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Antifungal Activity of Various Extracts of *Azadirachta indica* Leaf - an *In-Vitro* Study

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Abstract : Introduction: *Azadirachta indica* commonly called as Neem, known to be used in inflammation of gums, gingivitis, periodontitis, sores, boils, antiperiodic, malarial fever, antifertility, measles, smallpox, head scald and anthelmintic etc. The neem oil is useful as a female contraceptive also for the treatment of vaginal infections Neem oil is also used to eliminate the mosquitos in pest control management.

Aim and Objective: The aim of the study was to identify the extract which is having the maximum anti-fungal activity and also to identify the minimum dose required to inhibit the growth of the organism.

Materials and Methods: The antifungal activity of *Azadirachta indica* leaf was determined by using various sequential extracts like Hexane, benzene, ethylacetate, methanol, and aqueous against *Trichophyton rubrum*, *Microsporum gypseum*, *Epidermophyton floccosum*, and *Candida albicans*. The activity was evaluated by a disc-diffusion method and the minimum inhibitory concentration (MIC) by resazurinmicrotitre indicator method.

Results: The microbiological study was done for six times and the raw data was tabulated in excel spreadsheet and was subjected to statistics using IBM SPSS version 20 and the inhibition was found to be highly significant($P<0.05$).

It was determined that the aqueous extract of *Azadirachta indica* leaves have the maximum anti-fungal activity in *Trichophyton rubrum*(8.14±0.017 at 500 µl), *Microsporum gypseum*(10.69±0.021 at 500 µl), *Epidermophyton floccosum*(7.95±0.023 at 500 µl), and Candida(12.23±0.023 at 500 µl) and it was found that at minimum dose of *Azadirachta indica* against *Trichophyton rubrum*(125 mcg/ml), *Microsporum gypseum*(125 mcg/ml), Epidermophyton(250 mcg/ml), and Candida(62.5 mcg/ml) showing as Minimum Inhibitory Concentration(MIC).

Conclusion: The sensitivity of extracts was concentration dependent, when the extract concentration increases, the zone of inhibition also increased.

Keywords: Antifungal, *Azadirachta indica*, *Trichophyton rubrum*, *Microsporum gypseum*, Epidermophyton, *Candida albicans*, Resazurin.

Introduction:

Fungi are a diverse group of saprophytic and parasitic eukaryotic microorganisms. Infections caused by the fungi to the human skin are termed as dermatophytosis. Superficial mycosis includes infection of skin, nail, and hair. These infections generally caused by dermatophytes like *Trichophyton rubrum*, *Microsporum gypseum*, and *Epidermophyton floccosum* are called as cutaneous infective agents. Poor hygiene is one of the contributing factors for the emergence of dermatophytic infection. The resistance to the drugs is the major contributing factor for evolving the newer and also safer drugs especially in alternative medicine[1, 2].Indian traditional medicine is a plethora of healing herbs that heal many ailments like rheumatoid arthritis, liver diseases, skin diseases, inflammatory disorders, diabetes, etc. In the recent years, the evaluating therapeutic effects of plants and plant products has been enhanced dramatically[3, 4] as the majority of the world's population is depended alternative medicine[5, 6].

The system of Ayurvedic-ancient science of life originated in India about 3000 years ago. It is one of the oldest system of medicine identified exclusively with ancient Indian civilization and dealing with both preventive and curative aspects of life. Indian medicinal plants are the treasury for healing various ailments. These are well known for centuries from Charaka and Susruta.*Azadirachta indica* also called as *Melia azadirachta* Linn, belongs to family Meliaceae[7]. In English, it usually called as Neem tree, Margosa tree. In Ayurveda, it has many names like Nimba, Nimbaka, Arishta, Arishtaphala, Pichumarda, Pichumanda, Pichumandaka, Tiktaka, Sutiktak, Paaribhadra. In Siddha/Tamil, it is named as Vembu, Veppu, Veppan, Arulundi.Neem is a native of Asian countries and also countries of East and South African and in tropical Australia. *Azadirachta indica* as a whole is used as a medicinal source including the dried stem bark, root bark, leaves, and fruits.

Azadirachta indica was used as an ornamental boulevard tree in last century in arid zones of Africa and it is present in many Asian countries and in the tropical area of the world[8].*Azadirachta indica* is well grown in soil with poor nutrition and also capable of growing in extreme temperatures of severe hot and deep frost[9]. The leaves of *Azadirachta indica* constitutes 11-24% crude fibre, 48-51 % carbohydrates, 14-18 % crude protein, 2.3-6.9 % fat, 7.7-8.5% ash, 0.8-2.4% calcium, 0.13-0.24 % phosphorus[10] with myriad of aminoacids[11,12].A multiple number of sugars, carbohydrates, and polysaccharides have been found in the bark and gum of *Azadirachta indica*[13, 14].A two-dimensional TLC method has shown the availability of carotenoids and major constitutes in leaves[15].Neem has many pharmacological properties in the skin and soft tissue. It is clinically useful in various skin diseases without untoward adverse events.

A clinical trail was conducted in King George Medical College, Lucknow, India with *Azadirachta indica* lotion which was made from an extract of dried leaves which cures ring worm and scabies. Extracts of Neem oil and some pure isolates (Nimbidin, Nimbidiol, and Nimbin) can also inhibit fungal growth on animals[16,17,18] and humans[19]. Leaf extracts were shown good improvement within 15-20 days in chronic cases[20].

The neem plant leaf and bark are used in inflammation of gums, gingivitis, periodontitis, sores, boils, antiperiodic, malarial fever, antifertility, measles, smallpox, head scald and anthelmintic etc.The neem oil is useful as a female contraceptive also for the treatment of vaginal infections Neem oil is also used to eliminate the mosquitos in pest control management. The chemicals constituents present in the neem are numerous like complex tetranorterpenoid lactones Azadirachtin, Nimbin, Nimbidin, Salanin and Nimbolin B. Among Azadirachtin is the most active ingredient as antifeedant. Neem oil extracted from seeds contains many other compounds such as Nimbolides, Olichinolide B and Azadiradione along with above-said compounds. The leaves also contain Azadirachtin, Meliantrol, Salanin, Beta-Sitosterol, Stigmasterol and Flavonoids like Nimatone, Quercetin, Myrecetin, and Kaempferol.

This study was aimed to identify the extract which was having maximum anti-fungal activity and also to identify the minimum dose required to inhibit the growth of the organism which is safe alternative to drugs available.

Materials & Methods:

Collection of plant material:

The leaves of *Azadirachta indica* was collected, identified, and authenticated from the premises of Tagore Medical College & Hospital campus.

Preparation of Extraction:

The procured leaves were shade dried and pulverized by mechanical grinder then passed through a 20 mesh Sieve. The powdered leaf was sequentially extracted with Hexane, benzene, ethylacetate, methanol, using soxhlet apparatus and aqueous with cold maceration. The extraction was carried out for one day in normal room temperature with mild shaking. The extracts were filtered and concentrated at 35° centigrade, and the weight of each residue was recorded and stored in 4^{O} centigrade.

Collection of Microorganism:

The organisms were procured from the MTCC, Chandigarh.The antifungal activity was screened in the dermatophytes and the *Candida albicans* (MTCC 3017) species. The three dermatophytes were used for screening are *Trichophyton rubrum* (MTCC code no:7859), *Microsporum gypseum* (MTCC 4524), *Epidermophyton floccosum* (MTCC 7880).

Determination of antifungal activity:

Disc Diffusion Method:

The standard assay used for anti-microbial tests was carried out by disc-diffusion method[21] using 100 µl of a suspension containing 104 spores/ml of fungi spread on sabourand dextrose agar.

Each Petridish was inoculated with any one of the fungal cultures suitably diluted to containmore than 106 cells/ml by spreading 0.1ml suspension of the organism with a sterile cotton swab. In each plate, a 6 mm diameter of a non-contaminated sterile disc was placed. With the help of micropipette, the 10 µl (1mg/ml) of the extract was taken and placed on the disc. Then it was subjected to check the zone of inhibition. Clotrimazole was taken as a standard.

Minimum Inhibitory Concentration:

The minimum inhibitory concentration(MIC) was detected by the resazurinmicrotitre method[22].100 µl of Sabourd Dextrose Agar (SDA) broth was taken and placed in each well of 96 well microtitreplate which has a capacity of 200 µl.In well-sterilized conditions, the freshly grown microorganism was taken and placed in all 96 well microtitreplate and labeled properly. Serial dilutions of all the extracts were made and placed in an appropriate microtitre plate. Simultaneously 5 µl resazurin indicator (prepared by dissolving 270 mg tablet in 40 µl of sterile distilled water) was also added in all 96 wells. Shake it very gently to ensure that all wells are homogenously mixed. Finally the microtitre plate was kept in a dark place to avoid interference of light. It was once again shaken in 24 hours. After 48 hours, the color change from violet to colorless or any color was observed. Clotrimazole was mixed in 5% DMSO(Dimethylsulphoxide), used as a standard.

Results:

The present study shows that the plant *Azadirachta indica* was having anti-fungal activity in Hexane, benzene, ethylacetate, methanol, and aqueous extracts and it was observed that the dose dependant zone of inhibition against *Trichophyton rubrum*, *Microsporum gypseum*, *Epidermophyton floccosum*, and *Candida albicans*, the aqueous extract was having themaximim efficacy in all the *Trichophyton rubrum*, *Microsporum gypseum*, *Epidermophyton floccosum*, and *Candida albican s*organisms and in all the doses. The mean values were tabulated in Table: 1, the results were analyzedusing software, IBM SPSS Version: 20 and they showed as highly significant ($P<0.05$) by using one-way ANOVA (ANALYSIS OF VARIANCE).

The maximum zone of inhibition was observed in Hexaneextract and benzene extract of *Azadirachta indica* at 500 µg per ml is 13.14 mm, and 10.68 mm, but the aqueous extract at 250 µg per ml concentration shows 10.28 mm zone of inhibition against *Trichophyton rubrum.*

The maximum zone of inhibition was observed in aqueousextract,benzene extract and methanol extract of *Azadirachta indica* in 500 µg per ml is 10.69 mm, 10.54 mm, 10.30 mm respectively against *Microsporum gypseum*.

The maximum zone of inhibition was observed in aqueous extract of *Azadirachta indica* in 500 µg per ml is 7.95 mm, 8.44 mm in Hexane extract at 500 µg per ml against *Epidermophyton floccosum*.

The maximum zone of inhibition was observed in aqueous extract, ethylacetate extract of *Azadirachta indica* in 500 µg per ml is 12.23 mm, 9.65 mm respectively, but the aqueous extract at 250 µg per ml concentration shows 11.57 mm zone of inhibition against Candida.

The hexane extract of *Azadirachta indica* was not showing zone of inhibition even in dose escalation against *Candida albicans.* In benzene extract of *Azadirachta indica* was not showing zone of inhibition in 50 mcg/ml dose, statistically insignificant ($p>0.05$) but having efficacy in 100 mcg/ml, 250 mcg/ml, and 500 mcg/ml against *Candida albicans.* In ethylacetate extract of *Azadirachta indica* was not showing zone of inhibition in 50 mcg/ml and 100mcg/ml doses statistically insignificant ($p>0.05$), but having efficacy in 250 mcg/ml, and 500 mcg/ml against *Candida albicans* .In methanol extract of *Azadirachta indica* was not showing zone of inhibition in 50 mcg/ml dose statistically insignificant ($p>0.05$) but having efficacy in 100 mcg/ml, 250 mcg/ml, and 500 mcg/ml against *Candida albicans.* Only in aqueous extract of *Azadirachta indica* was showing efficacy in 50 mcg/ml, 100 mcg/ml, 250 mcg/ml, and 500 mcg/mlstatistically significant ($p<0.05$) against *Candida albicans.* We used clotrimazole as a standard in this study,zone of inhibition was 19.30 mcg/ml, 20.15 mcg/ml, 21.58 mcg/ml, and 19.99 mcg/ml against *Trichophyton rubrum*, *Microsporum gypseum*, *Epidermophyton floccosum*, and *Candida albicans* respectively.

Table: 1 - The average values of the diameter of the zone of inhibition with standard deviation.

Extract	**Organism**	**Diameter of Zone of Inhibition (mm) MEAN± SD**					
		50 mcg/ml	**100 mcg/ml**	**250 mcg/ml**	**500 mcg/ml**	**Vehicle Control mcg/ml**	**Std mcg/ml**
Hexane Extract of *Azadirachta indica*(HEAI)	Trichophyton	6.93±0.01	7.28±0.53	10.28±0.04	13.14±0.01	0.00	19.30±0.00
	Microsporum	6.34±0.04	6.73±0.01	6.99±0.01	7.93±0.01	0.00	20.15±0.00
	Epidermophyton	6.90±0.04	7.48±0.02	8.29±0.02	8.44±0.01	0.00	21.58±0.00
	Candida	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00	19.99±0.00
Benzene Extract of *Azadirachta indica*(BEAI)	Trichophyton	6.46±0.01	7.64±0.02	7.48±0.01	10.68±0.00	0.00	19.30±0.00
	Microsporum	6.19±0.01	7.37±0.02	7.70±0.01	10.54±0.01	0.00	20.15±0.00
	Epidermophyton	6.35±0.01	6.54±0.02	7.08±0.06	6.66±0.01	0.00	21.58±0.00
	Candida	0.00±0.00	6.53±0.03	6.74±0.01	7.77±0.01	0.00	19.99±0.00
Ethylacetate Extract of *Azadirachta indica*(EAEAI)	Trichophyton	6.06±0.01	6.55±0.02	6.87±0.01	8.32±0.01	0.00	19.30±0.00
	Microsporum	6.26±0.02	7.53±0.04	7.94±0.01	8.42±0.01	0.00	20.15±0.00
	Epidermophyton	6.55±0.05	7.86±0.04	8.13±0.03	7.42±0.03	0.00	21.58±0.00
	Candida	0.00±0.00	0.00±0.00	7.24±0.01	9.65±0.02	0.00	19.99±0.00
Methanol Extract of *Azadirachta indica*(MEAI)	Trichophyton	6.52±0.01	7.46±0.02	8.13±0.01	8.43±0.01	0.00	19.30±0.00
	Microsporum	6.53±0.023	8.93±0.025	9.97±0.01	10.30±0.061	0.00	20.15±0.00
	Epidermophyton	6.06±0.02	6.84±0.02	7.23±0.02	7.42±0.02	0.00	21.58±0.00
	Candida	0.00±0.00	6.82±0.01	7.41±0.01	8.16±0.02	0.00	19.99±0.00
Aqueous Extract of *Azadirachta indica*(AQEAI)	Trichophyton	7.13±0.02	7.80±0.01	8.03±0.02	8.14±0.01	0.00	19.30±0.00
	Microsporum	5.93±0.01	8.74±0.02	9.34±0.01	10.69±0.02	0.00	20.15±0.00
	Epidermophyton	6.33±0.05	7.57±0.02	7.83±0.02	7.95±0.02	0.00	21.58±0.00
	Candida	7.07±0.01	7.42±0.01	11.57±0.02	12.23±0.02	0.00	19.99±0.00

Figure:1 The antifungal activity of the Hexane and Benzene extract of *Azadirachta indica*in disc diffusion method.

The mean result obtained by the resazurinmicrotitre method was presented in **Table: 2**, the minimum dose required for inhibition of the growth of fungal organism is 125 µg per ml, 125 µg per ml, 250 µg per ml, and 62.5 µg per ml in aqueous extract of *Azadirachta indica* against *Trichophyton rubrum, Microsporum rubrum, Epidermophyton floccosum,* and *Candida albicans* respectively. The minimum dose required for inhibition of hexane extract of *Azadirachtaindica* is 250 µg per ml against *Candida albicans.* The minimum dose required for inhibition of ethyl acetate extract of *Azadirachta indica* is 250 µg per ml against *Microsporum gypseum.*The minimum dose required for inhibition of benzene extract and methanol extract of *Azadirachta indica* is 500 µg per ml against *Trichophyton rubrum, Microsporum gypseum, Epidermophyton floccosum,* and *Candida albicans.* Clotrimazole, standard, was 5 µg per ml, 20 µg per ml, 10µg per ml, and 10 µg per ml was observed against *Trichophyton rubrum, Microsporum gypseum, Epidermophyton floccosum*, and *Candida albicans* respectively.

Table no : 2 Comparison of Minimum Inhibitory Concentration (MIC) value determination with Resazurinmicrotitre detection method of various extracts in four fungal organisms.

Extract	**Minimum Inhibition Concentration (MIC) mcg/ml**			
	Trichophyton	**Microsporum**	**Epidermophyton**	**Candida**
Hexane Extract of *Azadirachta indica*(HEAI)	500	500	500	250
Benzene Extract of *Azadirachta indica* (BEAI)	500	500	500	500
Ethylacetate Extract of *Azadirachta indica* (EAEAI)	500	250	500	500
Methanol Extract of *Azadirachta indica* (MEAI)	500	500	500	500
Aqueous Extract of *Azadirachta indica*(AQEAI)	125	125	250	62.5
Standard	5	20	10	10

Discussion:

The plant *Azadirachta indica* as a whole used as anti-microbial, anti-fungal, insecticidal anti-viral, antipyretic antimalarial, mosquito larvicidal, anti-inflammatory, spemicidal, hypoglycemic.

Dermatophytosis is the commonest disease in worldwide with higher prevelance in tropical countries[23]especially in poor hygiene people.

All the extracts of *Azadirachta indica* used in this study are showing an anti-fungal activity as presented in results (as Table no: 1&Table no: 2 and interpreted), the effect of aqueous extract was showing in *Trichophyton rubrum, Microsporum gypseum*, *Epidermophyton floccosum*, and, *Candida albicans* organisms.

Singh. N et. al., reported that local application of a lotion prepared from 70% alcoholic extract of neem leaves was found to be effective in chronic skin diseases like ringworm infection, scabies, eczema.Charles. V et. al., clarified as the alcoholic neem leaf extract has been shown to control ringworm infection more effectively than Whitfield ointment (4:1 ratio of salicylic acid and benzoic acid). Natarajan. V[24], et. al., shows the anti-dermatophytic activity of neem leaf extract has been reported against different species of dermatophytes including *Trichophyton rubrum, Microsporum gypseum, Epidermophyton floccosum.* According to Khan. M, et.al., extracts of neem leaf are effective against certain fungi, like *Trichophyton rubrum, Microsporum gypseum, Epidermophyton floccosum*. According to Mahmound D.A[25], et.al., the aqueous extract of leaf of *Azadirachta indica* shows the very low percent of inhibition against *Candida albicans* and *Microsporum gypseum* but in my study, the aqueous extract is showing a high zone of inhibition.

The aqueous extract of *Azadirachta indica* was showing maximum efficacy with minimum dose and the further extension of the research can be carried to know the secondary metabolites which are having antifungal properties.

Conclusion:

The disc diffusion method is the standard method used for finding the zone of inhibition and also the resazurinmicrotitre method is relying assay based on the viability against fungal organisms. This study is showing the potential antifungal activity against the dermatophytes and also Candida.

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Conflict of Interest: No.

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