

Broad spectrum antimycotic plant as a potential source of therapeutic agent

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(Received 20 March 2010; Revised 16 April - 08 October 2010; Accepted 09 October 2010)

ABSTRACT

Antimicrobial evaluation of the essential oil(s) of some spp. of *Curcuma* viz., *Curcuma angustifolia*, *C. aromatica*, *C. domestica* and *C. zedoaria* –were screened against three common dermatophytic fungi causing ringworm infection in human beings. The essential oil of *Curcuma domestica* Valet. (Family- *Zingiberaceae*) was found strongest toxicant against the test fungi. The minimum inhibitory concentration (MIC) of the oil was 1.6µl/ml against *Epidermophyton floccosum* and 1.4µl/ml against *Microsporum gypseum* and *Trichophyton rubrum*; however, it was fungicidal at 1.6 µl/ml against *M. gypseum* and *T. rubrum*, and 2.0 µl/ml against *E. floccosum*, 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 hr against *T. rubrum*. The oils efficacy was thermo stable up to 80 °C and for 36 months of storage, the maximum unit taken into consideration. Moreover, the oil of *C. domestica* did not exhibit any adverse effect on mammalian skin up to 5% conc. The clinical trial of the oil in the form of ointment (at 1% V/V conc.) to topical testing on patients, attending outpatient department (OPD) of MLN Medical College, Allahabad is still in progress.

Keywords: Antimicrobial activity; Dermatophytes; Medicinal plants; MIC; Herbal drug.

INTRODUCTION

Fungal infections in human beings are a major problem in tropical and subtropical countries due to prevailing humidity and temperature regimes. The superficial fungal infection or dermatomycoses is the disease caused by a group of fungi known as dermatophytes. It involves superficial infections of keratinized tissue in human beings. Clinical surveys carried out in India have showed that ringworm is one of the most common dermatomycoses caused by the species of *Epidermophyton floccosum*, *Microsporum* and *Trichophyton*. 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 have been investigated to be an effective source of chemotherapeutic agents without undesirable side effects and with strong fungicidal activity. Consequently, in the present investigations, attempts have been made to explore the possibilities of *Curcuma* spp, as a protecting measurement against ringworm infections in human beings.

MATERIALS AND METHODS

In vitro investigation

Extraction and Isolation of Essential oil: The essential oils were extracted separately from the fresh leaves of *Curcuma angustifolia*, *C. aromatica*, *C. domestica* and *C. zedoaria* (Family- *Zingiberaceae*) by hydro distillation using Clevenger's apparatus (Clevenger, 1928). 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.

In-vitro antimicrobial investigations of the essential oil: 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., (2001), 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:

$$\text{FGI (\%)} = \frac{D_c - D_t}{D_c} \times 100$$

- D_c indicates colony diameter in control set, &
- D_t indicates colony diameter in treatment sets.

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 Shukla et al., (2001). Mycelial discs of 5mm diam of 7-day old 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).

Effect of some Physical Factors: Effect of some physical factors viz., temperature (40, 60 and 80 °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 following the method of Shukla et al., (2001) and Shahi et al., (2001). Samples of oil in small vials, each contains 1ml, were exposed at 40, 60 and 80⁰ C in hot water bath, respectively. Further, the oil's efficacy was tested against the test fungi at their respective MCCs (Table- 3).

Minimum Killing Time: The MKT of the pure oil and their respective MCCs of *C. domestica* against the test fungi was determined by using the method of Shahi, et al. (1999) (Table-4).

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 audouinii* 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 Shahi et al., (2001) (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 Shukla et al., (2001) (Table-5).

Comparison with some Synthetic Fungicides: The comparative efficacy of oil of *C. domestica* with some synthetic antifungal drugs was carried out by comparing MECs. This was done by using the method of Shahi, et al., (1999) (Table-6 & 7).

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

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.

RESULTS

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

The MEC of *Curcuma domestica* oil was 1.4 µl/ml against *M. gypseum* and *T. rubrum*, and 1.6 µl/ml against *E. floccosum*; however, it was fungicidal at 1.6 µl/ml against *M. gypseum* and *T. rubrum*, and 2.0 µl/ml against *E. floccosum*, 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 80⁰ 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 antifungals, MECs of the oil was more active than Dactrine, Nizalal and Tenaderm (Table- 6 & 7).

DISCUSSIONS

Essential oils obtained from the leaves of *Cymbopogon martini* var. *motia* (Dikshit, et al., 1980), *Hyptis leucodendron* (Dubey, et al., 1983); *Alpinia galangal* (Tripathi, et al., 1983) was found to contain fungistatic activity. However, some essential oils, *Cymbopogon flexuosus* (Pandey, et al., 1996); *Eucalyptus* oil (Shahi, et al., 2000); *C. flexuosus* (Shahi, et al., 2003); and *Homalomena aromatica* (Shukla, et al., 2009) prove to have fungistatic action at lower concentration and fungicidal action at higher concentration. Similarly, in the present investigation the oil of *Curcuma domestica* showed fungistatic activity at the lower concentration 1.4 µl/ml against *M. gypseum* and *T. rubrum*, and 1.6 µl/ml against *E. floccosum*; and fungicidal at the higher concentration 1.6 µl/ml against *M. gypseum* & *T. rubrum*, and 2.0 µl/ml against *E. floccosum*, 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, Nizal, Tenaderm.

A fungicide must not be affected by extreme temperatures. A few workers have studied the effect of temperature on antifungal activity of the essential oils. Singh et al., (1984) reported the oil of *Pepromia pellucida* was active up to 80 °C; Shahi et al., (2003) reported *C. flexuosus* activity up to 100 °C, and Shukla et al., (2009) reported the oil's efficacy of *H. aromatica* up to 80 °C. Similarly, in the present investigation the oil of *C. domestica* was not only thermostable up to 80 °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 (Singh, et al., 1980; Pandey, et al., 1982; Dubey, et al., 1983), but in the present study the oil of *C. domestica* exhibited broad antifungal spectrum.

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. domestica* 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 reveals that the oil of *Curcuma domestica*, 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 agents. 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.

Acknowledgements: The authors are thankful to the Head, Department of Botany, University of Allahabad for providing the research facilities; to Prof. A.K. Bajaj, Former-Head Dept of Dermatology, MLN Medical College, Allahabad and to Dr. Uma Banerjee, Division of Microbiology, All India Institute of Medical Sciences, New Delhi for providing their microbial expertise, as well as to the Department of Science & Technology, New Delhi (Ref. No. SR/ FTP/ LS- 227/ 2000) for financial assistance. The authors are also thankful to the authorities of the Mizoram University, Aizawl, for providing various kinds of support during the course of research.

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

<i>Curcuma</i> spp	Human Pathogenic Fungi		
	<i>Epidermophyton floccosum</i>	<i>Microsporium gypsum</i>	<i>Trichophyton rubrum</i>
<i>C. angustifolia</i>	2.6 µl/ml	2.2 µl/ml	2.4 µl/ml
<i>C. aromatica</i>	1.8 µl/ml	1.6 µl/ml	1.8 µl/ml
<i>C. domestica</i>	1.6 µl/ml	1.4 µl/ml	1.4 µl/ml
<i>C. zedoaria</i>	2.2 µl/ml	1.8 µl/ml	2.0 µl/ml

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

Concentration (µl/ml)	Human Pathogenic Fungi		
	<i>Epidermophyton floccosum</i>	<i>Microsporium gypsum</i>	<i>Trichophyton rubrum</i>
2.0	100 ^c	100 ^c	100 ^c
1.8	100 ^s	100 ^c	100 ^c
1.6	100 ^s	100 ^s	100 ^c
1.4	88	60	100 ^s
1.2	60	--	80
1.0	--	--	60

- ^c indicates cidal and ^s indicates static.

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

Properties studied	Observations		
	<i>Epidermophyton floccosum</i>	<i>Microsporium gypsum</i>	<i>Trichophyton rubrum</i>
Minimum Inhibitory Concentration			
MEC (µl/ml)	1.6 µl/ml	1.4 µl/ml	1.4 µl/ml
MFC (µl/ml)	2.0 µl/ml	1.6 µl/ml	1.6 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 °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.

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

Minimum Killing Time (MKT)	Mycelial Growth Inhibition (%)					
	<i>Epidermophyton floccosum</i>		<i>Microsporium gypseum</i>		<i>Trichophyton rubrum</i>	
	P.O.	MFC	P.O.	MFC	P.O.	M.F.C.
7.0	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	
1.00	100		100		100	
30 min	100		100		100	
15 min	100		100		100	
5 min	100		100		100	
60 sec	100		100		100	
30 sec	100		100		100	
20 sec	90		80		100	
10 sec	60	---	70	---	88	---

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

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

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

- ^s indicates static; ^c indicates cidal in nature.

Table- 6: Comparative MECs of the oil of *Curcuma domestica* with some synthetic anti-fungal.

Oil & Trade Name of Antifungal Drugs	Active Ingredients	Minimum Effective Concentration ($\mu\text{l/ml}$)		
		<i>Epidermophyton floccosum</i>	<i>Microsporum gypseum</i>	<i>Trichophyton rubrum</i>
<i>Curcuma domestica</i>	Essential oil	1.6	1.4	1.4
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 domestica* with some synthetic antifungal drugs.

Antimycotic Drugs	Drugs %	Cost (Rs.)		Adverse Effects	Expiry Duration (months)	Environmental impact
		ointment/gm	lotion/ml			
<i>C. domestica</i>	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	36	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----