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In vitro anti-fungal activity of few medicinal plants of Visakhapatnam region against clinically isolated dermatophytes

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Abstract: The aim of the study was to evaluate the antifungal activity of methanol extracts of twenty plant species used in traditional Indian medicine against the clinically isolated fungi. The plants were selected on the basis of their reported ethnobotanical uses. The studies on plants of South Indian medicinal plants collected from regions of Visakhapatnam and were investigated against three clinical fungal isolates viz., *Trichophyton mentagrophytes*, *Epidermophyton floccosum* and *Candida albicans* using agar well diffusion method. The plant extracts were prepared using the solvent methanol. It is clear from the results that, the extract of twenty plants used in this study acts as a good source of antibiotics against various fungal pathogens tested and exhibited a broad spectrum of antifungal activity. The results of this study support the use of all the selected twenty medicinal plants to discover bioactive natural products that may serve as leads in the development of new pharmaceuticals that address unmet therapeutic needs.

Key words: *Trichophyton mentagrophytes*; Anti-fungal; *Epidermophyton floccosum*

Introduction

Fungal diseases represent a critical problem to health and they are one of the main causes of morbidity and mortality worldwide (CSIR. 1998). Human infections, particularly those involving the skin and mucosal surfaces, constitute a serious problem, especially in tropical and subtropical developing countries (Portillo A. *et al.,* 2001). Dermatophytoses are one of the most frequent skin diseases of human, pets and livestock (Tsang P. *et al.,* 1996). Pathogenic fungi, dermatophytes, have the ability to invade keratinized tissues of animals and humans and cause a disease, dermatophytosis, which is the commonest human contagious fungal disease (Esquenazi D. *et al.,* 2004; Sidat MM. *et al.,* 2006). The disease is widely distributed all over the world with various degrees and more common in men than in women. There are three genera of fungi that cause dermatophytosis. They are *Epidermophyton, Trichophyton and Candida* spp. Contagiousness among animal communities, high cost of treatment, difficulty of control and the public health consequences explain their great importance (Chermette R. *et al.,* 2008). Different treatments have been recommended to control dermatophytes. A few antifungal agents are available and licensed for use in veterinary practice or human being treatment.

Large number of antifungal agents using in current day treatments against pathogenic microorganisms are constantly developing resistance to these agents (Al-Bari M.A. *et al.,* 2006). Many of the available antifungal drugs have undesirable side effects or very toxic, produce recurrence, show drug-drug interactions or lead to the development of resistance, some shows ineffectiveness (Muschietti L. *et al.,* 2005) and have become therefore less successful in therapeutic strategies. Increasing use of chemical antimicrobials have created a situation leading to an ecological imbalance and enrichment of multiple multi-resistant pathogenic microorganisms. Therefore, there is a need for the discovery of new safer and highly effective antifungal agents that would overcome these disadvantages. Herbal medicines used in traditional folk remedies may help to overcome the growing problem of resistance to antifungal drugs and their relative toxicity. Herbal medicines are in great demand in the developed countries primarily for their cost effectiveness and no side effects. Plant extracts are likely to provide a valuable source of new medicinal agents and are used traditionally to treat a number of infectious diseases including those caused by bacteria, fungi, protozoa and viruses (Soylu E.M. *et al.,* 2005; Yoshida M. *et al.,* 2005; Nejad B.S. and Deokule S.S., 2009). A number of reports are available in vitro and in vivo efficacy of plant extract against plant and human pathogens causing fungal infections (Natarajan V. *et al.,* 2003). In the present study, the medicinal plants were selected based on their common uses in Indian traditional systems of medicine (Chopra R.N., 1958; Kirtikar K.R. and Basu B.D., 1968; Nadkarani K.M., 1982; Satavat G.V. and Gupta A.K. 1987; Nadkarani K.M., 1989; Chopra R.N. *et al.,* 1992; Ahmad I. *et al.,* 1998). Keeping in view, an attempt has been made to evaluate whether the Indian indigenous plants have any antimicrobial activity against clinical fungal isolates. So the present investigation evaluates the in vitro antifungal activity of crude extracts of twenty medicinal plants against three

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clinical fungal isolates. The zone of inhibition, MIC and MFC are also recorded.

Materials and Methods

Plant Materials

Twenty different medicinal plants free from diseases were collected from the regions of Visakhapatnam, Andhra Pradesh, South India. The plant parts were cleaned of residual soil and air-dried at room temperature.

Preparation of plant extracts

The collected plant materials were dried with active ventilation at ambient temperature (25±1°C) and chopped into small pieces, shade dried and coarsely powdered in Willy mill. The coarsely powdered material weighed and extracted with Methanol using a soxhlet apparatus for five to six hours at temperature not exceeding the boiling point of the solvent. For each 100 grams of dry material 2 litres of solvent was used. The extracted solvents were concentrated under reduced pressure (in vacuo at 40°c) using a rotary evaporator. The residue obtained were designated as crude extracts and stored in a freezer at -20°c until assayed.

Fungal isolation and maintenance

Swab samples from patients were taken by gently rubbing a sterile cotton swab over the infected area and were incubated in saboured dextrose agar for primary isolation of the pathogens. These plates were then aerobically incubated for 24-72 h at 30°C in B.O.D. incubator for fungal species isolation. When growth was appeared on cultured plates, fungal pathogens were identified on the basis of carbohydrate assimilation or fermentation, their macroscopic and microscopic morphologic features after growth on specialized media. Moulds are identified primarily on the basis of their macroscopic and microscopic morphologic features. Newer chromogenic method that couple for the presumptive identification of yeasts and moulds from clinical specimens. All isolated pathogens were compared with MTCC standard strains like *Trichophyton mentagrophytes* (MTCC 7687), *Epidermophyton floccosum* (MTCC No. 613) and *Candida albicans* (MTCC No. 227) respectively.

Isolated clinical strains of fungi *Trichophyton mentagrophytes*, *Epidermophyton floccosum* and *Candida albicans* were taken for this study. The selected isolates were grown on sabouraud dextrose agar (SDA). Twenty-one-day old culture of dermatophytic fungi was scraped with a sterile scalpel and macerated with sterile distilled water. The suspension was adjusted spectrophotometrically to an absorbance of 0.600 at 450 nm. The strains were maintained on sabouraud dextrose agar slants and the cultures were kept under refrigerated conditions and were sub cultured at every 15 days.

Assay for antifungal activity

Fungal inoculums were prepared from overnight grown cultures (24 h) in peptone water (HiMedia, Mumbai, India), and the turbidity was adjusted equivalent to 0.5 McFarland units (equivalent to 1.5 X 10^5 or 10^6 CFU/ml). Flucanozole (10µg/ml) was taken as the positive control for antifungal activity. The DMSO was taken as negative control to determine possible inhibitory activity of the dilutant extract. The susceptibilities of the isolated pathogens were determined by using agar well diffusion method. 20ml of sabouraud dextrose agar (SDA) was taken into sterile universal bottles and these were then inoculated with 0.2ml of overnight culture of the test organism, mixed gently and poured into sterile petridishes. After setting wells of 6mm was punctured with the help of a sterilized cork borer into the pre-solidified sabouraud dextrose agar plates containing the test organism. Using the micropipette, 40 µl of each extract was poured into the different wells of the inoculated plates. Standard antifungal was used as positive control and fungal plates was incubated at 37°Cfor 72 h. The diameter of zone of inhibition was measured. Each experiment was done in triplicate and mean values were taken. Antifungal activity was measured by the diameter (mm) of the clear inhibitory zone formed around the well.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum fungicidal concentration (MFC)

The minimum inhibitory concentration (MIC) of different extracts was determined by microdilution method using serially diluted (2 folds) plant extract according to the National Committee for Clinical Laboratory Standards (NCCLS) (National Committee for Clinical Laboratory Standards, 2000). Equal volume of each extract and peptone broth were mixed in test tubes. Specifically, 0.1ml of standardized inoculums (1-2 ×10^5 cfu/ml) was added in each tube. The tube was incubated aerobically at 37°C for 24 to 48hours. Two control tubes were maintained for each test batch, these included antifungal control (tube containing extract and growth media without inoculums) and organism control (tube containing the growth medium, saline and the inoculums). The lowest concentration (highest dilution) of the extract that produced no visible fungal growth (no turbidity) when compared with the control tube was regarded as MIC. However, the MFC was determined by sub culturing the test dilution on to SDA medium and incubated further for 72 h, (Rubio, L.A. *et al.,* 2005). The highest dilution that yielded no fungal colony on the solid medium was taken as MFC.

Table 1: List of plant names

Plant Name	Common Name	Family	Part used
Acacia catechu	Kat or cacho	Fabaceae	Bark
Bacopa monnieri	Water hyssop, Neetibrahmi, Sambareni	Plantaginaceae	Whole Plant
Bauhinia purpurea	Devakanchanam, Camels foot tree	Leguminosae	Bark
Camellia sinensis	Tea plant	Theaceae	Leaf
Caralluma adscendens	Kundatikommulu	Asclepiadaceae	Aerial Parts
Catharanathus roseus	Periwinkle, Billaganneru	Apocynaceae	Aerial Parts
Cinnamomum zeylanicum	Cinnamon, Dalchina	Lauraceae	Bark
Cissus quadrangularis	Nalleru	Vitaceae	Aerial Parts
Curcuma longa	Turmeric	Zingiberaceae	Rhizome
Gymnema sylvestre	Podapatri	Apocynaceae	Whole Plant
Symplocos racemosa	Lodhra	Symplocaceae	Bark
Mentha arvensis	Pudina	Lamiaceae	Leaf
Mucuna gigantean	Enugu dulagondi, Elephant cow itch	Fabaceae	Leaf
Ocimum sanctum	Tulasi	Lamiaceae	Aerial Parts
Punica granatum	Pomegranate	Lythraceae	Peel
Rosmarinus officinalis	Rosemary	Lamiaceae	Aerial Parts
Senna alexandrina	Senna	Fabaceae	Leaf
Vitex negundo	Vavili, Nirgundi	Lamiaceae	Leaf
Vitis vinifera	Grape vine	Vitaceae	Seeds
Withania somnifera	Ashwagandha	Solanaceae	Root

Table 2: Antifungal activity of different concentrations of plant extracts on *Trichophyton mentagrophytes*

Plant Name	zone of inhibition in mm			MIC mg/ml	MFC /100ml
	500mg/ml	250mg/ml	100mg/ml		
Acacia catechu	14	12	9	100±1	0.01
Bacopa monnieri	18	14	10	100±0.5	0.01
Bauhinia purpurea	13	11	10	90±1.05	0.00625
Camellia sinensis	15	12	11	100±0.5	0.01
Caralluma adscendens	19	15	12	90±0.50	0.00625
Catharanathus roseus	24	21	18	25±1.5	0.00312
Cinnamomum zeylanicum	19	18	15	75±1.10	0.00625
Cissus quadrangularis	19	15	10	100±0.5	0.01
Curcuma longa	13	12	11	90± 0.5	0.01
Gymnema sylvestre	15	11	11	90±1	0.01
Symplocos racemosa	17	13	9	85±2	0.00625
Mentha arvensis	15	11	10	100±2	0.01
Mucuna gigantea	10	9	8	100±2.5	0.01
Ocimum sanctum	17	17	15	85±2.00	0.00625
Punica granatum	15	14	13	75±1.50	0.00625
Rosmarinus officinalis	11	9	8	100±1.15	0.01
Senna alexandrina	14	12	10	75±2.25	0.00625
Vitex negundo	9	8	8	100±2.00	0.01
Vitis vinifera	17	13	8	100±1.50	0.01
Withania somnifera	14	15	12	75±0.15	0.00625
Std. (Flucanazole)		37		10µg/ml	

6mm borer size

Table 3: Antifungal activity of different concentrations of plant extracts on *Epidermophyton floccosum*

Plant Name	zone of inhibition in mm			MIC mg/ml	MFC /100ml
	500mg/ml	250mg/ml	100mg/ml		
Acacia catechu	17	15	10	90±1	0.01
Bacopa monnieri	11	9	10	90±0.5	0.01
Bauhinia purpurea	15	11	11	75±1.05	0.00625
Camellia sinensis	13	10	10	100±0.5	0.01
Caralluma adscendens	11	13	10	90±0.50	0.00625
Catharanathus roseus	25	20	21	25±1.5	0.00312
Cinnamomum zeylanicum	15	11	12	90±1.10	0.00625
Cissus quadrangularis	11	09	8	100±0.5	0.01
Curcuma longa	16	13	11	90±0.5	0.01
Gymnema sylvestre	14	11	9	100±1	0.01
Symplocos racemosa	15	14	11	85±2	0.00625
Mentha arvensis	12	11	11	85±2	0.01
Mucuna gigantea	10	9	8	100±2.5	0.01
Ocimum sanctum	12	11	7	85±2.00	0.00625
Punica granatum	19	16	18	75±1.50	0.00625
Rosmarinus officinalis	12	13	11	100±1.15	0.01
Senna alexandrina	16	14	12	75±2.25	0.00625
Vitex negundo	9	9	7	100±2.00	0.01
Vitis vinifera	14	15	10	100±1.50	0.01
Withania somnifera	11	10	9	75±0.15	0.00625
Std. (Flucanazole)		37		10µg/ml	

6mm borer size

Table 4: Antifungal activity of different concentrations of plant extracts on *Candida albicans*

Plant Name	zone of inhibition in mm			MIC mg/ml	MFC /100ml
	500mg/ml	250mg/ml	100mg/ml		
Acacia catechu	25	21	16	50±1	0.00625
Bacopa monnieri	13	11	11	90±0.5	0.01
Bauhinia purpurea	9	7	8	100±1.05	0.00625
Camellia sinensis	16	11	11	100±0.5	0.01
Caralluma adscendens	18	15	12	90±0.50	0.00625
Catharanathus roseus	28	27	18	50±1.5	0.00312
Cinnamomum zeylanicum	17	12	8	100±1.10	0.01
Cissus quadrangularis	21	17	10	100±0.5	0.01
Curcuma longa	16	13	11	90±0.5	0.01
Gymnema sylvestre	17	15	11	100±1	0.01
Symplocos racemosa	21	17	11	75±2	0.00625
Mentha arvensis	13	9	7	100±2	0.01
Mucuna gigantea	18	13	10	100±2.5	0.01
Ocimum sanctum	23	19	10	100±2.00	0.00625
Punica granatum	25	19	15	75±1.50	0.00625
Rosmarinus officinalis	13	11	7	100±1.15	0.01
Senna alexandrina	18	13	10	85±2.25	0.00625
Vitex negundo	19	16	8	100±2.00	0.01
Vitis vinifera	21	18	8	100±1.50	0.01
Withania somnifera	17	12	10	85±0.15	0.01
Std. (Flucanazole)		37		10µg/ml	

6mm borer size

Results

In the present study the methanol extracts of twenty medicinal plants were screened against three fungal isolates. The details of the plants along with their family name, common name, part used have been listed in Table 1.

The antifungal activity of different plant extracts has been listed in Table 2, 3 and 4. *Catharanthus roseus* and *Punica granatum* revealed significant antifungal activity against all tested organisms. Our study revealed all chosen medicinal plant exhibited moderate to good antifungal activity against fungi studied. Out of selected medicinal plants *C.roseus* exhibited good antifungal activity.

In our study the percent activity i.e the total antifungal potency of studied plants extracts was evaluated with varying concentrations ranging from 10µg/ml to 500mg/ml. The effective MFC (25µg/ml) was observed with methanol extract of *C.roseus* against all the studied clinical isolates. The potency of the extract based on the zone of inhibition, was compared with standard commercial available antibiotic such as Flucanzole at (10 µg/ml) observed that the antifungal activity of crude extracts was less than those of the standard drug.

Discussion

Treatment used for the control and management of fungal infections resulting in emerging mutations and spread of resistant organisms. Many organisms acquire several resistance mechanisms; making them multi-drug-resistant (MDR) (Kenneth VIR, 2009). Therefore, the rapid propagation in antibiotic resistance and the increasing interest in natural products have placed medicinal plants in the front lights as a reliable source for the discovery of active anti-microbial agents and possibly even novel classes of antibiotics (Schultes RE, 1960).

In the present study significant antifungal activity was exhibited by the extract of *C.roseus* against *T.mentagrophytes*, *E.floccosum* and *C.albicans***.** Although numerous studies have in the past, focused on antimicrobial activity of different plants but very few studies have been focused on the antimicrobial activity of this medicinal plant against clinical isolates. According to Wankhede S.B. *et al.,* 2013, the methanol leaf extract of *Catharanthus roseus*, *Nerium oleander* and *Tabernemontana divaricata* showed activity against *C.albicans* ATCC 90028. In another study, the root extract of *Withania somnifera* (Ashwagandha) was also found to be very effective against *Candida albicans* (Haradeep Kaur *et al.,*). Rai M.K. and Upadhyay S. 1998, Extracts of leaves and stems of *Catharanthus roseus* showed good efficacy against *T.mentagrophytes*. Water extract of stem of *C.roseus* showed highest antifungal activity (77.72%) whereas water extract of leaves reported 40.12per cent antifungal activity. In another study, the leaf extracts of *Catharanthus roseus* were most active against the fungal cultures of *Trichophyton rubrum*, *T.mentagrophytes*, *Microsporum gypseum* and *Candida albicans*. Water and methanolic extracts of all plant parts were least effective against all fungi, but bound flavonoids of leaves and stem extracts showed good efficacy against all the four test fungi (Seema Bhadauria and Padma Kumar, 2011). To a large extent, the chronological age of the plant, percentage humidity of the harvested material, situation and time of harvest, solvent used and the method of extraction were possible sources of variation for the bioactivity of the extracts (Felix MT, 1982; Eloff JN, 1998; Nimri LF. *et al.,* 1999).

The results of our research highlights, the fact that the organic solvent extracts exhibited greater antimicrobial activity because the antimicrobial principles were either polar or non-polar and they were extracted only through the organic solvent medium (Mohanasundari C. *et al.,* 2007; Britto JS, 2001). So the present observation suggests that the organic solvent extraction was suitable to verify the antimicrobial properties of medicinal plants which are also supported by many other investigators (Krishna KT. *et al.,* 1997; Singh I and Singh VP, 2000; Natarajan E. *et al.,* 2003; Natarajan D. *et al.,* 2005). Apart from the studies earlier, the uses of *C.roseus, P. granatum, O.sanctum* and *W.somnifera* in the Indian traditional system of medicine to treat various diseases were justified by our study.

Conclusion

The present investigation reported that the medicinal plants used to study possess antimicrobial activity against the three clinical fungi which were used. These findings can form the basis for further studies of toxicity testing, isolate active compounds, elucidate the structures, and also evaluate them against wider range of resistant fungal strains with the goal to find new therapeutic principles.

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