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Chemical Characterization and Antifungal Activities of *Curcuma longa* and *Zingiber officinale* Essential Oils against Some Human Pathogenic Fungi

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Abstract

The essential oils from fresh rhizome of *Curcuma longa* and *Zingiber officinale* plants were isolated and standardized by their physico-chemical properties. The Gas-chromatography Mass spectrometry (GCMS) of the *Curcuma* and *Zingiber* oils indicated the mixture of 23 and 27 components respectively. The oils exhibited a broad fungitoxic spectrum against some ringworm disease causing fungi *viz*., *Microsporum gypsum, M. audouni, M. nannum, Trichophyton rubrum and T. violaceum.* The antimycotic activity of the oils was compared with 10 prevalent synthetic antifungal drugs. The volatile activity of the oils was studied by estimating the amount of oil vaporizing on fixed temperature in known time. The study reveals that Curcuma oil exhibited better evaporation in comparison to that of Zingiber oil. Skin sensitivity tests of essential oils have been evaluated and oils did not show any adverse symptoms on skin surface of human beings upto 5% concentration. On account of their broad antimycotic spectrum, superiority over some synthetic antimycotic drugs, better evaporating in nature and non toxic to skin, these plant products may be exploited as herbal antifungal agents in cure of ringworm disease.

Keywords: Essential oils, Human Pathogenic Fungi, Fungitoxicity, *Curcuma longa* and *Zingiber officinale.*

Introduction

Fungi are responsible for serious human pathogenic infections that have increased during the last decades (Pfaller *et al*, 2006; Arif *et al*, 2011). At present, amphotericin-B, caspofungin, flucytosine, itraconazole, variconazole and nystatin are commonly used as chemotherapeutic agents against mycosis (Dubey & Maheshwari, 1999). 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; Wajtulewski *et al.,* 1980) and invite scientists to search safer alternative of such drugs.

Today herbs are finding diverse uses in the society, from medicines to insecticides and many articles of daily uses (Sinha, 1996). It is perhaps for this reason that today over 25 percent of prescriptions issued in the developed countries are reported to contain one or more than one ingredients of plant origin. A number of international organizations such as UNIDO, UNESCO, Common Wealth Science Council and WHO have volunteered to support research programmes in this area. Undoubtedly nature has all along been with the diseases and it has created their cure. For every disease that arises on this planet, plant has a cure. Nature keeps in her green bag the secret of healthy life on this earth, perhaps in the luxuriant green cover, the biodiversity. Thus numerous plants and their products have been used economically in cure of different human diseases since long in traditional system of medicines. They have a very high level of safety mainly because they act through different modes of action than most of the organic chemicals. This safety advantage is very important to a large extent on the cost of development and registration of a new drug.

In the last years, research in aromatic and medicinal plants, and particularly their essential oils (EO), has attracted many investigators. EO have traditionally been used during centuries for their antifungal properties (Ríos & Recio, 2005) More recently, several studies have shown evidence of the huge potential of these natural products as antifungal agents (Inouye *et al*, 2006; Tullio *et al* ,2007; Zuzarte *et al*, 2009; Lima *et al*, 2011) justifying their current use in a number of pharmaceutical, food, and

cosmetic products. Therefore, it is not surprising that EO are one of the most promising groups of natural products, for the development of broad-spectrum, safer and cheaper antifungal agents.

Objective

In the present piece of investigation, it has been thought desirable to find out Physico-Chemical properties, range of fungitoxicity, comparative efficacy with some available standard synthetic antifungal drugs, evaporating nature and skin sensitivity test of *Curcuma longa* and *Zingiber officinale* oils, isolated from their rhizomes and may be exploited as herbal antifungal agents in cure of ringworm disease.

Material and Methods

The cultures of human pathogenic fungi were procured from Institute of Microbial Technology (IMTECH), Chandigarh and Department of Microbiology, MLN Medical College, Allahabad, India. Confirmation of their identity was also done with the help of relevant literature and culture on suitable fungal medium and stored in BOD incubator at 25±1oC for further experiments. Essential oils of *Curcuma longa* (rhizome) and *Zingiber officinale* (rhizome) were isolated by hydrodistillation through Clevenger's apparatus following Mishra and Dubey (1994)

Characterization of *Curcuma Longa* and *Zingiber Officinale* Oils

GCMS

The essential oils were analyzed by GCMS (Gas chromatography mass spectra) for their chemical composition. GCMS data of oils obtained with Shimadzu HR-1(Unless otherwise specified) equivalent to OV-1 fused silica capallary-0.25 mm x 50 m with film thickness-0.25 micron. The other conditions are given on the GCMS trace. An entry such as 100-6-10-250 means that the initial temperature was 100^{o}C for 6 minutes and then heated at the rate of 10^{o}C per minute to 250^{o}C. Carrier gas (Helium) flow: 2 ml per minute. The GCMS of oils was done at the Central Institute Medicinal Aromatic Plant (CIMAP), Lucknow, India.

Specific Gravity

Specific gravity of the oils was studied following Guenther (1972). A pycnometer was washed with chromic acid and was rinsed thoroughly with distilled water and finally with ether. It was dried in hot air oven at 30±1^{o}C upto 6 hours to remove the vapours of ether and then filled with distilled water and weighed. The weight of water was calculated. Similarly cleaned pycnometer was then filled with the oils separately and weighed. The weight of each oil was calculated. The specific gravity of oil was calculated by the following formula:

$$\text{Specific Gravity} = \frac{\text{Weight of Oil}}{\text{Weight of equal volume of water}}$$

The standard temperature at which, specific gravity of the essential oils is represented is 25^{o}C. A correction factor of 0.0008 per ^{o}C is used to convert the specific gravity determined at room temperature to 25^{o}C. If the room temperature is higher than 25^{o}C the correction factor is added in original value and vice-versa.

Optical Rotation

Lippeck type of polarimeter was used to determine the optical rotation 20 ml of acetone was pipetted to a flask containing one ml of the essential oil and the percentage of oil in the solution was determined (Guenther, 1972). It was filled in a polarimeter tube of 20 cm length. The tube was placed in the through of the instrument between the polorizer and the analyzer. The instrument was left for half an hour in sodium light in order to obtain equilibrium. The analyzer was slowly turned until both halves of the field showed, equal intensities of illumination when viewed through the telescope. The direction of the rotation was determined by readjusting analyzer until equal illumination of the two halves of the field was obtained. The eyepiece of the telescope was adjusted so as to give a clear sharp line between the two halves of the field and the field and the degree was read directly.

Refractive Index

Abb type of refracto meter with range 1.3 to 1.7 recommended for the routine analysis of the essential oil was used to determine the refractive index. The prism of the instrument was carefully cleaned with alcohol and then with ether. One drop of the each oil was placed separately in between the double prism of refracto meter and lightening the screw heads closed the prism. The instrument was allowed to stand for a few minutes so that the oil and the instrument attained the same temperature. The alilad was moved backward and forward to set a border line, which is band of colour. A sharp colour line was obtained by rotating the screw head of the compensator. The border line was adjusted so that it fell on the point of intersection of the cross hair. Refractive index was read directly on the scale of the sector (Guenther, 1972).

Solubility in Different Organic Solvents

One ml of oils was introduced into a 10 ml glass stoppered cylinder (Calibrated to 0.1 ml) different solvents were added to it drop by separately by shaking the cylinder after each addition. The volume of solvent used to obtain a clear solution was noted.

Acid Number

Acid number is defined as the number of milligrams of Potassium hydroxide required to neutralize the free acid in one gram of the oil. 2.5 gm the each oil were weighted separately Erlenmeyer flask. 15 ml of neutral 95% alcohol was added to it. Three drops of 1% phenolphthaline solution was then added as indicator free acids were triated with standard 0.1 N aqueous potassium hydroxide solution. The first appearance of a pink colouration that does not fade within 10 second is considered the end point (Guenther,1972).

$$\text{Acid Value} = \frac{\text{a} \times 0.00561 \times 1000}{\text{Weight of oil (in gm)}}$$

a = No. of ml of KOH required to neutralize free acids

Saponification Value

Saponification value of the oils was studied following Guenther (1972). Saponification value is defined as the number of miligrams of potassium hydroxide required to saponify (hydrolyze) esters

present in one gram of the oil following equation denotes the saponification value.

RCOOR' + KOH = RCOOK + R' OH

(R and R' may be aliphatic, aromatic or dicyclic radical. R may also be hydrogen atom) weighed amount of oil (2 gm) was taken in a 100 ml alkali resitant saponification flask. Ten ml of the 0.5 N alcoholic KOH solution was added to it through a pipette. A glass cooled condenser (one meter in length and one cm in diameter) was attached to the flask. The contents of the flask were refluxed for two hours on a steam bath (100°C). The flask was then cooled at room temperature for 15 minutes. Excess of alkali was titrated with 0.5 N aquous hydrochloric acid using three drops of alcoholic phenolphthalin as a indicator. The amount of acid used was noted (a). A blank without oil was run paralled to treatment sets and the amount of acid used during filteration was noted (b).

Saponification value was calculated by the following formula:

$$\text{Saponification value} = \frac{(b-a) \times 0.2805 \times 1000}{\text{Weight of Oil (in gm)}}$$

Ester Value

Ester value of the oils was studied following Guenther (1972). The ester value of a substance is the number of miligrams of potassium hydroxide required to neutralize the acids resulting from complete hydrolysis of one gram of the oil. The ester value of the oils was calculated by subtracting the acid value from the saponification value obtained in previous experiments (Ester value = saponification value - acid value).

Fungitoxic Spectrum of Oils

The range of fungitoxicity of the Curcuma and Zingiber oils were determined at 250 and 500 µg ml^{-1} concentrations with respect to 11 available human pathogenic fungi with usual poisoned food technique of Grover and Moore (1962) using SDA medium. The observations were recorded for percentage mycelial inhibition of tested fungi based on mean± SD with three replicates and the data are presented in **Table – 2.**

Comparison of Effectiveness of the Oils with Some Prevalent Synthetic Drugs

The comparative efficacy of the *Curcuma longa* and *Zingiber officinale* oils with some available standard synthetic antifungal drugs *viz.*, clotrimazole, griseofulvin, variconazole, caspofungin, nystatin, benzoic acid, salycelic acid, Itraconazole, flucytosin, and Amphotericin-B was studied against the test fungus *Trichophyton rubrum* by usual poisoned food technique. The synthetic drugs and oils in requisite amounts were dissolved separately in 0.5 ml acetone and mixed with 9.5 ml sabouroud dextrose agar medium to obtain different concentration. For control, requisite amount of sterilized water dissolved in 0.5 ml acetone was used in place of drugs/oils. The plates were inoculated aseptically with the assay disc and incubated for six days at 25±1°C. The observations were recorded on the seventh day and percentage mycelial inhibition was calculated **(fig. -1).**

Measurement of Volatile Activity of Oils

The volatile activity of *C. longa* and *Z. officinale* oil was studied for estimating the amount of oils vapourizing on fixed temperature in known time. The known volumes of oil were placed in cotton pads separately at 25±1°C. Oil containing cotton pads were kept in open petriplates in incubator at 25±1°C for 30 days. On 31st day volume of oils remaining on cotton pads were extracted by usual hydrodistillation method through Clevenger's apparatus. The percent volume of oils evaporated in days was recorded and based on mean value of three replicates ±SD.

Skin Sensitivity Test of Curcuma and Zingiber Oils on Skin Surface of Human Beings

Skin sensitivity test were carried out with skin surface of the control and four treatments groups (*viz.,* 5%, 10%, 25% and 50%). Requisite amounts of essential oils (i.e. 5%, 10%, 25% and 50%) were dissolved separately in acetone. The test was carried out by employing the method of Prasad and Stadelbacher (1973) with some modification. Evaluation of skin sensitivity test was conducted by twenty volunteers of 35 ± 5 years age numerical itching rating of 4 (equal to high itching), 3 (equal to moderate itching), 2 (equal to poor itching) and 1 (equal to no itching) were assigned for the judgments and mean scores were calculated for each treatment as presented in **Table-3**.

Results and Discussions

The oil of Curcuma was isolated in the month of March. The oil was orange, yellow in colour with pungent odour and was immiscible in water. GCMS of Curcuma oil indicated the mixture of 23 components namely *viz.* alpha. – pinene, beta. myrcene, l-phellandrene, alpha.-phellandren, benzene, methyl (1-methyethyl)-(cac), dl – limonene,, beta. – phellandrene, eucalyptol, gamma. – terpinene, benzene, (1,1- dimethylethyl) – (cac), phenol, 5-methyl-2 (1-methyethyl)-(cac), aceta-z-crysanthenyl, benzene, 1(1,5-dimethyl-4-hexenyl)-4-methyl-(cac), beta.–bisabolene, beta.-sesquiphellandrene, 2, 4-diphenyl-4-methyl-1 (e)-pentene, 1-deoxycapsidiol, beta.- tumerone, alpha. – tumerene, 6-(1',5'-dimethylhex-4'-enyl)-3-methylcycloh, bicyclo[4.3.0]-2, 9-nonadiene, acrylic acid, and 1,3,3-trimethyl-2-hydroxymethyl-3,3-dimethyl-4-(3-met).

The oil of Zingiber was isolated in the month of October. The oil was yellow in colour with pungent odour and was found to be immiscible in water. The GCMS of the Zingiber oil indicate the mixture of 27 components namely *viz.,* 2 – heptanol - (cac), alpha .-pinene, camphene, 6- methy – 5 - hepten – 2 - one, sabinene, beta. - myrcene, a - phellandrene, l - limonene, beta. - phellandrene, eucalyptol, alpha. – terpinolene, z-. beta.- ocimene, bornyl chloride, 1,3,6 – octatriene, 3,7 – dimethyl – (e), 5 – hepten – 1-ol, 2 – ethenyl–6–methyl, 2-cyclohexen-1ol, 2-methyl-5, (1-methylethenyl), benzene, 1 – (1,5 – dimethyl -4 – hexenyl) – 4 – nmethyl, alpha. – farnesene, beta. – bisabolene, germacrened, beta. – sesquiphellandrene – (cas), (z,z,) – alpha. – farnesene, *zingiber*ene, (z) – trans - alpha. bergamotene, ledene, (z) – cis – alpha. – berga motene & caryophyllene oxide.

The specific gravity, optical rotation, refractive index, acid number, saponification and ester value contained by oils are presented in **Table-1**. Moreover the oils were completely miscible with acetone, absolute alcohol, 90 percent alcohol,

benzene chloroform and petroleum ether in 1:1 concentration (**Table–1).** It has been well demonstrated that the concentration of different ingredients in the essential oils varies with growth stages, ecological conditions and the techniques used for isolation oil form the plant so that essential oils were standardized in this study.

The data presented in **Table -2** shows that Curcuma and Zingiber oils were found effective against all the fungi tested. However, Curcuma oil completely inhibited 5 fungi at 250 µg ml^{-1} and Zingiber oil completely inhibited 5 fungi at 500 µg ml^{-1}. It is also noteworthy that both the oils are absolutely effective against different species of Trichophyton and Microsporum, those are responsible for ring worm diseases .Thus *Curcuma* and *Zingiber* oils exhibited broad fungitoxic spectrum against ring worm disease causing fungi. Therefore *Trichophyton rubrum* was selected as test fungus for further studies. The data presented in **Fig.-1** show that the Curcuma oil and clotrimazole was 6 times superior to Nystatin & benzoic acid, 4 times to variconazole, 2 times to griseafulvin, caspofungin, salicylic acid, Itraconazole, flucytosin, amphotericin-B and Zingiber oil.

It was found that Percent evaporation (In Volume) of Curcuma oil (93.5±1) was more in comparison of Zingiber oil (83±1.32). The study indicated that oils exhibited better evaporating nature so there will be lesser chance for residual toxicity. It is evident from **Table - 3** that the effect of oils on skin surface of human, treatment sets 5% were found equal to those of control sets .and oils did not show any adverse symptoms on skin surface of human beings and the skin sensitivity is equal to control.

EO is natural complex mixtures of terpenic and non-terpenic compounds. In general monoterpenes and sesquiterpenes as well as their oxygenated derivatives are the predominant constituents but phenylpropanoids, fatty acids and their esters may also occur (Bakkali *et al* 2008). These secondary metabolites can be found in various plant organs being produced and stored in different secretory structures, which is closely related to the plant family (Upton *et al* 2011). Anatomical details of these structures are also very relevant to the market value of aromatic plants since they allow the verification of authenticity, detection of substitutions and/or adulterations (Svoboda & Svoboda 2000). In nature EO play important roles as signaling agents namely in the protection of plants against microorganisms, insects, and herbivores, as attractants of pollinators, and in allelopathic interactions (Bakkali *et al* 2008; Theis & Lerdau 2003). Aromatic plants and their EO have traditionally been used since antiquity for their biological properties as well as for cosmetic and medicinal applications (Bakkali *et al* 2008), Edris 2007) In recent years, research on aromatic plants has attracted many researchers and in vitro screening programs, based on ethnobotanical approaches, proved to be very efficient in validating traditional uses and providing new ways in the search for active compounds (Alviano & Alviano 2009).

Conclusions

The present investigation was undertaken with sole objective to explore a alternative therapy from the less explored world of natural antifungals against ringworm fungi, which can help the developing economy of a developing nation like India, by providing a cost effective fungal therapy but also providing with something more indigenous and safe product for early recovery from the fungal infections in India. The findings of the present study have been discussed in light of the previous fungitoxic investigations on higher plants and the requisite fundamental principles for development of an ideal therapeutant for the control of dermatomycoses.

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Table – 1
Physico - Chemical Properties of Essential Oils of *C. longa* and *Z. officinale*

Parameters	*Curcuma longa* oil	*Zingiber officinale* oil
Colour	Orange-Yellow	Yellow
Odour	Pungent	Pungent
Specific gravity	0.977	0.977
Optical rotation	-18°'	-32°'
Refractive index	1.514	1.489
Solubility		
Acetone	soluble (1:1 v/v)	soluble (1:1 v/v)
Absolute alcohol	soluble (1:1 v/v)	soluble (1:1 v/v)
90%	soluble (1:1 v/v)	soluble (1:1 v/v)
Benzene	soluble (1:1 v/v)	soluble (1:1 v/v)
Chloroform	soluble (1:1 v/v)	soluble (1:1 v/v)
Petroleum ether	soluble (1:1 v/v)	soluble (1:1 v/v)
Acid number	2.5	2
Saponification value	12.5	11
Ester value	10	9

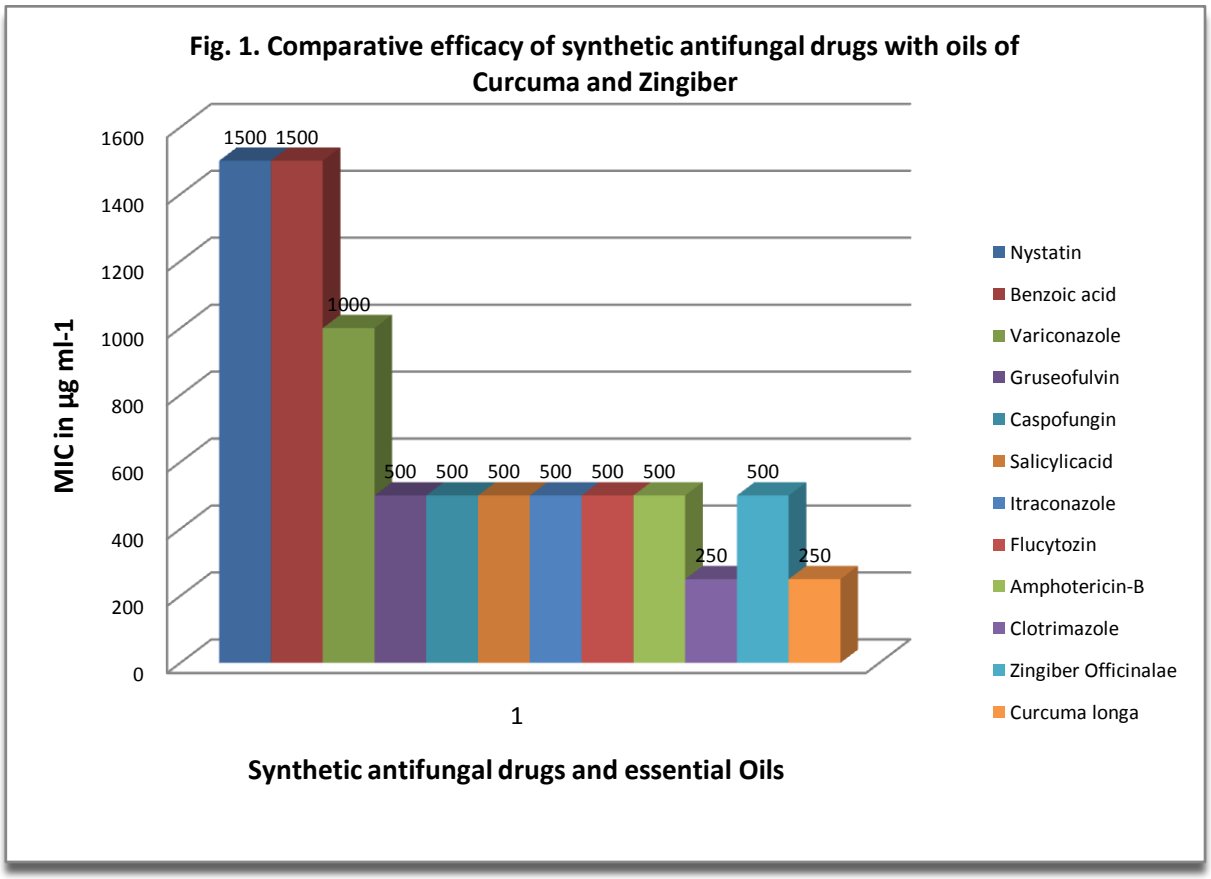
Table – 2
Fungitoxic Sepctrum of *Curcuma longa* and *Zingiber officinale* Oils

Fungi tested	Percent mycelial inhibition			
	C. longa oil		*Z. officinale* oil	
	250 µg ml^{-1}	500 µg ml^{-1}	250 µg ml^{-1}	500 µg ml^{-1}
Aspergillus fumigatus	18.33±7.63	21.66±5.13	26.66±2.88	33±7
Candida albicans	29.66±2.88	33±1.73	20.33±1.15	25±1.73
Cryptococcus neoforma	38.33±7.63	72.33±2.51	26.33±3.51	32±1.73
Microsporum gypseum	100	100	93.58±3.58	100
M. audouni	100	100	82±1.73	100
M. nannum	100	100	83±1.73	100
Trichophyton rubrum	100	100	73.33±2.88	100
Trichophyton violaceum	100	100	76.33±6.51	100
Trichothecium roseum	50.33±6.65	79.66±3.51	44.43±5.49	52.37±4.76
Aspergillus parasiticus	48.33±7.63	72.33±3.51	61±1.73	67.33±2.30
Trichoderma viride	44.43±5.49	52.37±4.76	38.33±7.63	48.33±7.63

Table - 3
Skin Sensitivity Test of Curcuma and Zingiber Oils on Skin Surface of Human Beings

Treatment sets								Control sets
C. longa				*Z. officinale*				
5%	10%	25%	50%	5%	10%	25%	50%	
1	1	1.95	2.2	1	1.5	2.25	2.8	1

Fig. 1. Comparative efficacy of synthetic antifungal drugs with oils of Curcuma and Zingiber



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