ulcer) showed prominent growth of CA (**Table 1**).

TABLE 1: DISTRIBUTION OF VARIOUS FUNGAL SPECIES IN ISOLATED SAMPLES:-

S. no.	Name of fungal species	No. of colonies	%age	Confirmatory test
1	**C. albicans*	35	77.77	Germ tube, **CF, CM
2	*C. glabrata*	3	5.88	CF and ¶CM
3	*C. krusei*	2	3.92	CF and CM
4	*C. pseudotropicalis*	2	3.92	CF and CM
5	*C. tropicalis*	1	1.96	CF and CM
6	*Penicillium marneffei*	1	1.96	¥Cm and Pigment production
7	*Chaetomium*	1	1.96	Cm & LCB preparation

*C- Candida, **CF- Carbohydrate fermentation, ¶CM-Corn Meal and ¥Cm- Colony morphology

Effect of CF leaves extract on isolated strains: Antifungal activity was checked by micro dilution method using isolated strains of *C. albicans*, *C. glabrata*, *C. krusei*, *C. pseudotropicalis* and *C. tropicalis*. For antifungal activity, fresh leaves of CF were grind with DMSO, pet ether, methanol and ethanol in concentration dependent manner (wt/vol). It was observed that DMSO and pet ether leaves extract has very potent anti-fungal action against isolates of *C.albicans* and *C.tropicalis* with IC_{50} of 0.8 mg/ml and 0.6 mg/ml respectively (**Table 2**).

Synergistic effect of CF leaves extract and fluconazole on Flu resistant strains of CA: A phenomenon referred to in this context as drug tolerance was observed with six isolated strains showed resistance against Flu and named as fluconazole resistance strains (FRS). The IC^{50} value of these six FRS was found to be in range form 35- 58 µg/ml (Flu). To further define whether Flu and CF leaves extract work synergistically, six FRS were examined under different combinations of Flu plus CF leaves extracts (at their IC_{50}) as depicted in **table 3**. It was found that cells of FRS that were exposed to Flu (52 µg/ml) were not killed during the overnight incubation (16hrs). On further, cells of FRS expose to combination treatment (Flu plus CF leaves), they did not grow up to the same density as that of control. Results of table 3 reveal that at least 75-85% growth inhibition of CA was observed even at ¼ IC_{50} of both drugs used in combination. It infers that both drugs are acting synergistically. Actively growing CA cells were killed rapidly and no viable cells among 5×10^5 cells were detected after 2 h. of expose to drugs in combination. Similarly, K. Amber *et al* 2010 showed that essential oil of *Ocimum sanctum* is an effective antifungal agent that inhibits Flu sensitive candida species (*C.albicans, C. tropicalis , C. parapsilosis*)but also Flu –resistant *C. albicans*, *C.tropicalis* isolates and *C. krusei* and *C. glabrata* which are intrinsically resistant to Flu or whose resistance is easily inducible and combination Flu/ketoconazole- *Ocimum sanctum* essential oil administered against the fungal strains under consideration is likely to augment the efficiency of these two azoles [23]. It is always true that basis of drug interactions are potentially multifactorial and complex.

TABLE 2: COMPARISON OF INHIBITION ACTIVITY OF *CF* LEAVES EXTRACT (IN DIFFERENT SOLVENTS) AGAINST ISOLATED *CANDIDA* SPECIES WITH THEIR IC_{50} VALVES:-

Leaves Extract	*C. albicans* (29*)	*C. glabrata* (3*)	*C. kruseii* (2*)	*C. pseudotropicalis*	*C. tropicalis*
1. DMSO (mg/ml)	0.8±0.06	2.8±0.65	4±0.23	1.6±0.034	1.1±0.024
2. Pet. Ether (mg/ml)	1±0.021	3.1±0.39	4.8±0.14	0.8±0.039	0.6±0.017
3. Methanol (mg/ml)	1.8±0.083	3.8±0.58	5±0.48	1.7±0.19	2.6±0.049
4. Ethanol (mg/ml)	2±0.15	3.9±0.46	5±0.17	1.9±0.28	2.8±0.068
5. Flu (µg/ml)	0.1±0.024	1.8±0.26	14±0.34	0.4±0.054	0.4±0.15

Note -Values are mean of 8 replicates (IC_{50}± SD valves), * = the number of isolates used for IC_{50} calculation, IC_{50}- 50% inhibitory concentration of leaves extract & Flu.

Synergistic effect of CF leaves extract and fluconazole on intracellular lipids of FRS B: Further, intracellular changes were studied by estimating the concentrations of ergosterol, phospholipid and total lipid of FRS B cells (shown in **table 4**) because this strain has highest value of IC_{50}i.e 52µg/ml (Table 2). These cells were grown in presence of IC_{50} of Flu, CF^{DMSO} and $CF^{Pet\ ether}$ extract and showed significant reduction in concentrations of total lipid, ergosterol and phopholipids. But the phospholipid and ergosterol contents were found to be statistically decreased with FRS-B were grown in combination (1/4 IC_{50} of Flu plus CF^{DMSO} and ¼ IC_{50} of Flu plus CF^{Pet}

ether). It was also observed that phospholipid and ergosterol content was 1.33 and 1.51 times decreased in FRS grown in the presence of CF^{DMSO}, Flu + CF^{DMSO}. But this effect was more pronounced (1.58 and 1.75 times) when FRSs were grown in combination of Flu + $CF^{Pet. ether}$. The decreased content of lipid was because of reduced rate of synthesis of these lipids. The correlation between loss of membrane permeability and leakage of intracellular DNA studies indicate that CF kills CA by damaging the cytoplasmic membrane, leading to an impairment of all cellular functions [24]. Similar observation by Irshad *et al* 2011 showed that *C. fistula* fruit pulp and seed extract possessed anticandidal activity. Therefore, the result of our study was significantly correlated between the MICs and ergosterol inhibition. It was concluded that the leaves extract is a promising source for anticandidal compounds. Sometimes compounds present in plant extract are responsible for their partition into the lipid bilayer of the cell membrane, leading to an alteration of permeability and a consequent leakage of cell contents [3, 4, 8, 25].

Synergistic effect of CF leaves extract and fluconazole on DNA synthesis of FRS B: To further elucidate the interaction of leaves extracts and to find out synergistic behaviour of leaves extract with the nuclear events, DNA content was measured in the isolated nuclei. Nuclear fraction was found to be significantly enriched with DNA indicative of its purity. DNA content also decreased markedly in cells grown in the presence of CF^{DMSO}, Flu + $CF^{Pet. ether}$, $CF^{Pet ether}$ and Flu + $CF^{Pet. ether}$ (shown in **fig. 1**). These finding were further substantiated by H^3 thymidine uptake studies, H^3 thymidine was decreased during all time period. Moreover, at 30 min significantly decline in the DNA synthesis was observed with cells grown in combination of Flu plus CF^{DMSO} (as shown on fig. 1).

TABLE 3: SELECTED PATIENTS WITH ISOLATED FLU RESISTANT STRAIN (FRS)

	FRS 1	FRS 2	FRS 3	FRS 4	FRS 5	FRS 6
Patient (Age/Sex/Area)	32/F/ § SU	44/M/SU	49/M/SU	37/F/SU	34/M/SU	52/M/SU
Oral Manifestation	*C	C	**U, C	U,C	U,C, ±X	U,C, ±X
CFU/mL	1674± 218	5920± 580	5388± 690	5448± 430	Confluent	Confluent
Type of colonies	One	Two	One	One	Three	Two
Gram staining	P	P for both	P	P	N for one	P for both
Grem tube	P	P for one	P	P	P for one	P for one
Clinically isolated fungal strains were confirmed by Carbohydrate fermentation test and Corn meal test						
Organism	**¶CA**	**CA and † CG**	**CA**	**CA**	**CA**	**CA and †† CP**
Flu IC_{50} (µg/mL)	52 ±2.8	35±1.2	40±1.5	48±5.0	58±2.7	40±1.5
CF^{DMSO} IC_{50} (mg/mL)	8.0±0.6	8.6±0.9	10.0±1.1	8.0±1.2	8.0±0.8	12±1.2
$CF^{Pet ether}$ IC_{50} (mg/mL)	9.0±0.9	11.0±0.7	13.0±0.9	9.0±1.1	10.0±0.9	10.0±0.9
Percentage growth inhibition of isolated fungal strains grown under combination of drugs and CF leaves extract in different solvents						
Flu IC_{50} + CF^{DMSO} IC_{50} (X)	98	95	98	95	99	98
½(X)	90	92	92	90	92	95
¼(X)	70	80	78	80	79	77
Flu IC_{50} + $CF^{Pet ether}$ IC_{50}(Y)	92	98	98	90	99	90
½ (Y)	82	85	85	85	87	85
¼(Y)	74	80	75	78	75	77

*- Candidiasis, **U- Ulcer, N-Negative, P-Positive, ±X- Xerostomia, ¶ CA-*Candida albicans*, †-CG *Candida glabrata*, ††CP-*Candida pseudotropicalis*, § SU- Sub Urban

TABLE 4: TOTAL LIPID, PHOSPHOLIPID AND ERGOSTEROL CONTENT OF ISOLATED STRAIN GROWN IN THE PRESENCE OF IC^{50} OF CF EXTRACT, FLU AND THEIR COMBINATIONS

FRS 2 Cells	Total lipid	Phospholipid	Percentage of Phospholipid	Ergosterol	Percentage of Ergosterol
Control	43.6 ± 1.8	15.8±1.2	36.2	20.8±3.2	47.7
Flu IC_{50}	28.1±1.9	9.7±0.6	34.5	8.6±1.2	30.5
CF DMSO IC_{50}	21.1±1.8	7.1±0.8	33.6	7.1±0.5	21.1
CF DMSO IC_{50}	23.2±0.7	5.9±0.7	25.4	11.2±0.8	48.2
¼ (X)	15.1±0.8	3.8±0.5	25.1	2.1±0.5	13.9
¼ (Y)	13.1±1.9	2.1±0.4	16.0	3.6±0.8	27.4

* Valves are expressed in mg/g of dry wt. ± S.D, Values are mean of 8 replicates.

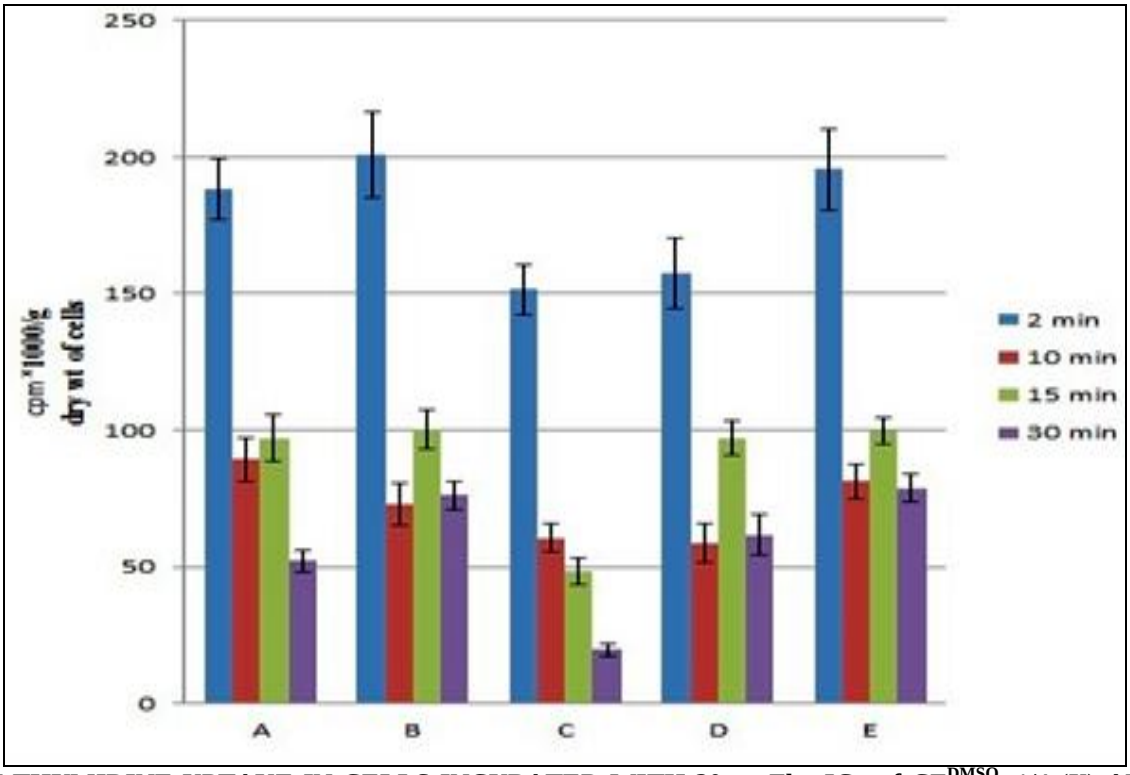

FIG. 1: ^{3}H THYMIDINE UPTAKE IN CELLS INCUBATED WITH 20µm Flu, IC_{50} of CF^{DMSO}, 1/4 (X) AND IC_{50} of $CF^{Pet\ Ether}$, 1/4 (Y)

CONCLUSION: This research work has shown that the DMSO leaves extract of CF has potent antifungal action and this action was much more pronounced with fluconazole. Combination of both drugs induced some intracellular changes that may be responsible for reduced growth of fluconazole resistant CA.

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