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Original Article

Dentistry Section

Invitro Anti-mycotic Activity of Hydro Alcoholic Extracts of Some Indian Medicinal Plants against Fluconazole Resistant *Candida albicans*

SARANYA VARADARAJAN[1], MALATHI NARASIMHAN[2], MALAIYANDI MALAISAMY[3], CHAMUNDEESWARI DURAIPANDIAN[4]

ABSTRACT

Background: Candidiasis is one of the most common opportunistic infections caused by *Candida albicans*. Fluconazole is the drug of choice for prevention and management of this condition. However, the emergence of fluconazole resistant candidal strains has become a major concern. Many herbs like fenugreek, cinnamon, papaya, oregano, garlic are rich in phytochemical constituents known to express antimycotic activity. With the available information, the present research study was carried out to assess the invitro anti-mycotic activity of hydro alcoholic extracts of *Trigonella foenum-graecum* seeds, *Cinnamomum verum* bark and *Carica papaya leaves* and seeds against fluconazole resistant *Candida albicans*

Materials and Methods: Hydro alcoholic extracts of *Trigonella foenum-graecum* (seeds), *Cinnamomum verum* (bark), *Carica papaya* CO.2 strain (male and female leaves) and *Carica papaya* CO.2 strain (seeds) were prepared by maceration. The anti-mycotic activity of the prepared extracts against *Candida albicans* was assessed by agar well diffusion method. Three independent experiments were performed in triplicates and the mean and standard deviation were calculated. Minimum inhibitory concentration was determined.

Results: The results of the present study revealed that all the extracts exhibited anti-mycotic activity in a dose dependent manner and minimum inhibitory concentration of all the extracts was found to be 15.62 µg/ml.

Conclusion: The results of the present study shed light on the fact that plant extracts could be used not only as an alternate drug for management of fluconazole resistant candidiasis but also explored further for oral cancer prevention as a therapeutic adjunct.

Keywords: *Carica papaya, Cinnamomum verum, Trigonella foenum graecum*

INTRODUCTION

Candida albicans formerly known as *Monilia albicans* is a yeast like fungus that belongs to the family Sacharomycetaceae. The other names include *Candida stellatoidea* and *Odium albicans*. This fungus exhibits three forms viz- yeast, pesudohyphae, and chlamydospore. It occurs as a commensal in the oral cavity and in the gastrointestinal tract of humans. Candidiasis is one of the most common opportunistic infections caused by the organism. In the oral cavity, candidiasis is termed as oral thrush due to the formation of white scrapable pseudomembrane. Oral candidal infection occurs in immunosuppressed conditions like acquired immunodeficiency syndrome, cancer chemotherapy and head and neck radiotherapy [1,2]. *Candida albicans* has also been implicated in oral carcinogenesis. *Candida albicans* metabolizes procarcinogens like ethanol and forms acetaldehyde. It also causes nitrosamine production. It also alters tumour microenvironment and induces chronic inflammation [3].

Fluconazole has been used as a "Gold Standard" for management of candidiasis as it has been found effective in both immune compromised and immunocompetent individuals. It has also been used prophylactically to prevent infections in patients receiving chemotherapy and radiotherapy [4]. Fluconazole exerts its antifungal activity by inhibition of 14 alpha lanosteroldemethylase. This leads to accumulation of lanosterol and 14 alpha methylated sterols in the cell membrane of fungi that alters membrane permeability ultimately leading to fungal death [5].

The various mechanisms of fluconazole resistance are explained as follows:

- Point mutations could occur in the ERG11 gene that codes for the enzyme lanosterol 14- alpha demethylase leading to reduced drug affinity to the enzyme product.
- There could also occur an overexpression of the ERG 11 gene leading to the increased synthesis of ergosterol and other steroids that support fungal growth.
- An overexpression of CDR gene (an ABC transporter) and MDR (a major faciltator) could also occur that causes reduction of fluconazole accumulation inside the fungal cell and reduced bioavailability of the same [6].

In this regard, herbs and naturally derived bioactive compounds have been explored for anti-mycotic therapy against resistant pathogens. Herbs are rich in phytochemical constituents like polyphenols, flavonoids, alkaloids, terpenoids, tannins, and glucosinolates that possess antioxidant, antimicrobial and immunomodulatory properties. *Trigonella foenum graceum* commonly called as fenugreek, *Cinnamomum verum* also called as Celyon cinnamon, *Carica papaya* commonly known as papaya possess phytochemicals that are known to exert antimicrobial activity. Moreover these herbs are a part of the normal Indian diet and can be procured in a cost effective manner. With the available information we set out to assess the anti-mycotic effect of hydro-alcoholic extracts of *Trigonella foenum-graecum* (seeds), *Cinnamomum verum* (bark) and *Carica papaya* (leaves and seeds) against fluconazole resistant *Candida albicans*.

MATERIALS AND METHODS

The study was conducted in 2014 in Faculty of Dental Sciences and Faculty of Pharmacy, Sri Ramachandra University. This study has an invitro design.

Collection of plant material and preparation of extracts was done as previously described [7].

Plant material: *Trigonella foenum-graecum* (seeds) *Cinnamomum verum* (bark) were collected from a reputed organic store in Chennai

and reputed spice market in Coimbatore respectively. *Carica papaya* CO.2 strain (*male and female leaves)* and *Carica papaya* CO.2 strain (seeds) were collected from Tamil Nadu Agricultural University Coimbatore. All the herbs were authenticated by Professor P. Jayaraman, Plant Anatomy Research Center, Tamil Nadu, Chennai, India.

Preparation of extracts: Preparation of extracts and fluconazole: Hydroalcoholic extracts of *Trigonella foenum-graecum* (seeds) (60 ethanol: 40 water; v/v), *Cinnamomum verum* bark (70 ethanol: 30 water; v/v), *Carica papaya* CO.2 strain (male and female leaves*)* (60 ethanol: 40 water; v/v) and *Carica papaya* CO.2 strain (seeds) (60 ethanol: 40 water; v/v) were prepared by maceration for 72, 48 and 24 hours. All the extracts individually were pooled together and concentrated using rotary flash followed by vacuum desiccator and stored at 2-4°C until use. Stock solution of the extracts was prepared by dissolving the extract in Dimethylsulfoxide (DMSO). Serial dilutions were done to obtain concentrations of 250, 500, 1000 µg/mL. Fluconazole was procured from Sigma Aldrich and a working concentration of 30 µg/mL was prepared in sterile distilled water.

Preparation of *Candia albicans Culture*: *Candida albicans* MTTC 227 was procured from Microbial Type Culture Collection and Gene Bank, Chandigarh. The Ampule was thawed in a water bath at 25°C for 2 minutes, which was wiped with 70% ethanol and was transferred to potato dextrose agar and incubated at 28°C. Drug resistance was induced according to the protocol of Yan L et al., with modifications [8]. Briefly a colony of *Candida albicans* culture was inoculated into potato dextrose broth and incubated overnight at 30°C in an orbit shaker at 200 rpm (revolutions per minute). An aliquot of this culture containing 10^6 cells was treated with twice the concentration of recently measured minimum inhibitory concentration of fluconazole and incubated 30ºC at in orbit shaker at 200 rpm. When the cultures attained a density of 10^8 cells an aliquot of 10^6 cells was taken and the procedure was repeated. Antibiotic sensitivity test for the last passage was performed to confirm fluconazole resistance and the strain was used for the study. Colonies of resistant strain of *C. albicans* were inoculated in potato dextrose broth incubated at 37ºC for 24 hours in an orbit shaker at 200 rpm. Standard inoculum of the fungus of 1.5×10^6 colony forming units (CFU mL-1) was diluted to 1:100 and turbidity was adjusted to match a McFarland standard [9].

Antimycotic activity: *Candida albicans* was swabbed onto potato dextrose agar using sterile swab sticks. Wells of 9 mm diameter were cut using sterile cork borer. The fungal cultures were treated with hydro alcoholic extracts of *T. foenum-graecum* (seeds), *C. verum* (bark), *C. papaya* CO.2 strain (*male and female leaves* and seeds) at different concentrations 250, 500, 1000 µg/mL using sterile microtips. Drug loaded plates were incubated at 37ºC for 24 hour. The zone of clearance was measured. Three independent experiments were performed in triplicates and mean and standard deviation was calculated.

The percentage inhibition was calculated using the formula [10].

$$\text{Percentage Inhibition (\%)} = \frac{\text{Zone of Inhibition (mm)}}{\text{Diameter of Pertiplate (mm)}} \times 100$$

Determination of Minimum inhibitory concentration: Hydroalcoholic extracts which showed significant zones of inhibition were chosen for the experiment at concentrations of 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 15.62 µg/ml and 7.81µg/ml. Minimum Inhibitory Concentration (MIC) was determined according to the standard protocol of Wariso and Ebong (1996) with slight modifications [11]. The antimycotic activity was classified based on MIC [12,13] as follows:

<100 µg/ml – good

100-500 µg/ml – moderate

500-1000 µg/ml – weak

>1000 µg/ml – inactive

RESULTS

All the extracts exhibited antimycotic activity in a dose dependent manner with zone of inhibition ranging between 10±0.7 to 26±1.82 µg/ml. Minimum Inhibitory concentration of all the extracts was found to be15.62 µg/ml. The results are depicted in [Table/Fig-1-3].

DISCUSSION

Candida albicans a commensal of human oral and gastro-intestinal flora and causes oral and systemic candidiasis in immunocompromised individuals, patients receiving chemotherapy and radiotherapy. It has also been implicated in oral carcinogenesis [3]. Fluconazole is one of the most common drugs used for prophylaxis as well as management of candidiasis as it has been found effective irrespective of the immune status of the individual receiving therapy. The emergence of fluconazole resistant strains warrants the need for alternate drugs.

Herbs and plant derived compounds have been used for the management of various diseases several hundred years ago by Charaka and Susrutha. Herbs and plants are rich in phytochemicals such as polyphenols, flavonoids, alkaloids that could exert antimycotic activity [14]. The antimicrobial and anti-mycotic activity *of Trigonella foenum graecum* (seeds), *Cinnamomum verum* (bark), *Carica papaya (leaves and seeds)* against different organisms has been demonstrated by various authors, however anti-mycotic activity against fluconazole resistant species has not been explored. With the available information we screened the invitro anti-mycotic activity of *Trigoenlla foenum graecum* (seeds), *Cinnamomum verum* (bark), *Carica papaya* (leaves and seeds) against fluconazole resistant *Cancida albicans*. The results of the present study revealed that all the extracts exhibited anti-mycotic activity in a dose dependent manner. The minimum inhibitory concentration of all the extracts depicts that all the extracts possesses good antimycotic activity [12,13].

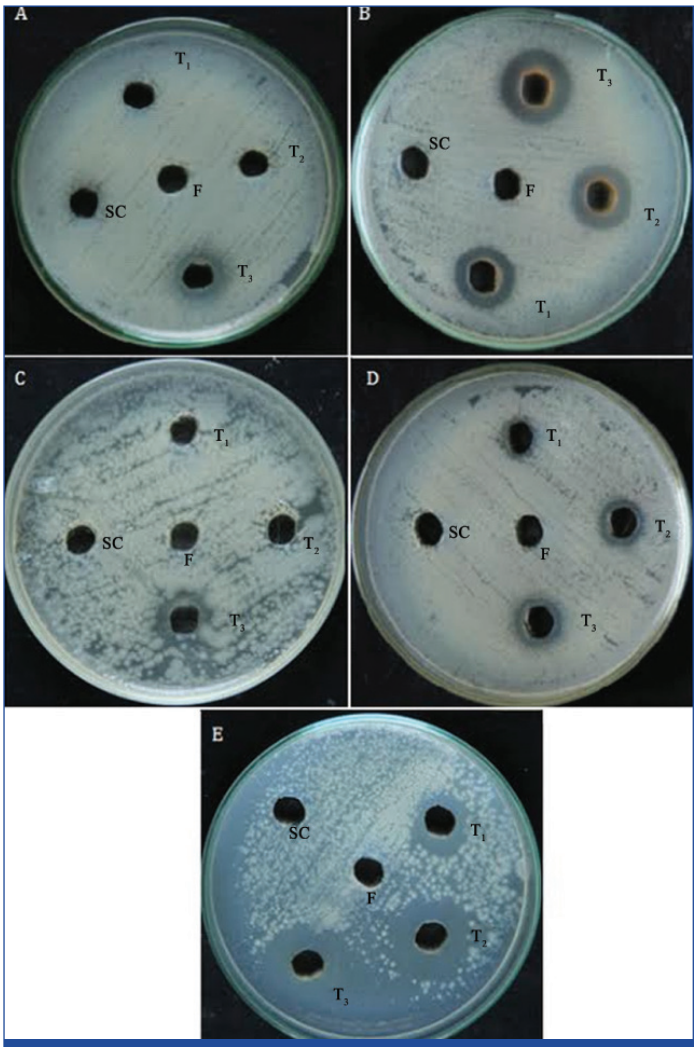
The bioactive toxic oils, volatile oils and alkaloids of *Trigonella foenum graecum* (seeds) exert toxic effects on bacteria, parasites and fungi [15]. The anti-mycotic activity of *Trigonella foenum graecum* (seeds) could also be attributed to polyphenols and flavonoids, however the exact mechanism remains unclear. Faten Omezzine et al., have reported the anti-mycotic activity of *Trigonella foenum graecum* at various stages of development and levels of ploidy and have shown that five novel compounds kaempferol 7-O-glucoside, kaempferol

Samples	Organisms	Zone of inhibition in mm			Percentage of Inhibition (%)		
		250 µg	500 µg	1000 µg	250 µg	500 µg	1000 µg
Trigonella foenum graceum (seeds)	*Candida albicans*	10±0.7	12±0.84	14±0.98	11.11±0.78	13.33±0.93	15.56±1.08
Cinnamomum verum (bark)		16±1.12	18±1.26	20±1.4	17.77±1.24	20.00±1.4	22.22±1.55
Carica papaya (male leaves)		-	-	15±1.05	-	-	16.67±1.16
Carica papaya (female leaves)		11±0.77	13±0.91	15±1.05	12.22±0.85	14.44±1.01	16.67±1.16
Carica papaya (seeds)		22±1.54	24±1.68	26±1.82	24.44±1.71	26.67±1.86	28.89±2.02

[Table/Fig-1]: Antifungal of hydroalcoholic extracts of *Trigonellafoenum-graecum*(seeds), *Cinnamomumverum*(bark) and *Carica papaya (leaves* and seeds) against fluconazole resistant *Candida albicans*. Values are mean ± standard deviation of triplicates of three independent experiments

Samples	MIC µg/mL
Trigonell afoenum-graceum (seeds)	15.62
Cinnamomum verum (bark)	15.62
Carica papaya (male leaves)	15.62
Carica papaya (female leaves)	15.62
Carica papaya (seeds)	15.62

[Table/Fig-2]: Minimum inhibitory concentration of selected medicinal plants



[Table/Fig-3]: Antifungal of hydroalcoholic extracts of *Trigonella foenum-graecum* (seeds), *Cinnamomum verum* (bark) and *Carica papaya (leaves* and seeds) against fluconazole resistant *Candida albicans*.
a- *Trigonellafoenum-graecum* (seeds).
b- *Cinnamomum verum* (bark).
c- CO.2 strain *Carica papaya (male leaves).*
d- CO.2 strain *Carica papaya (female leaves).*
e- CO.2 strain *Carica papaya (seeds).*
f- Fluconazole, SC- solvent control, T1- 250 µg/ml, T2 500 µg/ml, T3- 1000 µg/ml

3-O-β-d-glucopyranoside, kaempferol 7-O-β-d-glucopyranosyl (1–4) β-d-glucopyranoside, kaempferol 3-O-α-l-rhamnosyl (1→2) β-d-xyloside, vitexin hexoside and kaempferol 3-O-β-glucosyl (1→2) (6′-O-acetyl)-β-d-galactoside property of the herb [16].

The anti-mycotic property of *Cinnamomum verum* could be attributed to the presence of Cinnamaldehyde which is one of the most important phytochemical constituent of the plant. The anti-mycotic activity of Cinnamaldehyde has been reported by various authors. Cinnamaldehyde irreversibly affects sterol biosynthesis and ATPase activity in plasma membrane of fungal cell. This leads to accumulation of H^+ ions that alter cellular pH leading to cell death [17-21].

With regard to *Carcia papaya* (leaves), our results are concurrent with the findings of Nwachukwu et al., Pedro Chávez-Quintal et al.,. The terpenes, alkaloids and flavonoids of *Carica papaya* leaves could exert fungicidal effects by interaction with cell membrane of fungi, however the compounds responsible for the property have not been studied [22,23].

In the present study, *Carica papaya* (seeds) exhibited significant antimycotic activity. Onkar Singh et al., have reported the anti-mycotic activity of *Carica papaya* seeds and 2,3,4-trihydroxytoluene, a compound has been isolated from *Carica papaya* seeds with activity against *Candida albicans* [24]. Benzylisothiocyanate is another important phytochemical constituent in *Carica papaya* seeds that could exert antibacterial and fungicidal effects. Isothiocyanates form thiocarbazones and thioureas by reacting with thiol and amino group respectively which causes inhibition of proteins and enzymes essential for survival of bacterial and fungal cell. Derivatives of isothiocyantes also exert antimycotic property [25-27].

LIMITATION OF THE STUDY

It has an invitro design and clinical trials and toxicity studies have not been carried out.

CONCLUSION

The results of the present study shed light on the fact that plant extracts could be used not only as an alternate drug for management of fluconazole resistant candidiasis but also explored further for oral cancer prevention and therapeutic adjunct. Further studies have to be carried out to isolate the active compound and exact mechanism of fungicidal activity that would aid in development of newer drugs.

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PARTICULARS OF CONTRIBUTORS:
1. Senior Research Fellow, Department of Oral Pathology, Faculty of Dental sciences, Sri Ramachandra University, Chennai, India.
2. Professor and Head, Department of Oral Pathology, Faculty of Dental Sciences, Sri Ramachandra University, Chennai, India.
3. Research Scholar, Center for Advanced Studies in Botany, Guindy Campus, University of Madras, Chennai, India.
4. Principal, College of Pharmacy, Sri Ramachandra University.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Malathi Narasimhan,
Professor and Head, Department of Oral Pathology, Faculty of Dental Sciences, Sri Ramachandra University,
Porur, Chennai-600116, India.
E-mail: narasimhan.malathi@gmail.com

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