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Antifungal Properties of Some Essential Oils of Angiospermic Plants against *Trichophyton rubrum* Causing Dermatomycosis

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Abstract

During screening of 45 aqueous extracts of aromatic plant parts for their volatile toxicity against test fungus *Trichophyton rubrum,* the extracts of rhizomes of *Curcuma longa* and *Zingiber officinale* exhibited strong antifungal activity. The effect of some physical factors *viz.* temperature and storage on antifungal activity of rhizomes of *C. longa* and *Z. officinale* has been studied. The rhizomes of selected potent plants were subjected to hydrodistillation so as to collect volatile antifungal fractions which were found to be essential oils. The minimum inhibitory concentration (MIC) of the *Curcuma* and *Zingiber* oils were found to be 250 µg ml^{-1} and 500 µg ml^{-1} respectively against test fungus *T. rubrum.* Both oils were fungicidal up to 500 µg ml^{-1}. Minimum time required by oils to express fungicidal nature, effect of increased inoculum density of the test fungus, effect of storage and temperature on fungitoxicity of oils were also evaluated. On account of their strong efficacy against test fungus, long shelf life, thermostability of their potency, these plant products may be exploited as an herbal chemotherapeutents in place of synthetic drugs in cure of dermatomycoses.

Keywords: Essential oils, Dermatomycosis, Fungitoxicity, Trichophyton Rubrum, Curcuma Longa and Zingiber Officinale.

Introduction

In addition to the bacteria and viruses, fungi and protozoa also act as agents of disease (Pelczar *et al*., 1993). Of some 100,000 fungal species only about 100 have pathogenic potential for humans; of these, only a few species account for most clinically important fungal infections (Richard *et al.,* 2007). The dermatophytes constitute a group of superficial fungus infections of keratinized tissue *viz.,* the epidermis, hair and nails. The word dermatophytes literally mean “skin plants” (Chester *et al.,* 1970). Human fungal disease (mycoses) is classified by the location or in the body where the infection occurs. They are called cutaneous when limited to the epidermis, subcutaneous, when the infections penetrate significantly beneath the skin and systemic when the infection is deep within the body or disseminated to internal organs. These fungi spread radically in the dead keratinized layer of the skin by means of branching hyphae and occasional arthrospores. Inflammation of the living tissue below is very mild and only a little dry scaling is seen. Usually there is irritation, erythema, edema and inflammation at the spreading edge; this pinkish circle gave rise to the name ringworm. These diseases are widespread and difficult to control (Wedberg 1963).

Although some antibiotics have been developed from microorganism for this purpose but recently some of them after application have been found to induce side effects. Moreover, some fungi causing mycosis have developed resistant forms against these prevalent antibiotics and therefore it is sometimes difficult to control them completely (Spotts & Cervantes, 1986). Due to their various side effects on human systems, the use of most of such drugs has remained limited (Heel *et al*., 1978, Botter, 1980; Symoens *et al*., 1980, Wajtulewski *et al*., 1980).

In modern medicine also, plants occupy a very significant place as raw material for some important drugs, although synthetic drugs and antibiotics brought about a revolution in controlling different diseases, but these synthetic drugs are out of reach to millions of people. Those who live

in remote places depend on traditional healers, whom they know and trust. Judicious use of medicinal herbs can even cure deadly diseases that have long defied synthetic drugs (Shiva *et al.,* 2002). There is continuous search for more potent and cheaper raw material to feed the industry. With concerted research and development efforts, many medicinal plants could provide raw material in abundance to the indigenous pharmacies and local herbalists. Strong linkages should be developed between medicinal plant growers, health experts and pharmaceutical industries for developing scientific basis on which these systems of medicine are working. An integrated system of medicine based on natural products and synthetics may yield the most effective and cheap package for WHO's goal of "health for all".Because of the general awareness of the wide spread toxicity and harmful effects associated with the long use of synthetic drugs and antibiotics, the society prefers the drugs from natural sources than the synthetics (Shiva *et al*., 2002). Essential oils refer to fragrant oils extracted from plants chiefly through steam distillation .Essential oils, phytoncides and other natural compounds work in different ways. At the scent level they activate the limbic system and emotional centres of the brain. When applied to the skin (commonly in form of "massage oils" i.e. 1-10% solutions of EO in carrier oil) they activate thermal receptors, and kill microbes and fungi. Internal application of essential oil preparations (mainly in pharmacological drugs; generally not recommended for home use apart from dilution 1-5% in fats or mineral oils or hydrosols) may stimulate the immune system (Rho *et.al.,* 2006; Kim., 2007). Herbs are staging a comeback and an "herbal renaissance is blooming across the world". They have been prized for their medicinal, flavouring and aromatic qualities for centuries, yet for a while they were overshadowed by the synthetic products of the modern civilization. But after realizing the serious side effects of synthetic drugs people are going back to the nature (plant and their product) with hopes of safety and security.

Therefore, in the present piece of investigation, it has been thought desirable to find out potentiality of some plant products (essential oils) as antifungal agents against *Trichophyton rubrum* castellani (sab): causing dermatomycosis.

Materials and Methods

Procurements of test fungus *Trichophyton rubrum* (Castellani) Sab

The culture of dermatomycoses causing fungus *Trichophyton rubrum* was procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. Confirmation of its identity was also done with the help of relevant literature and culture on Sabouraud agar (SDA) medium and stored in BOD incubator at 25±1^{oC} for further experiments.

Screening of extracts of some angiospermic aromatic plants for their fungitoxic activity against *T.rubrum*.

Aromatic plants belonging to different families of angiosperms were collected from locality and identified with the help of floras (Duthie, 1903-1929; Bailey, 1958; Maheshwari, 1963; Santapau, 1967; Srivastava, 1976; Dubey, 2005). Confirmation of identity of the aromatic plants was done with the help of authentic herbarium specimens. Fresh leaves (25g) of each plant species were washed with water, macerated with 25 ml. of distilled water (1:1 W/V) in a pestle and mortar and then filtered through cheese cloth to collect the filterate. Two mg. of chloramphenicol was added to it in order to check bacterial contamination. The clear extract thus obtained was assayed for its antifungal activity against test fungus *T. rubrum* by the inverted petriplate method of of Bocher (1938). The Sabouraud agar medium (68 gm of SDA dissolved in 1000 ml of distilled water) was autoclaved and cooled to about 40^{oC}. Chloramphenicol at rate of 10 mg/l was added to the medium mixed thoroughly following Gupta and Banerjee (1970) in order to prevent bacterial contamination. Ten ml of medium was poured aseptically per petriplate (9.5 cm diameter). The petriplates containing solidified medium were aseptically inoculated with discs cut from the periphery of seven day old culture of the test fungus. The inoculated plates were inverted upside down. Five ml. of plant parts filterate was aseptically pipetted into the lid of petriplates. Control sets were prepared similarly using five ml sterilized distilled water in place of extract. The plates were incubated at 25±1^{oC} for six days. Diameter of fungal colony in control and treatment sets were measured in mutually perpendicular directions and fungitoxicity was recorded in terms of percent mycelial growth inhibition, calculated as per formula:

$$\text{Percent of mycelial inhibition} = \frac{dc - dt}{dc} \times 100$$

Where:

dc = Average diameter of fungal colony in control sets.

dt = Average diameter of fungal colony in treatment sets.

The experiment was run in triplicate and the mean values ±SD are presented in **Table -1,** where the plants are arranged alphabetically with their families.

Effect of storage and temperature on fungitoxicity of Rhizomes

Experiments on effects of storage and temperature on antifungal activity of rhizomes of *Curcuma longa* and *Zingiber officinale* were carried out in order to understand the nature of fungitoxicity.

Effect of Storage

To study the effect of storage, the freshly collected rhizomes were air dried and stored in through at room temperature (25-28^{o}C). Fungitoxicity of the stored rhizomes was tested at regular intervals of 5 days from the date of their collection by the usual inverted petriplate method and data were recorded in terms of percentage mycelial inhibition upto 30 days.

Effect of Temperature

To study the effect of temperature, four lots each containing 10 g of freshly collected rhizomes of *Curcuma longa* and *Zingiber officinale* were incubated separately at various temperatures *viz.* 40^{o}C, 80^{o}C

and 100°C for two hours. The fungitoxicity of the treated samples was tested separately by the usual inverted petriplate method and results recorded in terms of percent mycelial inhibition.

Isolation and screening of essential oils of potent antifungal angiospermic plant parts at 3000 µg ml^{-1} against *T.rubrum*

Essential oils of potent angiospermic plant parts *viz*, *Curcuma longa* (rhizome), *Cymbopogon citratus* (leaf), *Ocimum canum* (leaf), *O. basilicum* (leaf), *O. gratissimum* (leaf), *O. sanctum* (leaf) and Zingiber o*fficinale* (rhizome) were isolated by hydrodistillation through Clevenger's apparatus. Fresh effective plant parts (500 gm) were washed with water, cut out in small pieces and subjected to hydrodistillation. The isolated volatile essential oils showed two distinct layers - an upper oily layer and lower aqueous layer. Both the layers were seperated and stored for fungitoxicity test. However, the moisture content from the oily layer was removed with the help of anhydrous sodium sulphate. The percent recovery (W/V) of oils was determined following Mishra and Dubey (1994) by following formula:

$$\text{Percent recovery of oil} = \frac{\text{Volume of essential oil (ml)}}{\text{Weight of leaves(g)}} \times 100$$

The antifungal activity of essential oils was tested by poisoned food technique of Grover and Moore (1962). Requisite amounts of oils were dissolved separately in 0.5 ml of acetone in pre-sterilized petriplates. 9.5 ml of Sabouraud dextrose agar medium was pippeted to each petriplate so as obtain 3000 µg ml-1 concentration. The plates were swiveled thoroughly in order to obtain homogenous medium. For control sets, requisite amounts of sterilized water dissolved in 0.5 ml. acetone was added to medium in place of oils. Fungal discs (5 mm diameter) cut from the periphery of seven-day old culture of test fungus were placed aseptically to the centre of each petriplate containing medium. The petriplates were incubated at 25±1°C for six days. Diameters of fungal colony of treatment and control sets were measured in mutually perpendicular directions on the seventh day. The percent mycelial inhibition was calculated by the value of colony diameter by the mean±SD of colony diameter value based on three replicates as shown in **Table -2.**

Fungitoxic Properties of the Oils

Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration at which the oils showed absolute fungitoxicity was determined by the usual poisoned food technique. Different concentrations of the oils (2000, 1000, 500, 250, and 200 µg ml-1) were prepared by dissolving its requisite amount in 0.5 ml acetone and then mixing with 9.5 ml sabouraud dextrose agar medium separately. The medium of control set contain requisite amount of sterilized water and 0.5 ml acetone in place of the oils. As usual the plates were incubated aseptically with the assay discs of the test fungus and incubated for six days at 25 ± 1°C. The observation was recorded on the seventh day and percentage in form of mean ± SD based on three replicate in **Table -3.**

Nature of Toxicity

Nature of toxicity (fungistatic/fungicidal) of the oils against the test fungus was determined following Garber and Houston (1959) and Thompson (1989).Requisite amounts of *Curcuma longa* and *Zingiber officinale* oils were dissolved in 0.5 ml acetone and then mixed with 9.5 ml sabouraud dextrose agar medium separately to get requisite concentrations 5000, 4000, 3000, 2000, 1000, 500 and 250 µg ml-1 for *Curcuma longa* and 5000, 4000, 3000, 2000, 1000 and 500 µg ml-1 for *Zingiber officinale* oils respectively. Sterilized water and 0.5 ml acetone were used in control sets in place of the oils. The plates were inoculated aseptically with fungal disc (0.5 mm diameter taken from the periphery of a seven days old culture of the *Trichophyton rubrum*) and were incubated for six days. On seventh days the inhibited discs were taken out from the plates, washed with sterilized water and re-inoculated aseptically to plates containing fresh sabouraud dextrose agar medium. The percentage mycelial inhibition with respect to control was calculated on the seventh day and result are presented in **Table - 4**

Minimum Time Required by Oils to Express Fungicidal Nature

The extent of time required by oils to kill the test fungus at different cidal concentrations was studied with usual fumigation technique on sabouraud dextrose agar medium following the method adopted by Liu *et al.,* (2001 a, b). Different cidal concentrations of oils (500, 1000 and 1500 µg ml-1) were prepared with respect to the volume of petriplates (v/v). The inoculation method was the same described in previous experiment. After 24, 48, 72, 96 and 120 hrs. of incubation the fungal discs were placed on fresh medium to observe the revival of mycelial growth after five days. **(Table -5).**

Effect of Increased Inoculum on Toxicity of the Oils

The effect of increased inoculum density of *Trichophyton rubrum* on fungitoxicity of oils was studied following Pandey (2003). Requisite quantity of *C. longa* and *Z. officinale* oils were separately dissolved in 0.5 ml acetone and then mixed with to 9.5 ml of sabouraud dextrose broth medium to make the final concentration of 500 µg ml-1. Five sets thus prepared from each oil were inoculated separately by the assay discs (4mm) of the test fungus in geometrical progression of two i.e. 2, 4, 8, 16 and 32. For control sterilized water dissolved in acetone was mixed to the liquid medium. All the eleven flasks were incubated for six days at 25±1°C. Observation was recorded on the seventh day.

Effect of Storage and Temperature on Fungitoxicity of the Oils

C. longa and *Z. officinale* oils were stored in air tight specimen tubes separately at room temperature and their activity against the test fungus was tested at regular intervals on one month at their respective minimum inhibitory concentrations following poisoned food technique. The observation was recorded and percentage mycelial inhibition was calculated. To study the effects of temperature on

toxicity of Curcuma and Zingiber oils, 3 lots of oils each containing 2 ml of the oil separately were kept in air tight specimen tubes and were treated at different temperature (viz., 40°C, 60°C & 80°C) for three hours. The oils were cooled to room temperature and tested for fungitoxicity by the usual poisoned food technique at their MIC(s).

Results and Discussion

T. rubrum produces on sabouraud's agar a white to reddish, floccose or velvety colony. The cherry red pigment is most apparent on the reverse side of the colony. It is evident from **Table -1** that the 45 aqueous extracts of leaves and other plant parts of angiospermic aromatic plants belonging to 11 families, screened against the test fungus, most of plants parts showed either poor (below 50%) or moderate (above 50% and below 100%) activity. Moreover extracts of *Curcuma longa, Cymbopogon citratus, Ocimum basilicum, O. canum, O. gratissimum, O. sanctum* and *Zingiber officinale* were found to exhibit absolute toxicity inhibiting the growth of test fungus completely (100%). None of the plants accelerated the growth of the test fungus. Therefore, on account of absolute fungitoxicity of the *Curcuma longa* (rhizome), *Cymbopogon citratus* (leaf), *O. basilicum* (leaf), *O. canum* (leaf), *O. gratissimum* (leaf), *O. sanctum* (leaf) and *Zingiber officinale* (rhizome) were selected for further studies.

The fungitoxicity in stored rhizomes of *C. longa* and *Z. officinale* persisted upto 30 days, the maximum period taken into consideration and fungitoxicity of rhizomes remained unaltered up to 80^{o}C and then fungitoxicity declined on 100^{o}C.

It is evident from the **Table-2** that essential oils of *C. longa* (rhizome) and *Z. officinale* (rhizome) were found to exhibit absolute toxicity inhibiting the growth of the test fungus completely. Therefore among these essential oils, *C. longa* (rhizome) and *Z. officinale* (rhizome) were selected for further detailed studied. It is clear from **Table -3** that 250 µg ml-1 and above cocentrations the Curcuma oil inhibited the mycelial growth of test fungus completely. Whereas at 500 µg ml-1 and above of the *Zingiber* oil inhibited the mycelial growth of test fungus completely. Therefore the minimum inhibitory concentrations were recorded as 250 µg ml^{-1} and 500 µg ml^{-1} for *C. longa* and Z. *officinale* respectively.**Table -4** indicated that *C. longa* oil was fungistatic on its respective MIC (250 µg ml^{-1}), but fungicidal at above concentrations. However, *Z officinale* oil was fungicidal up to its MIC (500 µg ml^{-1}). The data of **Table-5** indicates that minimum 120 hrs were required by both the oils to kill *Trichophyton rubrum* at minimal cidal concentration i.e. 500 µg ml-1. However, 72 and 96 hrs was required by *C. longa* and *Z. officinale* oils to express cidal nature at 1000 and 1500 µg ml-1 concentrations respectively.

The oils inhibited the mycelial growth of the treatment sets containing even upto 32 fungal discs, indicating their capacity to withstand high inoculum density and oils remained effective upto 180 days of storage, the maximum period taken into consideration exhibiting long shelf life. The results also predict that the oils remained effective even up to 80°C the maximum temperature taken into consideration showing the thermostable nature of their fungitoxicity

Herbal medicines are being used by about 80% of the world population primarily in the developing countries for primary health care. They have stood the test of time for their safety, efficacy, cultural acceptability and lesser side effects. The chemical constituents present in them are a part of the physiological function of living flora and hence they are believed to have better compatibility with the human body. Ancient literature also mention herbal medicines for age related diseases namely memory loss, osteoporosis, diabetic wound, immune and liver disorder etc for which modern medicine or only palliative therapy is available (Rajasekharan, 2002)

Dermatomycoses (ring worm disease) a prevalent disease of the tropic and is common in India during rainy season and requires such type of preventive measures that would be cheaper and safer for the Indian people. Although, the preliminary testing with the essential oils against dermatophytes have been made (Pandey, et.al., 1983; Dubey & Mishra, 1990; Kishore *et al*, 1993; Yadav, 1997; Cassella *et.al.,* 2002; Benger *et.al.,* 2004) their detailed systematic investigation against ringworm fungi is lacking.

Thus these plant products (essential oils) may be exploited as indigenous, biodegradable, Eco-friendly, nontoxic, natural antifungal agents for control of superficial mycoses after detailed *in vivo* trials. The findings of the research will be useful for pharmaceutical trades for development of new antifungal agents with ayurvedic ethics.

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Table -1
Screening of Extracts of Angiospermic Aromatic Plants against *Trichophyton rubrum*

Name of Plants	Plant parts	Family	Percent inhibition of mycelial growth (mean ± SD)
Ageratum conyzoides L.	Leaf	Asteraceae	72.66±3.51
Aegle marmelos (L.) correa	Leaf	Rutaceae	83±3
Anethum graveolens L.	Leaf	Rutaceae	60.66±5.03
Anisomeles ovata R. Br.	Leaf	Asteraceae	65±5.56
Caesulia axillaris Roxb.	Leaf	Asteraceae	78.66±5.13
Caesulia axillaris Roxb.	Flower	Asteraceae	83±3
Chrysanthemum indicum DC	Leaf	Asteraceae	31±1.73
Chrysanthemum indicum DC	Flower	Asteraceae	22±3
Citrus aurentifolia (Cristmus) wingle	Leaf	Rutaceae	55±5
Citrus medica Linn.	Peel	Rutaceae	74.33±5.13
Citrus medica Linn.	Leaf	Rutaceae	82±1.73
Callistemon lanceolatus DC	Leaf	Myrtaceae	65.33±5.03
Cinnamomum zeylanicum Nees.	Leaf	Lauraceae	80.48±2.44
Cumin cyminum Linn.	Seed	Apiaceae	79.66±3.51
Curcuma longa Linn.	Leaf	*Zingiber*aceae	54.66±8.32
Curcuma longa Linn.	Rhizome	*Zingiber*aceae	100
Cymbopogon citratus Hook.	Leaf	Poaceae	100
Cymbopogon citratus Hook.	Root	Poaceae	15.86±2.74
Foeniculum vulgare Mill	Leaf	Apiaceae	72.66±9.71
Foeniculum vulgare Mill	Seed	Apiaceae	76.33±6.50
Hyptis suaveolens poir	Leaf	Lamiaceae	62.66±6.11
Lawsonia inermis Linn.	Leaf	Lythraceae	63.33±3.51
Lantana indica Roxb.	Leaf	Verbenaceae	30±5
Leucos aspara spreng.	Leaf	Lamiaceae	53.33±7.66
Lippia alba Linn.	Leaf	Verbenaceae	73±3
Mentha arvensis Linn.	Leaf	Lamiaceae	75±3
Murraya koenighii (L) Spreng.	Leaf	Rutaceae	76.3±6.50
Murraya koenighii(L) Spreng.	Seed	Rutaceae	83±1.73
Nepeta hindostana Roth Hains.	Leaf	Lamiaceae	76.66±2
Ocimum basilicum Linn.	Leaf	Lamiaceae	100
Ocimum canum Sims.	Leaf	Lamiaceae	100
Ocimum gratissimum Linn.	Leaf	Lamiaceae	100
Ocimum sanctum Linn.	Leaf	Lamiaceae	100
Rosa indica L. (Rose)	Flower	Rosaceae	20.37±2.30
Salvia leucantha Nees.	Leaf	Lamiaceae	50.33±6.65
Seseli indicum Wt. & Arn	Leaf	Asteraceae	81±4
Tagetes erecta Linn.	Leaf	Asteraceae	66±4
Tagetes erecta Linn.	Flower	Asteraceae	82±4.58
Trachyspermum ammi. L.	Leaf	Apiaceae	38.33±7.63
Trachyspermum ammi. L.	Seed	Apiaceae	74.66±4.04
Veteveria zinzanoides (L.) Nash	Root	Poaceae	80.33±2.30
Vitex negundo Linn.	Leaf	Verbenaceae	73±3
Vitex negundo Linn.	Stem	Verbenaceae	56±5.56
Zingiber officinale Rosc.	Leaf	*Zingiber*aceae	84±3
Zingiber officinale Rosc.	Rhizome	*Zingiber*aceae	100

Table -2
Screening of Essential Oils of Potent Angiospermic Plants at 3000 µg ml^{-1}against *T. rubrum*

Name of Plants	Plants Parts	Family	Percent recovery of oils ±SD	Percent mycelial inhibition of test fungus±SD
Curcuma longa (L.) Koenig	Rhizome	Zingiberaceae	0.29±0.01	100
Cymbopogon citrates (DC) Stapf	Leaf	Poaceae	0.48±0.02	76.33±3.51
Ocimum basilicum Linn.	Leaf	Lamiaceae	0.52±0.02	82±1.73
O.canum Sims	Leaf	Lamiaceae	0.18±0.01	93.58±5.55
O. gratissimum Linn.	Leaf	Lamiaceae	0.52±0.03	97.07±5.06
O. sanctum Linn.	Leaf	Lamiaceae	0.49±0.02	78.33±4.04
Zingiber officinale Rosc	Rhizome	Zingiberaceae	0.25±0.01	100

Table – 3
Minimum Inhibitory Concentrations of oils of *Curcuma longa* and *Zingiber officinale*

Concentration µg ml-1	Percent mycelial inhibition of *Trichophyton rubrum*	
	C.longa	***Z. officinale***
2000	100	100
1000	100	100
500	100	100
250	100	73.33 ± 2.88
200	89.33± 1.15	69.66 ± 3.51

Table – 4
Nature of toxicity of *C. longa* and *Z. officinale*

Concentration µg ml-1	Percent mycelial colony inhibition (mean ± SD)			
	C. longa		***Z. officinale***	
	Treated	**Reinoculated**	**Treated**	**Reinoculated**
5000	100	100	100	100
4000	100	100	100	100
3000	100	100	100	100
2000	100	100	100	100
1000	100	100	100	100
500	100	100	100	100
250	100	37.33 ± 2.51	-	-

Table -5
Time required by oils to kill the test fungus at different cidal concentrations (µg ml-1)

Time(h)	***Curcuma* oil**			***Zingiber* oil**		
	500	1000	1500	500	1000	1500
24	+	+	+	+	+	+
48	+	+	+	+	+	+
72	+	+	-	+	+	-
96	+	-	-	+	-	-
120	-	-	-	-	-	-

Where,

+ = fungal mycelial growth occurred
- = fungal mycelial growth not occurred

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