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Inhibition of nail infecting fungi of peoples of North Eastern U.P causing Tinea unguium through leaf essential oil of Ageratum houstonianum Mill

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ABSTRACT: *A Clinical surveys was carried out in North Eastern Uttar Pradesh,India and 200 samples of nail infections were collected.Seven fungal species were isolated from infected nails viz.,Aspergillus flavus,A.fumigatus,A.niger,Candida albicans,Epidermophyton floccosum,Fusarium oxysporum,Trichophyton rubrum. They were microscopically examined that showed the dominance of Candida and Trichophyton on the basis of percent occurrence on infected nails of peoples of different age groups.These species were also present in both sterilized and unsterilized infected nails. From the leaves of 32 higher plants essential oils were isolated and tested against Candida and Trichophyton.,found dominantly associated with nail infections in peoples of North Eastern U.P districts. The essential oil of Ageratum houstonianum Mill(Asteraceae)was found strongest toxicant against the test fungi inhibiting mycelial growth at 500ppm. The minimum inhibitory concentration (MIC) of the oil was 400 ppm against Candida albicans and Trichophyton rubrum. However, it was found fungicidal at 500ppm against both test fungi respectively.The oil was effective on heavy doses of inoculums (up to 10 discs of 5 mm each) at 400ppm.. The (MKT) of the oil was 30 sec against Candida albicans and 40 sec against T. rubrum.. The oils efficacy was thermo stable up to100°C and for 180 days of storage, the maximum taken into consideration. Moreover, on autoclaving there was no effect on fungitoxicity of oil.*

KEYWORDS: *Antifungal activity;essential oil;nail infecting fungi;plant drug.*

I. INTRODUCTION

Onychomycosis (also known as "dermatophytic onychomycosis, or "tinea unguium" eans fungal infection of the nail.(Rapini *et al*.,2007). It is the most common disease of the nails and constitutes about a half of all nail abnormalities (Szepietowski and Salomon,2007). This condition may affect toenails or fingernails, but toenail infections are particularly common. It occurs in about 10% of the adult population.(Westerberg, and Voyack,2013).The most common symptom of a fungal nail infection is the nail becoming thickened and discoloured: white, black, yellow or green. As the infection progresses the nail can become brittle, with pieces breaking off or coming away from the toe or finger completely. If left untreated, the skin can become inflamed and painful underneath and around the nail. The causative pathogens of onychomycosis include dermatophytes, *Candida*, and nondermatophytic molds. Dermatophytes are the fungi most commonly responsible for onychomycosis in the temperate western countries; while *Candida* and non dermatophytic molds are more frequently involved in the tropics and subtropics with a hot and humid climate. *Trichophyton rubrum* is the most common dermatophyte involved in onychomycosis. Other dermatophytes that may be involved are *T. interdigitale*, *Epidermophyton floccosum*, *T. violaceum*, *Microsporum gypseum*, *T. tonsurans*, *T. soudanense* (Chi *et al.*,2005 ;Westerberg and Voyack , 2013).Although there are number of synthetic antifungal are available in market but majority of them are fungi static in nature (Roxburg and Borrie, 1973). In recent years there has been a gradual revival of interest in the use of medicinal plants because herbal medicines have been reported to be safe and without any adverse side effects. Recent researches revealed that some products of plants origin in the form of essential oils have been investigated to be an effective source of chemotherapeutic agents without undesirable side effects and with strong fungicidal activity. So, in the present investigation, attempts have been made to explore the possibilities of use of endemic medicinal plants for isolating essential oils from leaves of some higher plants and testing their antifungal activity against dominant nail deteriorating fungi in peoples of Noth Eastern Uttar Pradesh.

II. MATERIALS AND METHODS

Collection of samples of infected nails-Infected nails were collected from five districts of North Eastern U.P districts in small presterilized polyethylene bags in order to study fungal infections in nails.

Isolation of Fungi-Fungus were isolated from infected nails by culturing infected nails on Sabouraud dextrose agar medium which included composition of 40g/L dextrose,10 g/L peptone, 20 g/L agar and a pH of 5.6. which was first created in 1892 by Raymond Sabouraud and the percent occurrence of each fungal species were calculated.

III. MICROSCOPIC DIAGNOSIS:

The clinical specimen consisting of nail was placed into a drop of 20% potassium hydroxide (KOH) preparation on a clean microscope slide. The slide is gently warmed and then examined with both the low and high-power objectives of the microscope**.**The Dermatophytes were identified by means of macroscopic as well as microscopic characteristics. Macroscopically colonies showed white, cream brown or pink with a distinctive bright pigment on the reverse.The presence of translucent, non pigmented, septate mycelium, and arthrospores were obseved. *Candida* spp. showed cluster of budding oval yeast with pseudo and true septate mycelium .

I**n vitro investigation**

Extraction and Isolation of Essential oil*:*

The leaf parts of 32 higher plants collected from North Eastern Uttar Pradesh regions were washed with sterilized water, macerated and hydrodistilled up to 6 h. in Clevenger's apparatus (Clevenger, J.F., 1928) for isolation of volatile constituents in the form of essential oils. The oils were dehydrated separately over anhydrous sodium sulphate to remove traces of moisture.

In-vitro antifungal investigations of the essential oil*:*

The toxicity of the oil was tested against *Candida albicans* and *Trichophyton rubrum* isolated from infected nails by Inverted petri plate technique (Bocher,1938). Each experiment repeated having five replicates. Fungal colony diameters of treatment and control sets were meausred on the seventh day. Fungitoxicity was recorded in terms of per cent inhibition of mycelial growth and calculated by following formula.

$$\text{Per cent inhibition of mycelial growth} = \frac{dc - dt \times 100}{dc}$$

Where, dc = average increase in diameter of fungal colony in control sets

dt = average increase in diameter of fungal colony in treatment sets

Physico-chemical properties of most active oil – leaf oil of *Ageratum houstonianum* :Freshly isolated oil was light yellow in colour and immiscible with water. The oil was characterized by determination of its various physico-chemical properties viz., specific gravity, specific rotation, refractive index, saponification number, ester number, and solubility (Langenau,1948)

Fungitoxic properties of *Ageratum houstonianum* oil:The minimum inhibitory concentration (MIC) of oil against test fungi was determined by poisoned food technique of Grover and Moore (1962). The nature (fungistatic/fungicidal) of the oil was studied following Garber and Houston (1959).The effect of spectrum,temperature, autoclaving and storage on the fungitoxicity of the oil was studied following Kumar and Tripathi (2002). The fungitoxicity was recorded in terms of percent inhibition of mycelial growth.

Effect of Inoculums Density*:* The effect of inoculums density on the minimum inhibitory concentration (MICs) of the oil against the test fungi was determined.. Mycelial discs of 5mm diam of 7-day old cultures were inoculated in culture tubes containing oil at their respective MICs. In controls, sterilized water were used in place of the oil and run simultaneously. The numbers of mycelial discs in the treatment as well as control sets were increased progressively up to 10 discs each having 5mm diam. Observations were recorded up to seventh day of incubation. Absence of mycelial growth in treatment sets up to 7th day exhibited the oil potential against heavy doses of inoculums.

IV. MINIMUM KILLING TIME:

The minimum killing time (MKT) of the oil was determined by the mycelial disc killing technique(MDKT) of Shahi *et al.*(1999) with some modification. Two sets were maintained, one with oil at its minimum inhibitory concentrations and other for control set.The treatment set using mic of the oil was prepared by mixing the required quantity of the oil in acetone and then adding this to the appropriate quantity of distilled water. Simultaneously, controls were maintained using sterilized water (in place of the oil) and acetone, adding into the distilled water in appropriate quantities.Mycelial discs of 5 mm diameter, cut out from the periphery of 7-day-old cultures of the test pathogens, were aseptically placed in the culture tubes of different treatment and control sets. These mycelial discs were taken out of the tubes at different time intervals and washed immediately in the washing solution (containing acetone: sterilized distilled water, 1:2) to remove the treatment solution. These washed mycelial discs were aseptically transferred upside-down to the sabouraud dextrose agar (SDA) medium (pH 5.6) in the petri plates.The same procedure was followed with the control sets.The inoculated petri plates were incubated at 28 ±2 ºC and the observations recorded as an average value of five replicates on the seventh day. The percentage of fungal growth inhibition was calculated following Mishra *et al.*(1995).

V. RESULTS ISOLATED FUNGAL ORGANISMS

Seven fungal species were isolated from infected nails viz.,*Aspergillus flavus,A.fumigatus,A.niger,Candida albicans,Epidermophyton floccosum,Fusarium oxysporum,Trichophyton rubrum* from peoples of North Eastern U.P districts.There was dominance of *Candida* and *Trichophyton* on the basis of percent occurrence on infected nails of peoples of different age groups.These species were also present in both sterilized and unsterilized infected nails(Table,1).

This showed the following characteristics-

Candida albicans.

On Sabouraud's Dextrose Agar medium colonies were white to cream colored, smooth, glabrous and yeast-like in appearance. Microscopic morphology showd spherical to subspherical budding yeast-like cells or blastoconidia, 2.0-6.0 x 3.0-7.5 um in size.

Candida albicans spp. was confirmed microscopically by the observation of production of chlamydospores .

Trichophyton rubrum

On Sabouraud's Dextrose Agar, colonies were flat to slightly raised, white to cream, suede-like to downy, with a yellow-brown to wine-red reverse. Mostly showed scanty to moderate numbers of slender clavate to pyriform microconidia. Macroconidia are usually absent, however closterospore-like projections was found to be present in some mounts.

Fungitoxicity testing

During evaluation of leaf essential oil of 32 higher plants *Ageratum houstonianum* was found to be highly fungitoxic at 500ppm against both the test fungi(Table,2).

Physico-chemical properties of most active oil

T he physic-chemical properties are recorded in Table,3.

Description of active plant:A short-lived (annual or biennial) herbaceous plant growing 0.3-1 m tall, with showy flower-heads.The stems are round, mostly green in colour, and softly hairy (pubescent). The leaves are often opposite, and may be alternate in upper parts of the stem. Leaves are borne on stalks (petioles) 0.5-3 cm long and vary from being almost triangular in shape (obovate) to egg-shaped in outline with broad end at base (ovate). These leaves (2-7 cm long and 1.5-6 cm wide) have bluntly toothed (crenate) margins and either blunt or pointed tips (obtuse to acute apices). Both surfaces of the leaves and the leaf stalks have a scattered covering of hairs (they are pubescent).The flower-heads (capitula) are arranged in dense clusters at the tips of the branches (in terminal corymbs) and do not have any obvious 'petals' (ray florets). Each flower-head (5-8 mm across) has numerous tiny tubular flowers (tubular florets) that are surrounded by two or three rows of greenish-coloured bracts (an involucre). The florets (2-3 mm long) range from pale lavender to blue, pink or purplish in colour and each has two elongated projections (style branches). The bracts at the base of the flower-head (3-5 mm long) are elongated in shape (linear-lanceolate) and covered in sticky hairs (glandular pubescent). Flowering occurs throughout most of the year.

Fungitoxic properties of *Ageratum houstonianum* oil

MIC of the oil

The minimum inhibitory concentration of the oil was found to be 400ppm.The oil was found to be fungicidal at 500ppm against both the test fungi viz.,*Candida albicans* and *Trichophyton rubrum(*Table,4).

Spectrum of oil

The fungitoxic spectrum testing revealed that oil was effective for 14 fungi including nail infecting and other plant fungi at 400ppm and at 600ppm this controlled all the fifteen fungi taken in present investigation(Table,5).

Effect of inoculums doses

Absence of mycelial growth in treatment sets up to 7th day exhibited the oil potential against heavy doses of inoculums.

Effect of physical factors on activity of oil

There was no adverse effect of physical factors such as temperature,autoclaving and storage upto 180 days on fungitoxicity of the oil.The oil was found to be thermostable upto 100°C (Table,6).

The (MKT) of the active oil

The (MKT) of the oil was 30 sec against *Candida albicans* and 40 sec against *T. rubrum*.(Table,7).

VI. DISCUSSION

The hands and fingernails are often affected by fungal and yeast infections, such as those caused by species of *Trichophyton* and *Candida*. In particular onychomycosis (nail infection) is the most common disease associated with the hands and feet, effectuating at least 50% of all fingernail infections.(Shemer at al.,2008) Onychomycosis is caused by dermatophytes (infectious fungi or yeast) invading the nail bed, which also cause ringworm and tinea, such as athletes foot. Most cases of onychomycosis is characterized by mild inflammations, resulting in the nail bed becoming cornified and losing its normal contour. Another form of onychomycosis results in the destruction of the nail plate and is often visible by a whitish yellow discoloration. Onychomycosis can also occur on the external nail plate, caused by an invasion of *Acremonium* and *Aspergillus*, which infect the superficial layers of the nail resulting in white patches on the nail.(Weitzman et al.,1995). Onychomycosis is increasingly viewed as a major medical concern as these infections can lead to secondary infections as well as being transferred to other bodily areas and other people (Kaur *et al.*,2008). These condition substantially increase the risk of onychomycosis and other related diseases. Sumbul *et al*(2005) *isolated Trichophyton mentagrophytes, Eidermophyton floccosum. Aspergillus glaucus, Aspergillus flavus, Aspergillus fumigatus,Candida albicans , Candida glabrata* and *Candida tropicalis from infected hair and nails.* In present investigation seven fungal species were isolated from infected nails viz.*Aspergillus flavus,A.fumigatus,A.niger,Candida albicans,Epidermophyton floccosum,Fusarium oxysporum,Trichophyton rubrum* from peoples of North Eastern U.P districts.There was dominance of *Candida* and *Trichophyton* on the basis of percent occurrence on infected nails of peoples of different age groups.This variation of fungal species may be due to different climatic conditions and isolation periods.

According to Wellman(1967) a fungicide must retain its fungitoxicity at the extreme of temperatures.The fungitoxicity of the oil of *Ageratum houstonianum* was found to be thermostable upto 100C like *Putranjiva roxburghii*(Kumar and Tripathi,2004),*Ageratum conyzoides*(Dixit *et al*.,1995) and *Nardostachys jatamansi*(Mishra *et al*.,1995).The oil retained its fungitoxicity on autoclaving(15lbs/square inch pressure).This quality of oil will facilitate the isolation of their constituents in active state.A fungicide should be able to retain its activity during long period of its storage(Wellman,1967).The fungitoxic factor in the oil of *Adenocalyma allicea* was lost within 21 d of storage(Chaturvedi,1979) while persisted for long period in the oil of *Ageratum conyzoides*(Dixit et al.,1995),*Trachyspermum ammi*(Singh and Tripathi,1999). The fungal toxicity was not affected by storage upto 180 days during present investigation.So this show that the oil can be safely stored at any ambient temperature for long periods without loss in toxicity.

VII. CONCLUSION

Thus,*Ageratum houstonianum* leaf oil shows potential as a potent botanical fungicide for the control of fungal infestation of nails of human beings as low cost and locally available and on the basis of its strong fungal toxicity at low MIC,themostability and long shelf life and minimum killing time.

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REFERENCES

[1]. Bocher,O.E(1938)Antibiotics.In;Modern methods of plant analysis.Eds.Peach K and Tracey M.V(ed.).Modern methods of plant analysis vol iii,651,Springer-Verlag,BerlinChaturvedi,R(1979)Evaluation of higher plants for their fungitoxicity against *Helminthosporium oryzae*.Ph.D Thesis Gorakhpur Uniersity,Gorakhpur,India.

[2]. Chi CC, Wang SH, Chou MC (2005). "The causative pathogens of onychomycosis in southern Taiwan". Mycoses **48** (6): 413–20.

[3]. Clevenger, J.F., (1928): Apparatus for the determination of volatile oil. J. Am. Pharm. Assoc.,17:346.

[4]. Dixit,S.N.,Chandra,H.,Tiwari,R and Dixit,V(1995)Development of botanical fungicide against blue mould of mandarins.J.Stored Prod.Res.**,31(2**);165-172.
[5]. Garber, R.H., Houston, B.R., (1959): An inhibitor of *Verticillium alboatrum* in cotton seed. Phytopathology, 49: 449-450.
[6]. Grover R.K and Moore J.D(1962)Toximetric studies of fungicides against brown rot organism,*Sclerotinia fructicol*a and *S.laxa*.Phytopath **52;**876-880.
[7]. Kaur, R., B. Kashyap and P Bhalla(2008) Onychomycosis-Epidemiology, Diagnosis and Management. Indian Journal of Medical Microbiology. **26**: 108-116.
[8]. Kumar,N and Tripathi,N.N(2004)Repellent property of volatile oil isolated from *Putranjiva roxburghii* against *Trogoderma granarium* associated with stored groundnut seeds.Proc.Nat.Acad.Sci India **74B(11**);179-187.
[9]. Langenau,E.E(1948)The examination and analysis of essential oils;synthetics and isolates.In;Guenther,E(Ed.)The essential oils Vol,1.Krieger Publishing Co.,Hutington,New York pp 227-348.
[10]. Mishra D.,Chaturvedi,R.V and Tripathi, S.C(1995)The fungitoxic effect of the essential oil of the herb *Nardostachys jatamansi* D.C. Tropical Agri **72(1);**48-52.
[11]. Rapini, Ronald P.; Bolognia, Jean L.; Jorizzo, Joseph L.(2007). *Dermatology: 2-Volume Set*. St. Louis: Mosby. p. 1135.
[12]. S umbul,S.S.,Waseemuddin .Siddiqui S.A and Azhar,I(2005)Superficial mycoses:a study performed for the isolation and identication of fungal species from infected patients.Pakistan Journal of Pharmacology,**22(1):**41-46.
[13]. Shahi, S.K., Shukla, A.C., Bajaj, A.K, Medgely, G., Diksit, A., (1999): Broad spectrum antimycotic drug for the control of fungal infection in human beings. Current Science, **76(6**): 836- 839.
[14]. Shemer, A., H. Trau, B. Davidovici, MH. Grundwald, B. Amichai(2008). Onycomycosis Due to Artificial Nails. JEADV. , **22**: 998-1000
[15]. Singh,J and Tripathi,N.N(1999)Inhibition of storage fungi of black gram(*Vigna mungo* L) by some essential oils.Flavour Fragrance J**. 14**;1-4.
[16]. Szepietowski JC, Salomon J (2007). "Do fungi play a role in psoriatic nails?". Mycoses ,**50 (6):** 437–42.
[17]. Weitzman, Irene, and Richard Summerbell.(1995) The Dermatophytes. Clinical Microbiology Reviews. **8**: 240-259.
[18]. Wellman,R.H(1967)Commercial development of fungicides.In;Plant pathology Problem and Progress Eds Holtan *et al*.,1908-1958.Indian University Press,Allahabad, India.
[19]. Westerberg, DP; Voyack, MJ (Dec 1, 2013). "Onychomycosis: current trends in diagnosis and treatment.". American family physician **88 (11**): 762–70.

Table1.Occurrence of different fungi on the infected nails of peoples of north Eastern U.P Districts

Fungal species	unsterilized	sterilized
Aspergillus .flavus	25.5	14.5
A.fumigatus	25.6	4.4
A.niger	21.4	18.6
Candida albicans	56.0	94.0
Epidermophyton floccosum	38.6	21.4
Fusarium oxysporums	10.2	9.8
Trichophyton rubrum	70.6	79.4

Table 2.Evaluation of essential oils of higher plants against *Candida albicans and Trichophyton rubrum*

Plant species	Per cent inhibition of mycelia growth of test fungi at 500ppm		
	Family	*Candida albicans*	*Trichophyton rubrum*
Aegle marmelos(L.)Corea	Rutaceae	37.3	52.3
Ageratum conyzoides L.	Asteraceae	90.0	80.3
*A. houstonianum*Mill	Asteraceae	100.0	100.0
Anetum graveolens L.	Umbelliferae	29.0	33.2
Anisomeles ovate R.Br.	Lamiaceae	54.3	60.2
Artabotrys hexpetalous(Lamm)Merr.	Annonaceae	43.2	46.0
Azadirachta indica A. Juss.	Meliaceae	43.1	38.0
Caesulia oxillaris Roxb.	Asteraceae	48.1	47.2
Callestemon lanceolatus DC	Myrtaceae	48.3	45.2
Cannabis sativa L.	Cannabinaceae	10.0	9.0
Cinnamomum tamla Nees and Bbrem	Lauraceae	29.0	23.5
Citrus aurantifolia Christm	Rutaceae	39.2	26.3
*C.medica var limonia(*L.)	Rutaceae	46.9	57.3
Eucalyptus citriodora Hook	Myrtaceae	48.1	34.8
E.globulus(l.) Herit	Myrtaceae	62.0	33.9
Eupatorium capillifolum (L.)	Asteraceae	42.0	33.9
Feronia elephantum Correa	Rutaceae	47.7	64.3

*F.limonia(*L.) Swingle	Rutaceae	51.8	63.4
*Hyptis suaveolens(***L.) Poit**	Lamiaceae	46.2	26.4
Lantana camera L.	Verbenaceae	57.3	37.1
L.indica Roxb.	Verbenaceae	54.7	44.0
Mentha arvensis L.	Lamiaceae	53.9	36.6
M.piperata L.	Lamiaceae	62.3	54.3
M.spicata L.	Lamiaceae	61.3	44.2
Murraya koenighii(L.)Spreng	Rutaceae	24.8	43.1
Ocimum adscendens Willd	Lamiaceae	52.0	54.9
O.basilicum L.	Lamiaceae	41.1	50.4
O.canum Sims	Lamiaceae	55.1	75.4
O.sanctum L.	Lamiaceae	59.1	52.6
Putranjiva roxburghii Wall	Euphorbiaceae	90.0	85.7
Tagetes erecta L.	Asteraceae	86.0	85.7
Thuja occidentalis L.	Cuppressaceae	20.0	46.5

Table 3. Physico chemical properties of leaf oil from *Ageratum houstonianum*

Parameters	Values
Specific gravity	0.943
Specific rotation	+10
Refractive index	1.549
Acid value	3.55
Saponification number	154.6
Ester number	151.05
Phenolic content	Nil
Solubility	Completely miscible with petroleum ether acetone and 90%ethanol in 1;1ratio but insoluble in water

Table 4. MIC of the leaf essential oil of *Ageratum houstonianum*

Dose of oil in ppm	*Candida albicans*	*Trichophyton rubrum*
200	30	40
300	70	80
400	100	100
500	100*	100*
600	100	100

*Fungicidal

Table 5.Fungitoxic spectrum of leaf essential oil of *Ageratum houstonianum* at sub lethal, lethal and hyperlethal doses

Fungal species	Per cent inhibition of mycelial growth of isolated fungi			
	Sublethal 200ppm	Lethal 400ppm	Hyperlethal 600ppm	Hyperlethal 800ppm
Alternaria alternata	45.6	100.0	100.0	100.0
Aspergillus candidus	49.6	100.0	100.0	100.0
A.flavus	50.0	100.0	100.0	100.0
A.niger	30.0	100.0	100.0	100.0
A.fumigatus	40.0	100.0	100.0	100.0
A.tamarii	48.0	100.0	100.0	100.0
Candida albicans	59.0	100.0	100.0	100.0
Epidermophyton floccosum	55.6	100.0	100.0	100.0
Fusarium moniliforme	40.0	100.0	100.0	100.0
F.oxysporum	42.0	100.0	100.0	100.0
F.solani	40.0	100.0	100.0	100.0
P.glabrum	59.0	100.0	100.0	100.0
Rhizopus nigricans	54.0	100.0	100.0	100.0
Trichoderma viride	55.0	80.0	100.0	100.0
Trichophyton rubrum	65.9	100.0	100.0	100.0

Table 6.Effect of physical factors on the fungitoxicity of leaf essential oil of *Ageratum houstonianum*

Physical factors	Per cent inhibition of mycelial growth at its MIC
Temperature(°C) Time of treatment-60min 40°C 60°C 80°C 100°C	 100 100 100 100
Autoclaving (15lbs/sq inch pressure at 120C) For 15 min	100
Storage in days 15 30 45 60 75 90 105 120 135 150 165 180	 100 100 100 100 100 100 100 100 100 100 100

Table- 7: Minimum killing time of the oil of *Ageratum houstonianum* Mill against *Candida albicans* and ***Trichophyton rubrum***

Killing time	*Candida albicans*	*Trichophyton rubrum*
	Percent mycelial inhibition	
5second	40.0	46.0
10econd	50.0	56.0
15 second	60.0	65.0
20 second	70.0	75.0
25 second	90.0	80.0
30 second	100.0	90.0
35 second	100.0	95.0
40 second	100.0	100.0
45 second	100.0	100.0