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Antifungal susceptibility testing of few medicinal plant extracts against *Aspergillus* spp. and *Microsporum* sp.

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ABSTRACT

Mycoses are fungal infections, the incidence of which in immunocompromised patients is currently devastating and the drugs available at hand are reported to exhibit side effects. To surmount the prevailing difficulty of complete eradication of the fungal infection, exploring new arena is a requisite and nature's wealth can be one area that may lead to a cure for fungal infections. In the present study, the whole methanolic extracts of few medicinal plants were evaluated against *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger* and the dermatophyte *Microsporum gypseum* isolated from the soil. *Enicostemma littorale* and *Wrightia tinctoria* exhibited significant (P<0.001) inhibition of about 48% against *A. niger*. *Eupatorium odoratum* inhibited both *A. fumigatus* (52%) and *A.flavus* (32%) whereas *Enicostemma littorale* showed about 54% of significant (P<0.05) inhibition against *A. fumigatus*. Significant inhibition of *M. gypseum* was exhibited only by *Sphaeranthus indicus* flower extracts (65% at P<0.05) which was even higher than the inhibition exhibited by positive control ketaconazole (49%) at 0.1 mg/ml concentration. This study demonstrates that among the medicinal plants evaluated *E.littorale*, *W.tinctoria*, *E.odoratum* and *S.indicus* flower exhibited significant antifungal activity against the tested organisms.

INTRODUCTION

Fungi are the eukaryotic organisms exist ubiquitously. They are significantly involved in many fungal infections and in the food spoilage thereby retarding its nutritive value. The toxin produced by them shows the effect of carcinogenicity, genotoxicity, teratogenicity, nephrotoxicity, hepatotoxicity, reproductive disorders and immunosuppression (Satish *et al.*, 2007) on consumption of spoiled food. The types of infections they cause are categorized as superficial, systemic and opportunistic and they are considered as one of the most dreadful diseases. Since the medications discovered until are reported in causing adverse side effects so far a suitable medicine was not identified. From ancient times, the usage of herbs and plants of medicinal value were in practice against several diseases including infections caused by fungi. This study focuses on few medicinal plants such as *Enicostemma littorale*, *Eupatorium odoratum*, *Holoptela integrifolia*, *Trichodesma indicum*, *Sphaeranthus indicus* flower, *Sphaeranthus indicus* aerial parts, *Vitex negundo* and *Wrightia tinctoria* which were evaluated for antifungal efficacy against the fungal species, *Aspergillus flavus*, *Aspergillus funigatus*, *Aspergillus niger* and dermatophyte *Microsporum gypseum*, through *in vitro* analysis. *Aspergillus* is a mold, found common and widespread and is a causative agent of aspergillosis which includes Invasive Pulmonary Aspergillosis (IPA), Non-invasive or Allergic Pulmonary Aspergillosis (ABPA), Chronic Pulmonary and Aspergilloma (CPA) and Severe Asthma with Fungal Sensitisation (SAFS) and Aspergilloma. These infections are life-threatening in immunocompromised patients, where *A. fumigatus* is the major causative organism in Invasive Aspergillosis (IA) cases (Chamilos and Kontoyiannis, 2005). Dermatophytes are a group of filamentous fungi that utilize keratin as the major source for growth and are the most common cause of cutaneous mycoses. They are of three genera *Trichophyton*, *Microsporum* and *Epidermophyton* and ecologically they are grouped as anthropophilic (human associated), zoophilic (animal associated) and geophilic (soil dwelling) (Achterman and White, 2012). The diseases are named according to the site of infection such as Tinea ungium (nail infection), Tinea pedis (athelete's foot) etc.

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Generally they are superficial infections but in immunocompromised patients they are experienced as disseminated disease. Despite the fact that the fungal infections are treatable, the complete eradication of the infection is uncertain due to the high rate of reinfection either as relapse or as a new infection.

Since the plants are rich source of bioactive secondary metabolites of wide variety such as tannins, terpenoids, saponins, alkaloids, flavonoids and other compounds, they become useful source of lead compounds of novel structure (Arif *et al.,* 2009). Therefore the present investigation could facilitate the determination of molecules with antifungal activity which is of great importance to human.

MATERIALS AND METHODS

Plant material and extraction

The fresh plant materials (Table 1) collected were washed under running tap water, shade dried and then homogenized to fine powder. The powdered plant samples were extracted with methanol solvent using soxhlet apparatus and evaporated at room temperature to obtain crude extracts which was further dissolved in Dimethyl sulfoxide (DMSO) to prepare stock solution of 250 µg/ml concentration.

Keratin baiting technique

Isolation of dermatophyte *Microsporum gypseum* from soil sample was carried out using hair-bait technique (Simpanya and Baxter, 1996). Soil sample was collected from Thoppampatti area of Coimbatore District around the cattle farm.

About 50 g of soil was placed into empty sterile petri dish and baited with sterilized human hair, cut into small pieces. The soil was moistened with 5-10 mL of sterilized distilled water containing antibiotics (ampicillin 0.1 µg/mL and cycloheximide (actidione) 0.1 µg/mL).

The baited samples were incubated at 25 °C in the dark and moistened at intervals of 3-4 days to prevent the soil from drying. The plates were inspected daily for up to 5 weeks before being discarded. The presence of keratinophilic fungi was confirmed by low power microscopic examination. Fragments of colonized hair were inoculated onto slopes of Sabouraud dextrose agar (SDA) containing cycloheximide and ampicillin and were incubated for 2 weeks at 25 °C.

Fungal strains and culture condition

The fungal strains *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus funigatus* and the geophilic dermatophyte *Microsporum gypseum* were grown and maintained in Potato dextrose agar (PDA) and SDA respectively.

The fungal strains were allowed to grow in respective agar medium and the fungal mycelia before the spore formation was used for antifungal assay in order to avoid the spreading of spores throughout the plate which will interrupt in the radial growth measurement.

Antifungal assay

Antifungal assay was determined using poisoned food technique (Grover and Moore, 1962). The sterilized PDA and SDA agar medium were poured into the sterile petri dishes and allowed to solidify. Normal and positive control (ketaconazole 0.4 µg/ml concentration) were maintained and the radial growth pattern of the organisms (*A.niger, A.flavus, A.fumigatus, M.gypseum)* at dilutions of 1000 µg/mL of the different plant methanolic extracts in SD agar were compared with the controls. 0.9 mL of the standard suspension of the organism was inoculated on test and control plates. Both the plates were incubated at room temperature and observed for growth on each day until the growth covers the control plate. A growth curve was plotted using plant extracts on X axis and extent of growth (cm) on Y axis. The experiments were carried out in triplicate.

Statistical analysis

Each *in vitro* experiment was performed in triplicate. Experimental results were expressed as mean ± SE (Standard error). The data were also analyzed statistically using One-way analysis of variance (ANOVA) and differences among the means were determined for significance at $P \leq 0.05$ and $P \leq 0.01$ using Windows excel.

RESULTS AND DISCUSSION

Right from its existence, the human race has depended on plants for food and then for both food and medicine. Scazzocchio *et al.* (2001) considered the bioactive chemical compounds from medicinal plants and found them to be most valuable alternatives to many sub-standard orthodox synthetic medicines that have side effects. Arif *et al.* (2009) have reviewed plant derived secondary metabolites possessing antifungal activity and recently, Vashist and Jindal (2012) have reviewed medicinal plants with antimicrobial activity which may aid the development of improved or novel chemotherapeutic agents against microbial infection. In order to combat the side effects caused by prolonged use of such drugs against microbial infections, researchers have been taking efforts to depend on natural sources.

Recently few of the Saudi Arabian desert plants were examined for their antimicrobial potency in which methanolic extract of *Tamarix aphylla* leaves exhibited significant inhibition against filamentous fungi *A. flavus*, *A. fumigatus* and *Penicillium chrysogenum* showing MIC at 2 mg/mL concentration (Zain *et al.,* 2012). The leaves of some of the garden plants, *Bougainvillea glabra choicy*, *Lantana camara* L. and *Delonix regia (Hook) Raf*, were also evaluated for antimicrobial activity against *Lactobacillus* sp., *Streptococcus mitis*, *Candida albicans* and *A.niger*, where the *Bougainvillea glabra choicy* exhibits excellent inhibition against all the tested organisms (Rani *et al.,* 2012). On these lines, an attempt had been made to evaluate the activity of few medicinal plants against *A.niger*, *A.flavus*, *A.fumigatus* and the dermatophyte *M.gypseum*. The methanolic extracts of the selected plants were obtained for 30gms of dry plant material using soxhlet

apparatus and the crude extract weight for each plant was about 1.4 mg, 2.1 mg, 0.6 mg, 2.0 mg, 0.9 mg, 0.4 mg, 1.2 mg and 2.3 mg for *E.littorale*, *E.odoratum*, *T.indicum*, *S.indicus* excluding flower and *S.indicus* flower alone, *W.tinctoria* and *V.negundo*, respectively. The percentage of inhibition exhibited by the methanolic extracts is summarized in Table 2. The methanolic extracts of the plants were used at a concentration of 1000 µg/ml of medium and the inhibition on the growth of the tested organisms differed for each plant extract. The concentration of the ketaconazole (KTZ) was determined as 0.4 µg/mL for the present study based on the reports of Therese *et al.,* (2006) on MIC of KTZ against few ocular fungal isolates. In the present study, the unfractionized methanolic extract of all the selected plants produced significant inhibition of growth on *A.niger* ranging from 40-48% which is comparable to the inhibition produced by KTZ (67%). Specifically both *E.littorale* and *W.tinctoria* exhibited 48% of inhibition against *A.niger* followed by the *S.indicus* flower (46%). The antifungal study for the whole plant extracts of *E.littorale* extracts was evaluated by Gopal *et al.* (2011) using three different concentrations 100µg/mL, 200µg/mL and 400 µg/mL and the inhibitory potency was compared with standard clotrimazole and ketaconazole each at 10µg/mL concentration. The inhibition was observed to be pronounced in ethanol extract against both *A. niger* (14mm zone of inhibition for 400 µg/mL concentration) and *Candida albicans* (15mm zone of inhibition for 400 µg/mL concentration) and hexane, chloroform extract showing moderate and pronounced activity against *A.niger* with 15mm and 16mm dia. of zone of inhibition, respectively. The inhibitory effect of lesser concentration (400 µg/mL) may be attributed to the fractionized ethanolic extract but in the present study the rationale behind the higher concentration (1000 µg/ml) required to produce significant inhibition may be ascribed to unfractionized methanolic extract. Inhibition against *A.fumigatus* exhibited by KTZ was about 64% whereas observation among the plant extracts were less than the drug, 42% and 39% of inhibition exhibited by *S.indicus* flower and *E.littorale*. Other extracts had insignificant inhibitory activity (<30%). In the case of *A.flavus*, KTZ showed 62% of inhibition and the plant extracts exhibited the percentage inhibition of about 32% by *E.odoratum* and 25% by *S.indicus* flower. Accordingly, the ethanol extracts of *E.odoratum* leaves showed significant inhibitory zone against *A.flavus* (2.8mm) than *A.niger* (2.3mm) at the concentration of 150µg/mL (Suriyavathana *et al.,* 2012). Inhibition of dermatophyte *M.gypseum* exhibited by the extract of *S.indicus* flower was observed to be 65% which is considered as significant, since it showed about 7.5 fold higher activity than the effect exhibited by KTZ (49%). *V.negundo* exhibited about 54% inhibition whereas other extracts produced negligible inhibition against *M.gypseum*. In a previous report, aerial parts and flowers of *S.indicus* were extracted with hexane, benzene, choloroform, ethylacetate and acetone and evaluated for its antimicrobial activity against few bacterial species and *Candida albicans*. In which, the hexane extract of both aerial parts and flowers exhibited effective inhibition (Duraipandiyan *et al.,* 2009). Even the leaf parts of *S.indicus* extracted with ethanol showed inhibition against *B.subtilis*, *S.aureus* and *C.albicans* with the MIC 0.5mg/mL, 2.5mg/mL and 0.5mg/mL, respectively (Meher *et al.,* 2013). The growth pattern of the tested organism on control plate and extract treated plates were observed (Figure. 1) on consecutive days. Owing to the prevalence of cancer, AIDS and other immunocompromise infections among the human race, the incidence of fungal infections has been on the increase. Despite the availability of numerous antifungal agents, it has been seen that continuous intake results in development of drug resistance. Therefore, identification of new classes of drugs derived from natural sources is an immediate necessity. Since the plant kingdom provides novel lead compounds, a wide-scale investigation is required. Under the circumstance, the current study provides an insight into the antifungal and antidermatophytic activity of the collected plants. Hence, it is concluded that medication based on natural sources could lead to cure without side effects and better health of the humankind in near future. More efforts have to be undertaken by the scientific community to bring about effective remedies in every possible form to serve humanity.

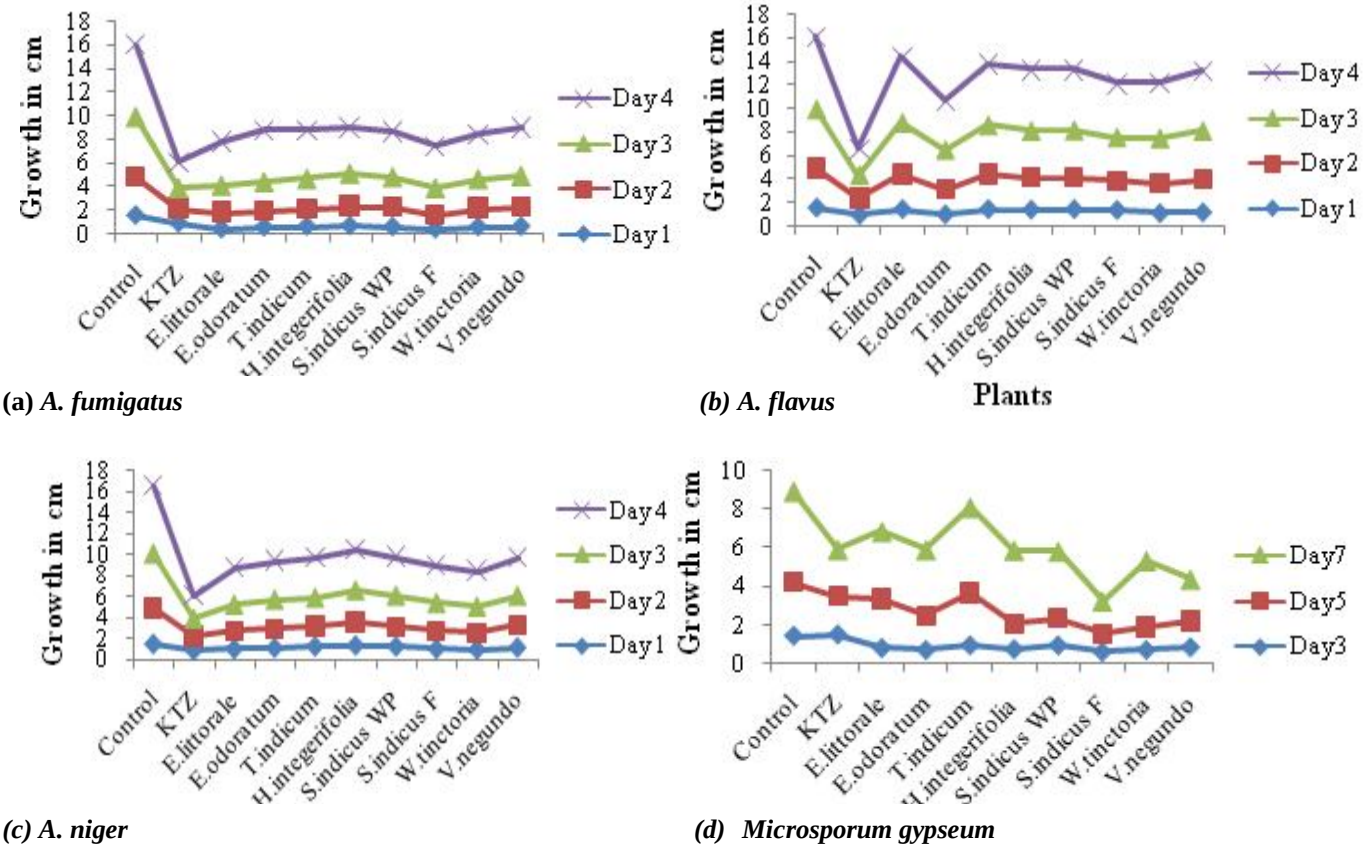
Table. 1: List of plant species selected and studied for its antifungal potency.

S.No.	Plant Botanical Name	Common Name	Tamil Name	Family Name	Parts Used	Traditional Use
1.	*Enicostemma littorale*	White head	Vellarugu	Gentianaceae	Whole aerial parts	Leave decoction used in rheumatism, abdominal ulcers, hernia, swelling, itches and insect poisoning (Sankaranarayanan *et al.*, 2010)
2.	*Eupatorium odoratum*	Bitter bush	Aana vanthan	Compositae	Leaves	Leaves crushed to treat skin wounds in Indonesia (Ayyanar and Ignacimuthu, 2009)
3.	*Holoptelea integrifolia*	Indian Elm	Aavimaram	Ulmaceae	Leaves	Leaves and bark are used in skin diseases (Saxena, 2012)
4.	*Trichodesma indicum*	Indian Borage	Kallutaitumapi	Boraginaceae	Whole Plant	Roots are made into paste with water and applied externally to swollen joints, inflammations and superficial skin injuries (Periyanayagam *et al.*, 2012)
5.	*Sphaeranthus indicus*	East Indian Globe Thistle	Kottakkarandai	Compositae	Flower, whole aerial parts excluding flower	Leaf, Flower and seeds, are ground into paste and applied topically to treat skin disease (Shiddamallayya *et al.*, 2010)
6.	*Wrightia tinctoria*	Pala Indigo	Paalai	Apocynaceae	Leaves	Powder of leaf, root and bark with leaves is made into a paste with water and applied externally to cure skin diseases (Dhanabal *et al.*, 2012)
7.	*Vitex negundo*	Five-leaved chaste tree	Nochhi	Lamiaceae	Whole plant	Essential oil of the leaves is effective in treatment of venereal diseases and other syphilitic skin disorders (Vishwanathan and Basavaraju, 2010)

Table. 2: Effect of methanolic extracts (1000 µg/mL) on the growth of *Aspergillus* spp. and *Microsporum gypseum*.

Plant species	*A.niger*		*A.fumigatus*		*A.flavus*		*M. gypseum*	
	Growth in cms	Inhibition %age	Growth in cms	Inhibition %age	Growth in cms	Inhibition %age	Growth in cms	Inhibition %age
Control	6.43 ± 0.07	-	6.15 ± 0.09		6.10 ± 0.06	-	4.50 ± 0.09	-
Positive control	2.13 ± 0.07	67	2.20 ±0.06	64	2.30 ± 0.06	62	2.43 ± 0.02	49
Enicostemma littorale	3.37 ± 0.03*	48	3.73 ± 0.03*	39	5.57 ± 0.07	9	3.50 ± 0.06	26
Eupatorium odoratum	3.63 ± 0.03	44	4.35 ± 0.09	29	4.13 ± 0.07*	32	3.43 ± 0.03	27
Trichodesma indicum	3.67 ± 0.07	43	4.13 ± 0.03	33	5.20 ± 0.10	15	4.43 ± 0.00	6
Holoptela integrifolia	3.83 ± 0.07	40	3.90 ± 0.06	37	5.20 ± 0.10	15	3.83 ± 0.07	19
Sphaeranthus indicus aerial parts excluding flower	3.63 ± 0.03	44	3.87 ± 0.09	37	5.17 ± 0.13	15	3.47 ± 0.03	27
Sphaeranthus indicus flower	3.47 ± 0.03	46	3.55 ± 0.03*	42	4.57 ± 0.07*	25	1.67 ± 0.03*	65
Wrightia tinctoria	3.33 ± 0.03*	48	3.77 ± 0.04	39	4.73 ± 0.13	22	3.43 ± 0.03	27
Vitex negundo	3.57 ± 0.03	45	4.07 ± 0.09	33	5.13 ± 0.03	16	2.17 ± 0.07*	54

Note: ***A. niger*- *P*<0.01; *A. flavus*- *P*≤0.01; *A. fumigatus*- *P*<0.05; *M. gypseum*- *P*<0.05; * indicates significant inhibition.**



(a) *A. fumigatus* **(b) *A. flavus***

(c) A. niger ***(d) Microsporum gypseum***

Fig. 1: Comparison of Growth pattern of (a) *A. fumigatus*, (b) *A. flavus*, (c) *A. niger* and (d) *M. gypseum* with control and plant extract treated plates.

CONCLUSION

Rapid research on the evaluation of antifungal property exhibited by the medicinal plants would in near future greatly assist the mankind in the identification of novel compounds against the ailing diseases. In the case, the current study has given focus on the plants *E. littorale*, *W. tinctoria*, *S. indicus* flower with determined ability against the fungal species used.

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