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Evaluation of Five Indigenous Medicinal Plants of Sindh, Pakistan for their Antifungal Potential

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Abstract. Candidiasis and systemic mycosis due to opportunistic pathogens is frequently reported in Sindh, especially in rural areas. In search of local antifungal medicinal plants, methanol, petroleum ether and aqueous extracts of five native medicinal plants *Trachyspermum ammi*, *Hyoscyamus niger*, *Carum roxburgianum*, *Linum usitatissimum* and *Centella asiatica* were screened against five *Candida* strains including three strains of *Candida albicans* and one strain of *C. glabrata* and *C. tropicalis*, each. Antimicrobial screening of five filamentous fungal strains of clinical origin comprising of three strains of *Aspergillus niger*, one species of *A. flavus* and *Penicillium* each, revealed 100% activity of methanolic extract of *T. ammi*; petroleum ether extract of *T. ammi* and *H. niger* and methanolic extracts of *H. niger*, *C. asiatica* and *C. roxburgianum* produced 60, 20, 50, 50 and 10% inhibition, respectively, whereas, *L. usitatissimum* was inactive. Reference antibiotics were Nystatin and Amphotericin-B for yeast species and filamentous fungi, respectively. Least minimum inhibitory concentration (125 mg/disc) against *Candida* sp. was produced by the methanolic extract of *T. ammi* and *H. niger* and 500 and 1000 mg/disc against *Aspergillus* species, respectively. Results indicated that *T. ammi* and *H. niger* may be considered as potential future antifungal agents.

Keywords: mycosis, candidiasis, *Trachyspermum ammi*, *Hyoscyamus niger*, *Carum roxburgianum*, *Linum usitatissimum*, *Centella asiatica*, filamentous fungi

Introduction

During the last 20 years, risk of opportunistic fungal infections has highly increased in patients who are severely compromised due to cancer chemotherapy, organ or bone marrow transplantation and human immunodeficiency (Wingard *et al*., 1993; 1979). Candidiasis is an acute or chronic, superficial or deep infection with a very wide clinical spectrum; it occurs mostly in patients who are predisposed to an overgrowth of their own yeast flora. Candidal vaginitis is predominantly caused by strains of *Candida albicans* (Sobel *et al*., 2003; 1995) and is a common problem in immunocompetent or healthy women. Human mycosis, which may be life threatening in immuno compromised patients, is not effectively treated due to inefficiency and toxicity of the available drugs (Feresin *et al*., 2001).

About 50% of all small molecules approved as therapeutic drugs during 2000-2006 were of natural origin (Newmann and Cragg, 2007). *Trachyspermum ammi* (Umbliferacae) is reported to possess thymol (39.1%) as major constituent and some antifungal potential (Singh, 2004). Three withanolide class steroids were isolated from the seeds of *Hyoscyamus niger* (Solanaceae). Two of them were identified as datura-lactone-4 and Nic-3 (which is now named hyoscyamilactol). The new compound was elucidated as 16a-acetoxyhyoscyamilactol on the basis of spectroscopic properties and X-ray crystallographic analysis (Ma *et al*., 1999). The major components of *C. roxburgianum* were thymol (49.0%), g-terpinene (30.8%), *p*-cymene (15.7), b-pinene (2.1%), myrcene (0.8%) and limonene (0.7%) (Khajeh *et al*., 2003). Leaves of *C. asiatica* (Umbliferacae), also known as barhami boti, were used for pediatric complaints relating to bowel and fever and applied externally for bruises. Sharma (1987) reported that *C. asiatica* possessed anti-leprosy properties.

During recent years very few researches were carried out on the remedies of leprosy. This situation highlights the need for finding safe, novel and effective antifungal compounds. The aim of the present study was to investigate any possible antifungal properties of the native phytoflora of Sindh, Pakistan, based upon the suggestions of local healers.

Materials and Methods

Plant Material. Plants used in this study (Table 1) were purchased from local herb dealers of Nawab Shah, Sindh, Pakistan, except *L. usitatissimum* which was acquired from Liaquatabad, Karachi, Pakistan. These herbs were recently plucked by the sellers and identified by Department of

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Pharmacognosy, Faculty of Pharmacy, University of Karachi, Karachi, Pakistan; voucher specimens (*T. ammi*, T-004), (*H. niger*, K-005), (*C. roxburgianum*, Aj-006), (*L. usitatissimum*, A-007) and (*C. asiatica*, A-008) were deposited in the Herbarium of Department of Pharmacognosy, University of Karachi, Karachi, Pakistan.

Extraction and isolation. All materials were shade-dried and pulverized in grinder to powder (2 mm dia. mesh). The dried and powdered plant material (250 g) was macerated in one litre methanol (Merck) for one week. Filtrate, obtained using Whatman filter paper (# 1), was concentrated under vacuum. Same procedure was applied to get the petroleum ether extract (PE). Aqueous extracts of herbs were prepared by adding 25 g of dry, ground herb to 100 ml distilled water, boiling the solution and letting it simmer for 45 min. Afterwards, the solution was cooled, the extract was carefully decanted out, centrifuged for 5 min at 1500 rpm, filtered through 0.45 µm filter and stored at 4 °C. An aliquot of each sample of the aqueous extract was dried down under vacuum to determine the concentration (Kuete *et al*., 2007).

Microorganisms used. Ten strains were used, which included five yeast strains, i.e. three strains of *Candida albicans* (CA1, CA2 & CA3*)*, one of *C. glabrata* (C.*g*) and one of *C. tropicalis* (C.*t*), and five strains of filamentous fungi including 3 strains of *Aspergillus niger* (AN1, AN2 & AN3), one of *Aspergillus flavus* (AF) and one *Penicillium* sp.(P). All these cultures were collected from reference clinical labs and assayed.

Antifungal susceptibility assay. Methanol, aqueous and petroleum ether extracts of the selected plants were tested for antifungal activity using agar disc diffusion technique on Sabouraud dextrose agar (SDA). Inocula of the microorganisms were prepared from 12 h broth cultures (Sabouraud dextrose broth) and incubated at 37 °C. All culture media and distilled water were sterilized at 115 °C for 15 min in autoclave. The inocula were diluted with sterilized distilled water to obtain the density corresponding approximately to 0.5 of Mcfarland standard turbidity scale. The dried organic plant extracts were dissolved in DMSO and the aqueous extract was made in sterile distilled water. Antifungal tests were then carried out by disc diffusion method (Murray *et al*., 1995), using 100 µl of suspension containing 10^6 CFU/ml of yeast and 10^4 spore/ml of filamentous fungi. Antifungal activity was determined by agar disc diffusion assay using Whatman # 3, 6 mm dia, soaked in 20 ml of the extracts giving 2000 mg/disc concentration. Zones of inhibition were recorded in mm. Each experiment was performed in triplicate (McGinnies, 1980; Bauer *et al*., 1966).

Minimum inhibitory concentration analysis. Minimum inhibitory concentration (MIC) analysis was carried out by the same method. Active extracts of 100 mg/ml (2000 mg/disc) were diluted up to five consecutive serial two-fold dilutions. Each dilution corresponded to the concentrations of 1000, 500, 250, 125 and 62.5 mg/disc. Filter paper discs were soaked in these diluted samples and the protocol, same as above, was followed. Least concentration of test samples able to produce 9 mm zone of inhibition was considered as MIC.

Results and Discussion

The investigations covered 15 plants extracts, (methanol, petroleum ether and aqueous of each five native medicinal plants) (Table 1), against five yeast and five filamentous fungal isolates (Table 2) from different clinical sources. Out of the five plants studied, four were found active while one i.e. *L. usitatissimum* remained inactive.

As far as spectrum of activity is concerned, zones of 8 mm, 9-10 mm and >10 mm were considered as low, moderate and good activity, respectively.

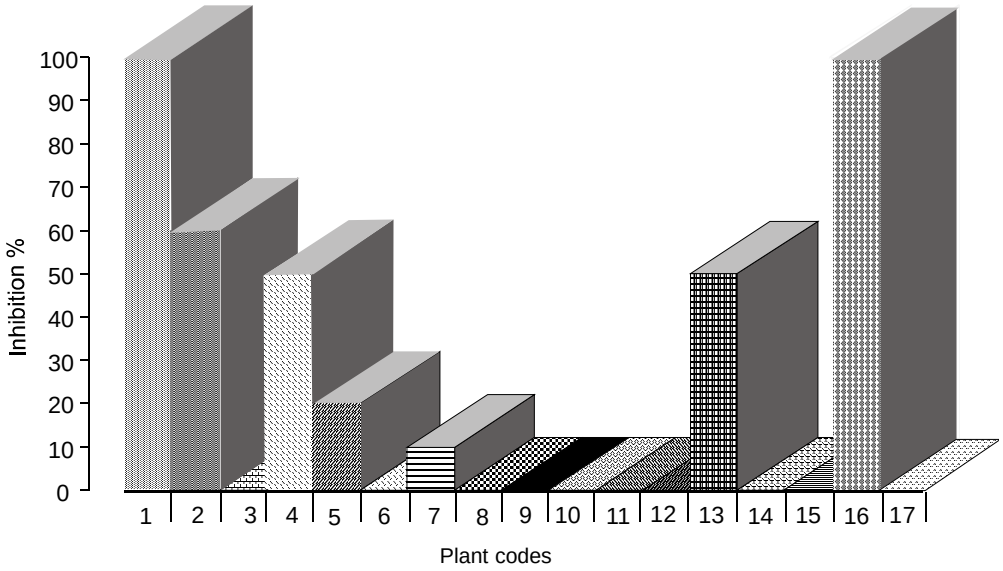
T. ammi appeared as the most active plant against all test fungi with varying potential (Fig. 1), while *H. niger* produced 25 mm zone against *Candida* sp; other plant extracts were moderate to low active whereas *L. usitatissimum* was inactive.

Some extracts (Table 2) produced ≤ 9 mm zones and were not considered as therapeutically active against that particular pathogen but still cannot be regarded as inactive. Extracts without any zone of inhibition were considered inactive. Extracts showing 9 mm zones of inhibition at initial screening at concentrations of 2000 mg/disc were subjected to further MIC analysis.

Standard reference antibiotics used were Nystatin for yeast and Amphotericin B for filamentous fungi. Antimycotic

Table 1. Botanical description of the plants studied

Common names of plants	Botanical names of plants	English names of plant	Parts used	Family
Ajwain desi	*Trachyspermum ammi*	Ajowan	Seeds	Umbliferaceae
Ajwain khurassani	*Hyoscyamus niger*	Henbane	Seeds	Solanaceae
Ajmud/ rhanduni seed	*Carum roxburgianum*	Wild celary	Seeds	Apiaceae
Alsi	*Linum usitatissimum*	Common flex, linseed	Seeds	Linaceae
Barhami boti Gotu-cola	*Centella asiatica*	Centella	Shoots	Mackinlayacae



T. ammi: (1) Meth. ext. (2) Pet. eth. ext. (3) Aq. ext.; *H. niger*: (4) Meth. ext. (5) Pet. eth. ext. (6) Aq. ext.; *C. roxburgianum*: (7) Meth. ext. (8) Pet. eth. ext. (9) Aq. ext.; *L. usitatissimum*: (10) Meth. ext. (11) Pet. eth. ext. (12) Aq. ext.; *C. asiatica*: (13) Meth. ext. (14) Pet. eth. ext. (15) Aq. ext. (16) Reference antibiotic (17) DMSO

Fig. 1. Percentage of fungal inhibition from test extracts

Table 2. Antifungal activity of plant extracts against fungal pathogens

Plants and extracts	Yeast sp. inhibition zone (mm)					Filamentous fungi inhibition zone (mm)					Cumulative SEM	Inhibition (%)
	C. albicans Str. 1	*C. albicans* Str. 2	*C. albicans* Str. 3	*C. glabrata*	*C. tropicalis*	*A. niger* Str. 1	*A. niger* Str. 2	*A. niger* Str. 3	*A. flavus*	*Penicillium* sp.		
T. ammi												
-Meth	20	15	18	18	14	10	10	10	10	12	13.7±1.171	100
-Pet. eth.	12	10	10	10	10	8	-	-	-	-	6±2.34	60
-Aq.	-	-	-	-	-	-	-	-	-	-	-	0
H. niger												
-Meth	20	-	25	20	10	10	-	-	-	-	8.5±2.78	50
-Pet. eth.	9	-	-	-	-	9	-	-	-	-	1.8±0.424	20
-Aq.	-	-	-	-	-	-	-	-	-	-	-	0
C. roxburgianum												
-Meth.	-	-	-	-	10	-	-	-	-	-	-	10
-Pet. eth.	-	-	-	-	-	-	-	-	-	-	-	0
-Aq.	-	-	-	-	-	-	-	-	-	-	-	0
L. usitatissimum												
-Meth	-	-	-	-	-	-	-	-	-	-	-	0
-Pet. eth.	-	-	-	-	-	-	-	-	-	-	-	0
-Aq.	-	-	-	-	-	-	-	-	-	-	-	0
C. asiatica												
-Meth	10	9	10	9	10	-	-	-	-	-	4.8±2.3	50
-Pet. eth.	-	-	-	-	-	-	-	-	-	-	+	0
-Aq.	-	-	-	-	-	-	-	-	-	-	+	0
Reference antibiotic	18	18	15	15	18	18	18	18	18	18	17.7±5.44	100
DMSO	-	-	-	-	-	-	-	-	-	-	-	0

Meth. = methanolic extract; Pet. eth. = petroleum ether extract; Aq. = aqueous extract; reference antibiotics: Nystatin for yeast and Amphotreicin B for filamentous fungi.

screening revealed that maximum zone was produced by the methanolic extract of *H. niger* i.e. 25 mm (Table 2) against *C. albicans* str. 3. Methanolic extract of *T. ammi* produced zones of 20 mm, 18 mm, 18 mm, 15 mm and 14 mm against *C. albicans* str. 1, str. 3, *C. glabrata*, *C. albicans* str. 2 and *C. tropicalis*, respectively.

Petroleum ether extract of *T. ammi* produced 10 mm inhibition zones against *C. albicans* strains 2 and 3, *C. glabrata* and *C. tropicalis*, while 12 mm zone against *C. albicans* str. 1. Methanolic extract (Meth. ex.) of *H. niger* appeared more active producing 25, 20, 20 and 10 mm zones against *C. albicans* str. 3 and 1, *C. glabrata* and *C. tropicalis*, respectively. Petroleum ether extract of *H. niger* produced 9 mm zone against *C. albicans* str. 1 only. Meth. ext. of *C. asiatica* produced 9-10 mm zone against *Candida* sp. while its pet. eth. extract remained inactive. In overall study, aqueous extracts appeared inactive.

Activity of test extracts against filamentous fungi revealed that meth. ex. of *T. ammi* inhibited all test pathogens, producing 10 mm zones against all test *Aspergillus* sp. And 12 mm against *Penicillium* sp. whereas its pet. eth. extract displayed low activity against the filamentous fungi with 8 mm zone of inhibition against *A. niger* str. 1 only. Methanolic and pet. eth. extracts of *H. niger* produced 10 mm and 9 mm zones of inhibition against *A. niger*, respectively, but it remained inactive against *Penicillium* sp. Methanolic extract of *C. roxburgianum* produced 10 mm inhibition zone against *C. tropicalis*. Methanolic and pet. ether extracts of both *T. ammi* and *C. asiatica* and pet. ether extract of *C. roxburgianun* remained inactive against filamentous fungi. All aqueous extracts were inactive (Table 2). Extracts producing 9 mm zone of inhibition at concentration 2000 mg/disc were considered as moderately active and were subjected to further MIC analysis.

Minimum inhibitory concentration analysis (MIC). In minimum inhibitory concentration analysis, different activity patterns were observed against fungal sp. (Table 3). Methanolic and pet. eth. extract of *T. ammi* showed MIC of 125-500 mg/disc and 1000-2000 mg/disc, respectively, while methanolic and pet. ether extracts of *H. niger* and meth. extract of *C. asiatica* indicated 125-500 mg/disc, 2000 mg/disc and 1000 mg/disc MIC, respectively (Table 3). Reference antibiotics, Nystatin and Amphotericin B, also displayed different patterns of inhibition against filamentous fungi.

Table 3. Minimum inhibitory concentration (MIC) of active plant extracts of medicinal plants against yeast and fungal pathogens (in mg/disc.)

Plants	Yeast sp.					Filamentous fungi				
and extracts	*C. albicans* Str. 1	*C. albicans* Str. 2	*C. albicans* Str. 3	*C. glabrata*	*C. tropicalis*	*A. niger* Str. 1	*A. niger* Str. 2	*A. niger* Str. 3	*A. flavus*	*Penicillium* sp.
T. ammi										
-Meth.	125	500	250	500	500	500	1000	1000	2000	1000
-Pet. eth.	1000	2000	2000	2000	2000	-	-	-	-	-
-Aq.	-	-	-	-	-	-	-	-	-	-
H. niger										
-Meth.	125	-	125	500	125	1000	-	-	-	-
-Pet. wth.	2000	-	-	-	-	2000	2000	-	-	-
-Aq.	-	-	-	-	-	-	-	-	-	-
C. roxpurgianum										
-Meth.	-	-	-	-	-	-	-	-	-	-
-Pet. eth.	-	-	-	-	-	-	-	-	-	-
-Aq.	-	-	-	-	-	-	-	-	-	
*L. usitatis*simum										
-Meth.	-	-	-	-	-	-	-	-	-	-
-Pet. eth.	-	-	-	-	-	-	-	-	-	-
-Aq.	-	-	-	-	-	-	-	-	-	-
C. asiatica										
-Meth.	1000	1000	1000	1000	1000	-	-	-	-	-
-Pet. eth.	-	-	-	-	-	-	-	-	-	-
-Aq.	-	-	-	-	-	-	-	-	-	-
Reference antibiotic	12.5	12.5	12.5	12.5	25	6.25	6.25	6.25	6.25	6.25
DMSO	-	-	-	-	-	-	-	-	-	-

Meth. = methanolic extract; Pet. eth. = petroleum ether extract; Aq. = aqueous extract; reference antibiotics: Nystatin for yeast and Amphotericin B for filamentous fungi.

Methanolic and pet. eth. extract of *H. niger* showed 1000 mg/disc and 2000 mg/disc MIC against *A. niger* str. 1 and 2, respectively, (Table 3) while all other test extracts were inactive against filamentous fungi.

Percentage activity of test samples was also examined wherein *T. ammi* appeared the most active (100%) while others exhibited relatively moderate to low activity (Fig. 1). Petroleum ether extract of *T. ammi*, met. extract of *H. niger* and *C. asiatica*, pet. eth. extract of *H. niger* and meth. extract of *C. roxburgianum* exhibited 60, 50, 40, 20, 10% inhibition activity, respectively (Tables 2 and 3).

Our investigation is related to local phytoflora of Sindh which has therapeutic potential and is used in folklore medicines. According to the investigation there are several indigenous plants still unexplored for the treatment of mycotic infections. Remote areas of Pakistan have high rate of fungal infections including Candidiasis, Aspergillosis and Dermatomycosis. In the present work, five medicinal plants and their 15 organic and aqueous extracts were studied. Selection of herbs was made under the guidance of local healers. In our study, some of the test fungi were inhibited more as compared to the existing commercially available antibiotics (Table 2).

Main reasons for the higher rate of fungal infection incidents are poor sanitary conditions and lack of awareness about cleanliness. Use of open polluted bodies of water has also increased the frequency of fungal infections. Common brands of drugs used against mycotic infections of dermal origin contain volatile chemicals (Singh, 2004). *T. ammi* and *H. niger* used in the present study have also been reported to contain volatile components e.g. thymol (Ma *et al*., 1999).

6-7.2% of the vaginal isolates of *Candida albicans* from women with Candidal vaginitis is resistant to Flucanazole (Sobel *et al*., 2003). Combination therapies have been introduced as alternatives during the past ten years but still eradication of fungal infections remained difficult as compared to the bacterial infections (Patterson, 2005; Kartsonis *et al*., 2003).

Candida albicans is a dimorphic yeast. Its ability to switch from yeast cells to hyphae are considered important for the interaction of *Candida albicans* to its host (Cutler, 1991). Both yeast cells and hyphae are present in the host during the growth and infection. Hyphae are thought to be important virulent factor that promotes invasion of cells into the mucosa, allowing *Candida* cells to resist macrophage and neutrophil engulfment (Yang, 2003). Isolation of *C. albicans* from the oral lesions is reported to be up to 76.08%. People with poor oral hygiene may be the oral yeast carrier, at a higher rate. Also oral yeast carriage rates are generally higher in the patients receiving medical attention (Rouhi, 2003). Oropharyngeal Candidiasis occurs in patients with diabetes mellitus, receiving antibacterial antibiotics. Despite advances in antifungal therapies, many problems remain to be solved in this respect as the use of the most available antifungal drugs e.g. Amphotericin-B, known as the gold standard is limited because of its infusion related reactions and nephrotoxicity (Faons and Cataldi, 2001; Grasela *et al*., 1990) and resistant strains have also been reported. In addition, these antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune suppression and allergic reactions (Ahmad *et al*., 1998). The use of azoles, such as flucanazole, ketoconazole and miconazole, has resulted in clinically resistant strains of *Candida* species (Sojakova *et al*., 2004; Lyman and Walsh, 1992). Medicinal plants may represent valuable, untapped source of novel antifungal drugs especially protoberberine alkaloids, which are readily extractable from Chinese and Korean medicinal plants; these have been found to possess diverse biochemical and pharmacological properties, while being non-toxic to man even in high concentrations. Their antifungal activity has been demonstrated against some *Candida* and filamentous fungal species (Park *et al*., 1999). *Heterothalamus alienus* is reported for good antifungal activity against fungal dermal infections of *Aspergillus* sp. (Adriena *et al*., 2008).

The study indicates that several indigenous plants of Sindh (Pakistan) can be explored for treatment of mycotic infections as methanolic and petroleum ether extracts of *T. ammi* inhibited growth of 100% and 60% test fungi at 125 and 1000 µg/disc concentration, respectively, while methanolic extracts of *H. niger* and *C. asiatica* showed 50% inhibition with 125-1000 mg/disc MIC (Tables 2 and 3); methanolic extract of *C. roxburgianum* produced 10% inhibition with 1000 mg/disc concentration against *C. glabrata*. Previously, good antifungal inhibition by *T. ammi* against dermatophytes and filamentous fungi has been reported (Singh *et al*., 2004).

Among all the test plants *L. usitatissimum* appeared inactive; it may be due to the presence of tissue-specific soluble (1>4)-ɒ-galactan (Tatyana *et al*., 2004) in its composition which has not displayed good antimicrobial activity. All aqueous extracts appeared inactive, this may be attributed to incapability of water to extract antifungal constituents. Our results suggest that *T. ammi* and *H. niger* can be used in the treatment of Candidiasis and superficial mycosis etc.

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