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Antifungal Potential of Leaves and Barks of *Azadirachta indica*

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Abstract. The morbidity of fungal infection is very high all over the world. The treatment of fungal infections is becoming difficult due to expensive antifungal drugs, which have limited efficacy with side effects. *Azadirachta indica* (neem) tree has been traditionally used for medicinal purposes. Keeping in view the folklore medicinal importance, we determined the antifungal effect of leaves and bark decoctions and infusions in this work. Agar well diffusion method is used for the determination of antifungal potential. Decoctions and infusions prepared from dried and fresh leaves of neem tree showed promising anti-dermatophytic activity against *Microsporum canis* and *Trichophyton tonsurans* which was found to be better than antifungal drug fluconazole. This study supports the traditional use of neem trees to treat fungal infections.

Keywords: antifungal activity, dermatophytes, neem tree, infusions, decoctions

Introduction

The prevalence of fungal infection has been estimated to be very high and affects about 25% peoples of the world and the causative agents of these fungal infections depend on the geographical locations. The delay of diagnosis or mis-diagnosis of serious systemic fungal infections may result in the death of patients. About 1.7 billion humans are infected by superficial skin and nail fungal infections (Brown *et al*., 2012).

These superficial skin infections are usually caused by dermatophytes (Ghannoum *et al*., 2013). Saprophytic fungi are known to cause infections in both humans and animals. Moisture content, high temperatures and heavy rain may favour fungal infections (Mongalo *et al*., 2018). Immune compromised patients with AIDS, a kidney transplant or persons of old age are at high risk of fungal infections. The incidence of dermatomycosis is increasing day by day (Straten *et al*., 2003). Although dermatophytes mostly cause infections in the superficial layer of skin and are non-life-threatening, they are the most communicable disease among human beings (Foss *et al*., 2014). The pathogenesis of fungi may include the formation of biofilms, capable of resistance to the environment, ability to attach a variety of surfaces, formation of hydrolytic enzymes and morphological transitions (Raghavendra and Balsarf, 2014). Dermatophytes have the ability to secrete sulfite, endo-proteases and exo-proteases. The sulphite can break the bond of keratin and make it easy for the dermatophytes to digest it by secreting endo-proteases and exo-proteases (Monod, 2008). Dermatophytes can easily be transmitted through direct contact and are also considered a zoonotic disease, spread through especially contact between humans and animals such as pocket pets, cats, dogs, birds and small rodents (Javed *et al*., 2015).

Neem trees have been known to possess antimicrobial and insecticidal activities. A well known compound of neem tree named, *Azadirachtin* has been reported to possess toxicity against insects. Ethanolic extract of neem leaves has been claimed to have antibacterial potential against pus-producing bacterial pathogens *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) (Javed *et al*., 2020).

The neem tree's different parts consist of various phytochemicals such as quercetin, *Azadirachtin*, a number of liminoids and nimbosterol. Leaves are known to possess nimbin, nimbanene, 6-desacetylnimbinene, nimbandiol, nimbolide, ascorbic acid, *n*-hexacosanol and different amino acids and nimbiol *etc*., the barks are reported to contain nimbin, nimbinin and nimbidin. All of these phytochemicals in literature have been claimed to have a role in the cure of various ailments (Rahmani *et al*., 2018). Leaves are used in the treatment of leprosy and helminth infections and also as diuretics and insecticides. The roots of this plant have also been reported to cure ulcers, rheumatism, malaria and cancer (Bakht *et al*., 2021).

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The cure of fungal infections is costly, with limited efficacy and many side effects (Santos *et al*., 2013). The excessive use of antifungal drugs in the past years has increased the emergence of multidrug resistant fungi (Lopes *et al*., 2013). Amphotericin B and azole are the main drugs that have been effectively used for fungal infections (Masoka *et al*., 2007). Widespread use of antibiotics and immuno-suppressive drugs leads to an increased incidence of systematic fungal infections due to the development of resistance in some fungal strains. (Sagar, 2013). The side effect of currently used antifungal drugs and the development of multidrug resistant fungi is the reason why researchers now shift their focus of attention toward medicinal plants to cure fungal infections. Several plants are known to possess antifungal potential (Pandey and Gupta, 2016).

Azadirachta indica (locally known as Neem or Margosa tree) is the fast growing tree that belongs to the mahogany family Meliaceae. It is commonly found in dry forest areas in southeast Asia, including Pakistan, Sri Lanka, India, Thailand, Malaysia and Indonesia. In our subcontinent, the neem tree has been traditionally used for the cure of a variety of ailments. Neem leaves and bark have been known to possess inhibitory activities against inflammation, hyperglycemic condition, ulcer, malaria, cancer, viral, fungal and bacterial infections and also possess antioxidant, antimutagenic and immunomodulatory potentials (Arumugam *et al*., 2015). The medicinal property of plants may be related to external factors such as climate, geographical location, season, nature of the soil and growth conditions and cause variation in the concentration of their bioactive phytochemicals. The bioactivity of the same plant varies due to the collection from the different geographical locations at other times or from different areas that may yield new active compounds (Muraina *et al*., 2008). Therefore, the same medicinal plants from different locations may not consistently produce the same phytochemicals in the exact quantities. By keeping in mind the medicinal importance of the neem tree, this study was designed to study the antifungal effects of decoctions and infusions prepared from leaves and barks of the neem tree to explore less expensive and effective alternate antifungal agents.

Materials and Methods

Collection of samples. The leaves and barks of healthy neem (*Azadirachta indica*) tree from the garden of Federal Urdu University (FUUAST) Gulshan Iqbal campus, Karachi.

Preparation of infusion. The infusions of 10% concentration were prepared by taking five grams of fresh and dried neem tree leaves and barks paste and powdered respectively and each of them was soaked in 50 mL distilled water separately in a bottle and left in the room temperature for two days and then strained and then filtered through 0.22 um membrane filter and then this clear infusion were kept save in the freezer for further testing.

Preparation of decoction. The aqueous decoctions were prepared by taking 5 g of each dried and fresh leaves and bark of neem tree paste and powdered respectively and boiling it in 50 mL distilled water for 15-20 min. After cooling at room temperature, the decoction is strained and filtered by passing through a 0.22 µm filter and kept in a freezer for antifungal testing (Sherwani *et al*., 2013).

Collection of micro-organisms. The organisms used in the study were procured from the Microbiology Department of FUUAST. The antifungal potential was determined against four dermatophytes, *Trichophyton tonsurans, Trichophyton mentagrophytes, Microsporum canis* and *Microsporum gypseum.* Four saprophytic fungi, *Aspergillus flavus, Aspergillus niger, Penicillium* sp. *Rhizopus* sp. and three yeast, *Saccharomyces* sp. *Candida albicans* and *Candida tropicalis*.

Antifungal activity by agar well diffusion method. Agar well diffusion method was used to study the antifungal potential (Magaldi *et al*., 2004). A small amount of fungal culture was added to 2 mL of normal saline in a screw-capped tube with a few glass beads (1 mm in diameter) and vortex for 5 min to make a homogeneous suspension. This fungal suspension was seeded in the sabouraud dextrose agar (SDA) plates. Wells were constructed in the fungal seeded plates by a sterile borer of 6 mm size, 30 µL of each sample (10%) was added to each well (concentration of sample = 30 µg/well). Plates were incubated at room temperature for one week. Antifungal activity was evaluated by measuring the zone of inhibitions in millimeter (mm). Antifungal drug fluconazole 30 mg/well was used as the positive control. Distilled water was used as the negative control.

Determination of minimum inhibitory concentration (MIC). The infusion and decoctions showing zones of

inhibition of 15 mm or more were considered good antifungal agents and selected for determination of their MIC, two-fold dilutions of the sample having concentrations varying from 3 mg/well to 0.0025 mg/well were prepared. The MIC was determined as the lowest concentration of the sample, giving the zone of inhibitions of 10 mm.

Statistical analysis. Each reading of the zone of inhibitions is the mean of triplet readings (N = 3) ± SD. A comparison of MIC values of neem tree infusion and decoctions and MIC values of standard drug fluconazole was made by using the student's t-test. A P-value of < 0.05 was considered statistically significant.

Results and Discussion

Almost every part of the neem tree has been applied for the management of different ailments in folklore (Rasool *et al*., 2017). The neem tree is known to possess a large number of bioactive compounds that are diverse structurally as well as chemically (Mahmoud *et al*., 2011). Although most of the previous works has been described the antifungal activity of neem trees *in vitro*, one of the main reasons for performing this study was to observe the antifungal activity of decoction and infusion of neem trees grown in Pakistan against a number of different dermatophytes, saprophytic fungi and yeast. The weather and soil constituents factor play an essential role in the presence of phytochemicals composition of neem trees grown in different geographical locations (Arumugam *et al*., 2015). Therefore, the present study tested the antifungal potential of infusion and decoctions of fresh and dried leaves and barks against eleven different fungal cultures.

For testing antifungal activity, 30 µL of each prepared solution of decoction and infusion (10%) was added to each well (the concentration used was 30 µg/well). In the case of dried neem tree leaves the decoction was found more effective than infusion. Decoction of dried leaves was active against many fungi such as *Microsporum canis, Trichophyton tonsurans, Penicillium* sp. and *Saccharomyces* sp., while in the case of neem barks, the decoction was found inactive, while infusion was found active only against *Trichophyton tonsurans*. Results are seen in Table 1 and Fig. 1. The present results show fresh leaves infusion and decoction are better than fresh bark infusion and decoction. Fresh leaves infusion inhibited the growth of dermatophytes, *Microsporum canis* and *Trichophyton tonsurans*, while fresh leaves infusion strongly inhibited the growth of yeast *Saccharomyces cerevisiae.* Results are shown in Table 2. The dried leaves decoction and fresh leaves infusion of neem tree was highly active against dermatophytes. MIC values are many folds better than fluconazole drugs. The MIC values of neem tree decoctions and infusions were significantly better than fluconazole (P-value = 0.036). There was a negative correlation between the values of MICs and the zone of inhibitions, as the high values of MIC were associated with smaller values of the zone of inhibitions (Table 3 and Fig. 2).

However, in the case of barks, both dried and fresh infusion and decoctions didn't show good antifungal activity except for dried bark infusion, which effectively inhibited *Trichophyton tonsurans.* These values are better than the known antifungal drug fluconazole. MIC values are shown in Table 3. This work is also in

Table 1. Antifungal activity of dried leaf and bark of neem tree (zones of inhibition (mean±S.D) in mm)

Organisms	D. NLD	D. NLI	D. NBD	D. NBI	Flucanazole 30 µg/well)
Aspergillus niger	-	-	-	-	15 ± 0.2
Aspergillus flavus	-	20 ± 0.4	-	-	20 ± 0.1
Candida tropicalis	-	-	-	-	45 ± 0.2
Candida albicans	-	-	-	-	30 ± 0.4
Microsporum gypseum	-	-	-	-	25 ± 0.01
Microsporum canis	33 ± 0.2	-	-	-	20 ± 0.2
Penicillium sp.	-	10 ± 0.2	-	-	13 ± 0.1
Rhizopus sp.	-	-	-	-	15 ± 0.2
Saccharomyces sp.	-	15± 0.1	-	-	47 ± 0.3
Trichophyton mentagrophytes	-	-	-	-	25 ± 0.1
Trichophyton tonsurans	35 ± 0.1	-	-	40 ± 0.2	30 ± 0.1

D. NLD = dried neem leaves decoction; D. NLI = dried neem leaves infusion; D. NBD = dried neem bark decoction; D. NBI = dried neem bark infusion; each reading is the mean of triplet readings (n=3).

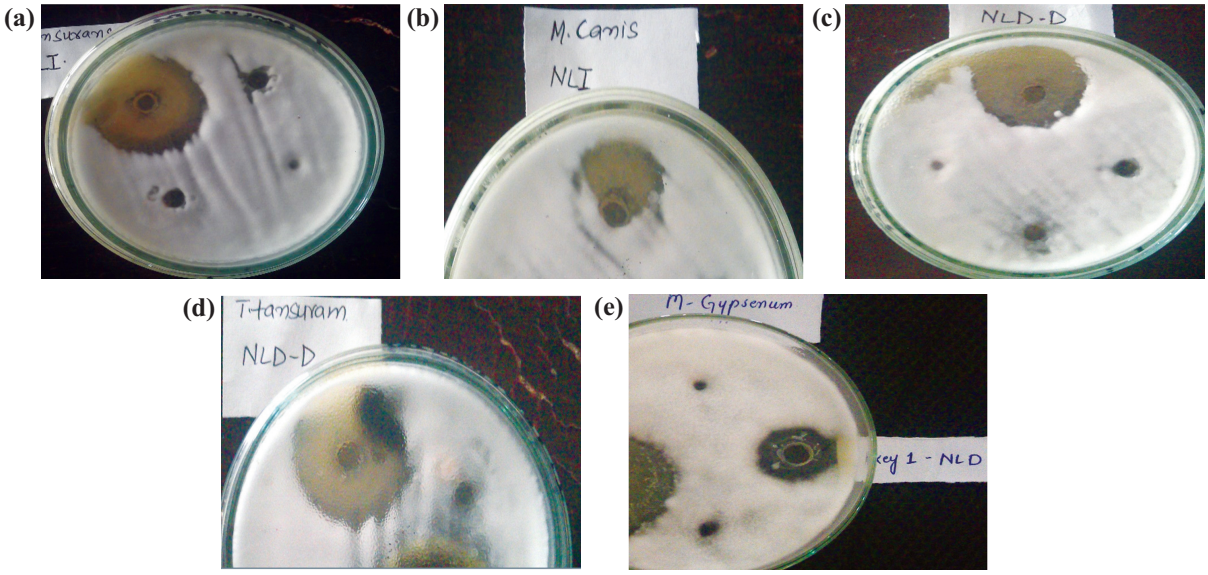

Fig. 1. Zone of inhibition a *T. mentagrophytes* with fresh neem leaves decoctions **(a)** for *T. tonsurans* with the infusion of fresh neem leaves, **(b)** for *M. canis* with the decoction of dried neem leaves, **(c)** for *T. tonsurans* with the decoction of neem leaves, **(d)** for *M. gypseum* with the decoction of fresh neem leaves, **(e)** for *M. canis* with the infusion of fresh neem leaves.

accordance with (Salazar *et al*., 2015) that found that the neem leaves had antidermophytic potential against *Trichophyton mentagrophytes, Trichophyton rubrum, Epidermatophyton floccosum* and *Microsporum canis*, they correlated this activity of leaves due to high concentration of terpenoids. The phytoconstituents alkaloids, glycosides, flavonoids and saponins are the main phytochemicals of the neem tree known to possess antimicrobial effects. These phyto-chemicals protect the plants by attacking different pathogens (Hancock, 1999).

Dermatomytosis is the most common infection worldwide and its prevalence is found high, especially in tropical countries and is related to poor hygiene people. Dermatophytes cause infections of hair, skin, subcutaneous and nails in humans and animals (Bokhari, 2009) by utilizing keratin of keratinized tissues. These infections are more common in males than females, especially in children. These infections can easily be transmitted from person to person through direct contact with animals and indirectly through fomites of infected patients such as combs and clothes, etc. (Kakande *et al*., 2019).

This work did not support the previous work of (Arumugam *et al*., 2015) that has reported the activity of neem extract against *Aspergillus niger* and *Candida albicans* previously. In the current study, the infusion and decoctions of neem tree leaves and barks did not exhibit antifungal potential against these organisms.

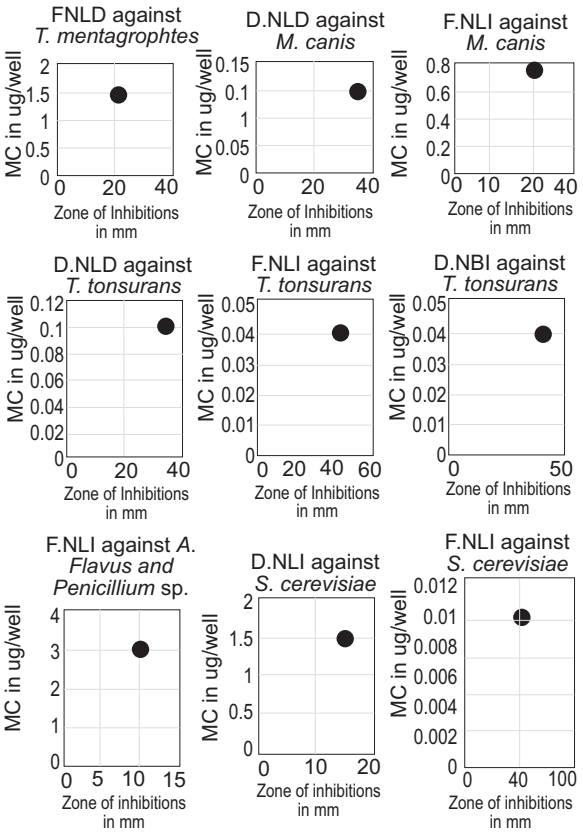

Fig. 2. Scatter plot graphs show the correlation between minimal inhibitory concentrations (MICs) and the zone of inhibitions in mm.

Table 2. Antifungal activity of fresh bark and leaves of neem tree (zones of inhibitions (mean±S.D) in mm)

Organisms	D. NLD	D. NLI	D. NBD	D. NBI	Flucanazole 30 µg/well)
Aspergillus flavus	-	-	-	-	20 ± 0.1
Aspergillus niger	-	-	-	-	15 ± 0.2
Candida albicans	-	-	-	-	30 ± 0.4
Candida tropicalis	-	-	-	-	45 ± 0.2
Microsporum canis	-	20 ± 0.1	-	-	20 ± 0.2
Microsporum gypseum	-	-	-	-	25 ± 0.01
Penicillium sp.	-	-	-	-	13 ± 0.1
Rhizopus sp.	-	-	-	-	15 ± 0.2
Saccharomyces cerevisiae	55 ± 0.2	-	-	-	47 ± 0.3
Trichophyton mentagrophytes	20 ± 0.1	-	-	-	25 ± 0.1
Trichophyton tonsurans	-	40 ± 0.3	-	-	30 ± 0.1

F. NLD = fresh neem leaves decoction; F. NLI = fresh neem leaves infusion; F. NBD = fresh neem bark decoction; F. NBI = fresh neem bark infusion; each reading is the mean of triplet readings (n=3).

Table 3. MIC (minimum inhibitory concentration) in µg/well

Samples code	Zone of inhibitions in mm								MIC in	MIC of
Conc in µg/well	3	1.5	0.1	0.5	0.25	0.1	0.01	0.05	µg/well	fluconazole µg/well
Aspergillus flavus										
D. NLI	10	-	-	-	-	-	-	-	3	30
Microsporum canis										
D. NLD	16	13	10	-	-	-	-	-	0.1	0.1
F. NLI	10	-	-	-	-	-	-	-	3	
Saccharomyces cerevisiae										
F. NLD	25	23	21	17	15	12	10	-	0.01	
Trichophyton mentagrophytes										
F. NLD	13	10	-	-	-	-	-	1.5	3	
Trichophyton tonsurans										
F. NLI	30	28	25	22	18	16	13	10	0.05	1.5
D. NBI	28	25	23	19	16	14	12	10	0.05	
P-value	0.036*									

P-value = not detected; D. NLD = dried neem leaves decoction; D. NLI = dried neem leaves infusion; D. NBD = dried neem bark decoction; D. NBI = dried neem bark infusion; F. NLD = fresh neem leaves decoction; F. NLI = fresh neem leaves infusion.

The differences in the antifungal potential of different neem extracts could be due to the presence of different types of active phytochemicals. The concentration of phytochemicals depends on several factors such as the age of the plant and geographic location of the plant, method of extraction and the use of the extracting solvent (Arumugam *et al*., 2015).

Conclusion

The present study revealed that the leaves of the neem plant had antifungal potential against *Microsporum canis*, *Trichophyton tonsurans* and *Sacchromyces cerevisiae*. The neem tree leaves were found to possess better antifungal potential than barks. The present study supports the traditional use of neem trees against fungi,

especially dermatophytes. However, further studies are required to purify and identify its active constituent and determine their toxic studies, pharmacological potentials and the molecular mechanisms of their actions to develop alternative cheap and effective antifungal agents.

Conflict of Interest. The authors declare that they have no conflict of interest.

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