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'Turmeric' An Age-Old Panacea for many ills can be a Potential Source of Antidermatophytic Agent

Amritesh C. Shukla[1*], Awadhesh Kumar[2], Archana Verma[3], R. K. Mishra[4], A. Dikshit[5] , Om Prakash[6] and Neetu Shukla[7]

[1]*Associate Professor, Department of Horticulture, Aromatic & Medicinal Plants, Mizoram University, Aizawl-796 004, India*
[2]*Research Scholar, Department of Horticulture, Aromatic & Medicinal Plants, Mizoram University, Aizawl-796 004, India*
[3]*DST- Women Scientist, Department of Horticulture, Aromatic & Medicinal Plants, Mizoram University, Aizawl-796 004, India*
[4]*DST- Research Scientist, Biological Product Laboratory, Department of Botany, University of Allahabad-211 002, India*
[5]*Professor & Director, Biological Product Laboratory, Department of Botany, University of Allahabad-211 002, India*
[6]Project Manager, Indian Institute of Information Technology (IIIT), Allahabad- 211002, India
[7]*Resident Doctor, Ekta Institute of Child Health, Raipur-492 001, India*

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*Corresponding Author

Tel : +91-389-2330273
Fax : +91-389-2330214

Email:
amritesh.shukla@rediffmail.com

Abstract

Antimicrobial evaluation of the essential oil(s) of some spp. of *Curcuma* viz., *Curcuma angustifolia, C. aromatica* and *C. zedoaria* – "an age old panacea for many ills", were screened against three common dermatophytic fungi causing ringworm infection in human beings. The essential oil of *Curcuma aromatica* Salisb.(Family- *Zingiberaceae*) was found strongest toxicant against the test fungi. The minimum inhibitory concentration (MIC) of the oil was 1.8µl/ml against *Epidermophyton floccosum* and *Trichophyton rubrum,* and 1.6µl/ml against *Microsporum gypseum*; however, it was fungicidal at 2.0 µl/ml against *E. floccosum* and *T. rubrum*, and 1.8 µl/ml against *M. gypseum,* respectively. The efficacy contains heavy doses of inoculums (25 discs of 5 mm each.). The (MKT) of the oil was 30 sec against *E. floccosum* & *Microsporum gypseum* and 20 sec against *T. rubrum*, while, its MFCs required 6.30 hrs against *E. floccosum* & *Microsporum gypseum* and 5.30 hrs against *T. rubrum.* The oils efficacy was thermo stable up to 100 ^{0}C and for 36 months of storage, the maximum unit taken into consideration. Moreover, the oil of *C. aromatica* did not exhibit any adverse effect on mammalian skin up to 5% conc. Relationship of the dermatophytes to the toxicity of the oil vis-a vis phylogeny using molecular data of the pathogens have also been discussed. Further, the clinical trial of the oil in the form of ointment (at 1% v/v conc) to topical testing on patients, attending out patient department (OPD) of MLN Medical College, Allahabad is still in progress.

Key Words: Antimicrobial activity, Dermatophtes, Medicinal plants, Minimum inhibitory concentration, Herbal drug

Introduction

The World Health Organization (WHO) estimated that 80% of the population of developing countries rely on traditional medicine mostly plant drugs, for their primary health care needs [1]. Medicinal plants being natural, non-narcotic, having no side effects, safe, cost effective, preventive and curative therapies which could be useful in achieving the goal of "Health for all" in a coat effective manner. Demand for medicinal plants is increasing in both developing and developed countries but 90% malarial la harvested from wild sources without applying scientific management hence many species are under threat to become extinct [1, 2].

In fact the traditional herbal remedies led the Scientists to the development of numerous modern drugs [3-5]. At this point the discovery of reserpine from *Rauvolfia serpentine* can be cited as an example of how a plant utilized by the indigenous people eventually becomes the source of one of the most important pharmaceuticals of the world [1].

Keeping these views in mind, in the present investigations, a scientific attempt has been made to explore the possibilities of *Curcuma* spp, as a protecting measurement against the ringworm infection on human beings.

Materials & Methods

In vitro investigation

(a). Extraction and Isolation of Essential oil:

The essential oil(s) were extracted separately from the fresh leaves of *Curcuma angustifolia, C. aromatica* and *C. zedoaria* (Family- Zingiberaceae) by hydro distillation using Clevenger's apparatus [6]. A clear light yellow colored oily layer was obtained on the top of the aqueous distillate, later which was separated and dried over anhydrous sodium sulphate. The oils thus obtained were subjected to various antimicrobial investigations.

(b). In- vitro antimicrobial investigations of the essential oil(s):

The minimum effective concentration (MEC) of the oil against some common human pathogenic fungi *Epidermophyton floccosum* Hartz, *Microsporum gypseum*

(Bodin) Guiart et Grigorakis and *Trichophyton rubrum* Castellani, was determined by using the technique of Shahi et. al. [10], with a slight modification. Two sets were maintained; one for the treatment set and another for the control. The treatment set at different concentration of the oil was prepared by mixing the required quantity of the oil samples in acetone (2% of the total quantity of the medium) and then added in pre-sterilized sabourad dextrose agar medium (SDA). In control set, sterilized water (in place of the oil) and acetone were used in the medium in appropriate amount. The fungi-static/ fungicidal (MSC/ MCC) action of the oil was tested by aseptically re-inoculating the fungi in culture tubes containing sabourad dextrose broth (Table 1-3). The data recorded was the mean of triplicates, repeated twice. The percentage of fungal growth inhibition (FGI) was calculated as per formula:

$$FGI\ (\%) = \frac{Dc - Dt}{Dc} \times 100$$

Where, Dc indicates colony diameter in control set, &
Dt indicates colony diameter in treatment sets.

Table-1: Minimum effective concentration of four different spp of Curcuma against some common human pathogenic fungi

Curcuma spp	Human Pathogenic Fungi		
	Epidermophyton floccosum	*Microsporum gypsum*	*Trichophyton rubrum*
Curcuma angustifolia	2.6 µl/ml	2.2 µl/ml	2.4 µl/ml
C. aromatica	1.8 µl/ml	1.6 µl/ml	1.8 µl/ml
C. zedoaria	2.2 µl/ml	1.8 µl/ml	2.0 µl/ml

Table- 2: Minimum effective concentration of the oil of *Curcuma aromatica* against test fungi

Concentration (µl/ml)	Human Pathogenic Fungi		
	Epidermophyton floccosum	*Microsporum gypsum*	*Trichophyton rubrum*
2.0	100^{C}	100^{C}	100^{C}
1.8	100^{s}	100^{C}	100^{s}
1.6	92	100^{s}	96
1.4	88	60	80
1.2	60	--	76
1.0	--	--	60

^{c} indicates cidal and ^{s} indicates static

Table- 3: Detailed in-vitro investigations of *Curcuma aromatica* against the test fungi

Properties studied	Observations		
	Epidermophyton floccosum	*Microsporum gypsum*	*Trichophyton rubrum*
Minimum Inhibitory Concentration			
MEC *	1.8 µl/ml	1.6 µl/ml	1.8 µl/ml
MFC	2.0 µl/ml	1.8 µl/ml	2.0 µl/ml
Minimum Killing Time			
Pure oil	30 sec	30 sec	20 sec
MFC	6.30 hrs	6.30 hrs	5.30 hrs
Inoculum Density (25 disc, 5mm diam)	No Growth	No Growth	No Growth
Thermostability (up to 100 ^{0}C)	No Growth	No Growth	No Growth
Effect of Storage (36 months)	No Growth	No Growth	No Growth

*MEC *indicates Minimum Effective Conc.;* MFC *indicates Minimum Fungicidal Concentration*

(c). Effect of Inoculums Density:

The effect of inoculums density on the minimum cidal concentration (MCCs) of the oil against the test fungi was determined using the method of [20]. Mycelial discs of 5mm diam of 7-day oil cultures were inoculated in culture tubes containing oil at their respective MCCs. 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 25 discs, in multiply of five. 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 (Table- 3).

(d). Effect of some Physical Factors:

Effect of some physical factors viz., temperature (40, 60 and 80^{0} C respectively) and autoclaving (up to 15 lb/ sq inch pressure for 30 min) on efficacy of the oil, at minimum cidal concentration, was also determined. It was determined following the method of [7, 8]. Samples of oil in small vials, each contains 1ml, were exposed at 40, 60 and 80^{0} C in hot water bath, respectively. Further, the oil's efficacy was tested against the test fungi at their respective MCCs (Table- 3).

(e). Minimum Killing Time:

The MKT of the pure oil and their respective MCCs of *C. aromatica* against the test fungi was determined by using the method of [9], (Table-4).

Table- 4: Minimum killing time of the oil of *Curcuma aromatica* against test fungi

	Mycelial Growth Inhibition (%)					
Minimum	*Epidermophyton floccosum*		*Microsporum gypseum*		*Trichophyton rubrum*	
Killing Time (MKT)	P.O.	MFC	P.O.	MFC	P.O.	M.F.C.
7.0 hrs	100	100	100	100	100	100
6.30	100	100	100	100	100	100
6.0	100	60	100	80	100	100
5.30	100	---	100	---	100	100
5.0	100		100		100	80
2.30	100		100		100	---
2.0	100		100		100	
1.30	100		100		100	
1.0	100		100		100	
30 min	100		100		100	
15.0	100		100		100	
5 .0	100		100		100	
60 sec	100		100		100	
30	100		100		100	
20	90		80		100	
10	60	---	70	---	88	---

*P.O. *indicates Pure Oil;* MFC *indicates Minimum Fungicidal Concentration*

Table-5: Fungi toxic spectrum of the oil of *Curcuma aromatica* against some common pathogenic fungi

Fungi Tested	Lethal Conc (2.0 µl/ml)	Hyper Lethal Conc (4.0 µl/ml
Human Pathogens		
Microsporum auddouinii	100[s]	100[c]
M. canis	100[s]	100[c]
M. nanum	100[c]	100[c]
Trichophyton mentagrophytes	100[c]	100[c]
T. tonsurans	100[c]	100[c]
T. violaceum	100[c]	100[c]
Plant Pathogens		
Aspergillus parasiticus	100[s]	100[c]
Cladosporium cladosporioides	100[c]	100[c]
Curvularia lunata	100[c]	100[c]
Colletotrichum capsici	100[c]	100[c]
C. falcatum	100[c]	100[c]
Fusarium oxysporum	100[c]	100[c]
F. udum	100[c]	100[c]
Helminthosporium maydis	100[c]	100[c]
H. oryzae	100[c]	100[c]
Penicillium implicatum	100[c]	100[c]
P. minio-luteum	100[c]	100[c]

[s] indicates static; [c] indicates cidal in nature

Table- 6: Comparative MECs of the oil of *Curcuma aromatica* with some Synthetic Antifungals

Oil & Trade Name of Antifungal Drugs	Active Ingredients	Minimum Effective Concentration (µl/ml)		
		Epidermophyton floccosum	*Microsporum gypseum*	*Trichophyton rubrum*
Curcuma aromatica	Essential oil	1.8	1.6	1.8
Dactrine	Miconazole nitrate	6.0	6.0	6.0
Nizaral	Ketoconazole	6.0	0.5	5.0
Tenaderm	Tolnaftate	2.0	1.5	0.8

Table- 7: Comparative Efficacy of the oil of *Curcuma aromatica* with some Synthetic Antifungal Drugs

Antimycotic Drugs	Drugs %	Cost (Rs.)		Adverse Effects	Expiry Duration (months)	Environmental impact
		ointment/g	lotion/ ml			
C. aromatica	1%v/v	0.90	0.70	No adverse effects	24-36	Renewable, biodegradable, non-residual toxicity.
Dactrine	2%w/w	2.80	---	Occasionally produced gastrointestinal side effects viz., nausea, vomiting, diarrhea	35	Non-renewable, non-biodegradable and residual toxicity
Nizaral	2%w/w	3.75	3.17	Adverse reaction observed were mainly burning, irritation. Drug may block testosterone synthesis	24	-----do-----
Tenaderm	1%w/v	1.06	1.30	Adverse effects were fever, nausea, vomiting, diarrhoea & skin rash, rarely produced irritation	24	-----do-----
Batrafine	1%w/v	1.50	1.60	----do----	24	----do----

(f). Fungi-toxic Spectrum:

The fungi-toxic spectrum of the oil at lethal and hyper lethal concentration (i.e. 2.0 µl/ml and 4.0 µl/ml respectively) was determined against some common human pathogenic fungi viz., *Microsporum auddouinii* Gruby*, M. canis* Bodin*, M. nanum* Fuentes*, Trichophyton mentagrophytes* (Robin) Blanchard*, T. tonsurans* Malmstem, and *T. violaceum* Bodin. This was done by using the method of [9], (Table-5).

Besides, the oil's efficacy was also tested against some plant pathogenic fungi viz., *Aspergillus parasiticus* Speare, *Cladosporium cladosporioides* (Fresenius) de Vries, *Curvularia lunata* (Wakker) Boedijin, *Colletotrichum capsici* (Syd.) Butler & Bisby, *C. falcatum* Went, *Fusarium oxysporum* Schlecht, *F. udum* de vries, *Helminthosporium maydis* Nisikado & Miyakel, *H. oryzae* Breda de Haan, *Penicillium implicatum* Biourge and *P. minio-luteum* Dierckx; by using the technique of [7] (Table-5).

(g). Comparison with some Synthetic Fungicides:

The comparative efficacy of oil of *C. aromatica* with some synthetic antifungal drugs was carried out by comparing MECs. This was done by using the method of [10], (Table-6 & 7).

All the experiments were repeated twice and each contained three replicates; the data presented in the tables are the mean values.

(h). Statistical analysis:

Analysis of variance (ANOVA) was used to determine the significance ($P \leq 0.05$) of the data obtained in all experiments. All results were determined to be within the 95% confidence level for reproducibility. The ANOVA was computed using the SPSS version 16.0 software package.

(i). Phylogenetic study of dermatophytes

To find out the reason why the *Curcuma aromatica* is more effective against certain pathogenic fungi, phylogenetic relationship of the dermatophytes were studied including the genera *Trichophyton*, *Microsporum*, and *Epidermophyton* and identified the species using the base pair sequences of ITS1 [11], Fig. 1. The ITS1 sequences of the standard strains used in this study and members of the *Trichophyton* spp complex (*T. rubrum*, accession no. AB011453; *Microsporum gypsem*, accession no. AB017177 and *Epidermatophyton floccosum*, accession no. AB017181), were aligned using the Clustal W computer program [12,13] and GENETYX-MAC 10.1 software (Software Development Co., Ltd., Tokyo, Japan). Phylogenetic trees were then constructed by the DNA maximum-likelihood (ML) method in the PHYLIP program (Phylogeny Inference Package), version 3.5c [14], and the neighbor-joining (NJ) method in the NJPLOT program [15]. Bootstrap [14] analysis with the Clustal W program was performed by taking 1,000 random samples from the multiple alignments.

Results

On comparing the minimum effective concentration (MEC) of oils of *Curcuma angustifolia, C. aromatica* and *C. zedoaria* against the test fungi, the MEC of the oil of *C. aromatica* was found most effective (Table- 1).

The MEC of *Curcuma aromatica* oil was 1.6 µl/ml against *M. gypseum,* 1.8 µl/ml against *T. rubrum* and *E. floccosum;* however, it was fungicidal at 1.8 µl/ml against *M. gypseum,* and 2.0 µl/ml against *E. floccosum* and *T. rubrum*, and respectively (Table- 2).

The oil's efficacy contains heavy doses of inoculums (i.e. up to 25 discs, each of 5mm), thermo stable up to 80^0 C and

also persisted after autoclaving at 15 lb/ sq inch pressure for 30 min. (Table- 3).

The pure oil kills the test fungi within 30 second; however, its MCC ranges 5.30 to 6.30 hrs to kill all the fungi (Table- 4).

Fungi toxic spectrum of the oil at lethal and hyper lethal concentration (i.e. 2.0 µl/ml and 4.0 µl/ml), against some common pathogenic fungi reveals that the oil contains a broad fungicidal spectrum (Table- 5).

Furthermore, on comparing MECs of the oil with some synthetic antifungal drugs, MECs of the oil was more active than Dactrine, Nizaral and Tenaderm (Table- 6 & 7).

The phylogenetic relationship of dermatophytic genera *Trichophyton*, *Microsporum*, and *Epidermophyton* were basis of their ITS1 sequences. The NJ tree was constructed with data for standard strains of dermatophytes (11) demonstrated by using internal transcribed spacer 1 (ITS1) region ribosomal DNA sequences. *Trichophyton* spp. and *Microsporum* spp. form a cluster in the phylogenetic tree with *Epidermophyton*. All strains were successfully identified by comparison of their base sequences with those in the ITS1 DNA sequence database [11] NJ tree of dermatophytes is shown in Fig. 2.

The relationship of the toxicity of the essential oil vis-à-vis phylogeny was analysed using molecular data. The effectiveness of the oil was equal in dermatophytes that are close in phylogenetic tree (Fig. 2).

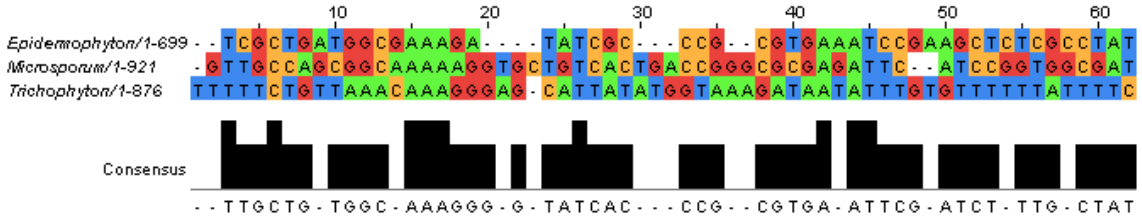

Fig. 1: Alignment of ITS1 sequences of standard strains of dermatophytes

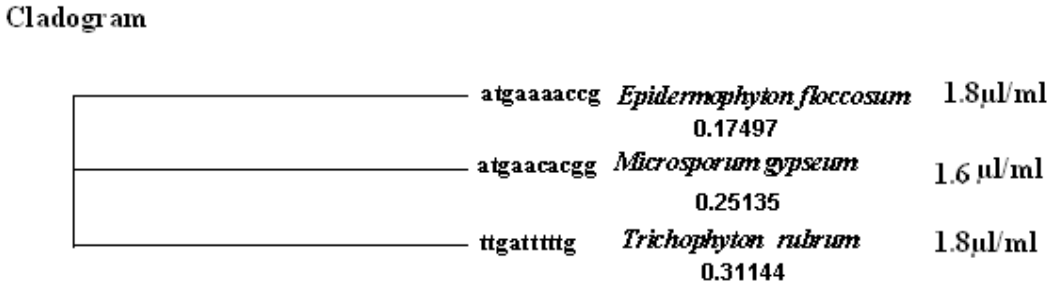

Fig. 2: Result of Cladogram (Neigbour Joining Tree plot) standard strains of dermatophytes on the basis of their ITS1 sequences.

Discussions

Essential oils obtained from the leaves of *Cymbopogon martini* var. motia [16], *Hyptis leucodendron* [17]; *Alpinia galangal* [18] was found to contain fungistatic activity. However, some essential oils, *Cymbopogon flexuosus* [19]; *Eucalyptus* oil [20]; *Citrus sinensis* [21] and *Homalomena aromatica* [22] prove to have fungistatic action at lower concentration and fungicidal action at higher concentration. Similarly, in the present investigation the oil of *Curcuma aromatica* showed fungistatic activity at the lower concentration 1.8 µl/ml against *E. floccosum* and *T. rubrum*, and 1.6 µl/ml against *M. gypseum;* and fungicidal at the higher concentration 2.0 µl/ml against *E. floccosum* & *T. rubrum*, and 1.8 µl/ml against *M. gypseum,* respectively. The fungicidal efficacy of the oil persisted heavy inoculums density with quick killing activity as well as having an edge over some synthetic antifungals viz., Dactrine, Nizaral, Tenaderm.

A fungicide must not be affected by extreme temperatures. Only a few workers have studied the effect of temperature on antifungal activity of the essential oils. [23] reported the oil of *Pepromia pellucida* was active up to 80 ^{0}C; Shahi et al., [24] reported *C. flexuosus* activity up to 100 ^{0}C, and Shukla et al., [22] reported the oil's efficacy of *H. aromatica* up to 80 ^{0}C. Similarly, in the present investigation the oil of *C. aromatica* was not only thermostable up to 80^{0}C but also autoclavable up to 15 lb/ sq inch pressure for 30 min.

A substance may behave as a strong fungicidal against certain fungi yet may be ineffective against the other pathogens. Therefore, a clear picture about the toxicity of a fungicide comes only after it is tested against the large number of fungi. The literature showed that essential oils have been found to exhibit narrow or wide range of activity [22, 25-27], but in the present study the oil of *C. aromatica* exhibited broad antifungal spectrum.

The effectiveness of the oil was equal to those dermatophytes which are close in phylogenetic tree. To understand the relationship of the DNA sequences of the tested fungal strains and their variable response to the different concentrations of active fractions (extracted in the form of essential oil from the leaf of *Curcuma aromatica*) have been critically analyzed. Further, evaluation of the phylogenetic analysis and identification system, both of which are based on

ITS1 rDNA sequences, are continuing in our laboratory with other species and strains.

A toxicant should be tested under both *in vitro* and *in vivo* conditions in order to prove its potential as promising antifungals for the control of disease. Since, detailed *in vitro* studies on the essential oil of *C. aromatica* indicate their potentiality to be as ideal antifungal agent against the dermatophytic fungi; hence, the same was further subjected for detailed *in vivo* investigations as well as clinical trials in the form of ointment (at 1% V/V conc.), which is still in progress.

Conclusions

The preliminary *in vitro* investigations revels that the oil of *Curcuma aromatica*, due to its strong fungicidal efficacy, inhibiting heavy doses of inocula, quick killing activity, broad fungicidal spectrum, long shelf life, and having an edge over some synthetic antifungal, can be used successfully in the form of broad spectrum herbal anti-dermatophytic agent(s). The commercial viability of the same can be determined after detailed *in vivo* as well as successful multi central clinical trials, which is in progress.

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