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Tradition to technology: an approach to drug development against human pathogenic fungi

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Abstract

During antidermatophytic investigations of the plant secondary metabolites of some common ethnomedicinal plants, the essential oil of *Curcuma longa* Linn (family Zingiberaceae) was found to be the strongest toxicant against the pathogenic fungi *Trichophyton mentagrophytes* and *T. rubrum*, dermatophytes causing ringworm infection in human beings. The minimum inhibitory concentration (MIC) of the oil was recorded at 1.9 mg/ml against *T. mentagrophytes* and 2.1 mg/ml against *T. rubrum*. However, it was fungicidal at 2.4 mg/ml against *T. mentagrophytes* and 2.5 mg/ml against *T. rubrum* respectively. The effective concentration contains heavy doses of inoculums (25 discs of 5 mm each.). The minimum killing time (MKT) of the oil was 45 sec against *T. mentagrophytes* and 30 sec against *T. rubrum*, while it's MFCs required 7.00 hrs against *T. mentagrophytes* and 5.30 hrs against *T. rubrum*. The efficacy was thermo stable up to 80°C and for 36 months of storage, the maximum unit taken into consideration. Moreover, the oil did not exhibit any adverse effect on human skin up to 5% conc. Further detail *in vivo* investigations as well as clinical trial are required with the formulation(s) of the oil to fully develop an alternative to the synthetics.

Key words: Antimicrobial; dermatophytes; ethnomedicinal; minimum inhibitory concentration; herbal drug.

Introduction

Curcuma is an important genus in the family Zingiberaceae and several species are used as medicine, as a yellow dye for cloth, and as spices since time immemorial. Its generic name originated from the Arabic word *kurkum* meaning "yellow", and most likely refers to the deep yellow rhizome color of the true turmeric *(Curcuma longa* L.). Besides *C. longa,* there are several species of medicinal importance, such as *C. aromatica* Salisb., *C. amada* Roxb., *C. caesia* Roxb., *C. aeruginosa* Roxb., and *C. zanthorrhiza* Roxb. Others are beautiful and splendid plants of great ornamental value, such as *C. alismatifolia* Gagnep., *C. elata* Roxb., and *C. roscoeana* Wall. Locals and tribal people in most Asian countries

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use *Curcuma* species in religious rituals, as a foodstuff, and as medicinal plants.[1]

Turmeric has been valued as a source of medicine and color in the whole of South Asia, from ancient times. Probably man would have been attracted to this plant due to its attractive color and in due course, it acquired many religious and socio cultural associations. For the ancient people of India, turmeric was the *Oushadhi* – the medicinal herb, and possibly it might have played a great role in the day-to-day life of ancient Indians as a wound healer, as a medicine for stomach ache, flatulence, poison, etc., for dyeing clothes and yarns, and for worshipping their gods and goddesses. This plant has acquired great importance in the present-day world with its anti-aging, anti-cancer, anti-Alzheimer's, antioxidant, and a variety of other medicinal properties.[2]

Traditional uses of turmeric

The earliest reference about turmeric can be seen in *Atharvaveda* (ca. 6000 yr B.P.), in which turmeric is prescribed to charm away jaundice. It was also prescribed in the treatment of leprosy. Reference to turmeric has also been made in the *Yajnavalkyasamhita* (composed ca. 4000 yr B.P.) at the time of the epic Ramayana. Turmeric was listed as a colouring plant in an Assyrian herbal dating about 2600 yr B.P. Marco Polo, in 1280 A.D., mentioned turmeric as growing in China.[3] Evidences indicate that turmeric was under cultivation in India from ancient times. Fluckiger and Hanbury[4] wrote "several varieties of turmeric, distinguished by the names of the countries or districts in which they are produced are found in the English market; although they present differences that are sufficiently appreciable to the eye of the experienced dealer, the characters of each sort are scarcely so marked or so constant as to be recognizable by mere verbal descriptions." Linschoten,[5] while describing with the utmost details the trade in Cochin makes no mention of turmeric.

The Hindus, both tribal and civilized, consider turmeric as sacred and auspicious. It is associated with several rituals from ancient period and the tradition still goes on. In some tribal communities in Tamil Nadu, Andhra Pradesh, and in the Northeast regions of India, a piece of turmeric tied to a thread, dyed yellow with turmeric powder, is used as the nuptial string (*mangalsutra*). Even now, in the village and urban Hindu community, this practice is very much prevalent; rich people use gold chain also along with the natural yellow rhizome, but the poor depend on turmeric alone. Turmeric is also used as an amulet and a piece of turmeric tied on the hand is believed to keep away evil spirits.

Table 1. *Curcuma longa* L. in Mizoram*

Local name: **Aieng**

Cultivated in large scale in jhum lands, throughout the Mizoram.

The plant is popular among the tribal communities' viz., Hmar, Paite, Lai, Mara, Chakma, Bru, Bawm, Tlanglau/ Pang, Mog and the dominant tribals of the Mizos.

Ethnomedicinal uses:

Fresh rhizome is crushed and applied externally on sprains, wounds and swellings and then bandaged.

Fresh rhizome in combination with *Centella asiatica* and 5-10 clones of *Allium sativa* are grinded and taken with water against asthma.

Ethnoveterinary uses:

Use to treat wounds, ulcers and sores of domestic animals like dogs and goats.

Juice of fresh rhizome is applied externally.

*Source: Lalramnghinglova[6-7]

Keeping these views in mind, an attempt has been made to compile the traditional information on *C. longa* and their scientific validation/ bioefficacy against two common dermatophytes *Trichophyton mentagrophytes* and *T. rubrum*; which cause the ring worm infections/ skin disease in human beings.

Turmeric production in NER

Turmeric can be grown in diverse tropical

conditions from sea level to 1500 m above sea level, at a temperature range of 20-35°C with an annual rainfall of 1500 mm or more, under rain-fed or irrigated conditions. Though it can be grown on different types of soils, it thrives best in well-drained sandy or clay loam soils with a pH range of 4.5-7.5 with good organic status.

Among the North Eastern states; production of turmeric is highest in Meghalaya followed by Assam, Tripura and Nagaland but the productivity is highest in Mizoram (Table 2). The most popular cultivated variety in the region is Lakadong (7.5%) and Megha Turmeric-1 (6.8%) that possesses higher curcumin content and has maximum demand.

Materials and Methods

Extraction and isolation of essential oil

The essential oil was extracted from the fresh leaves of *C. longa* using hydro distillation technique through Clevenger's apparatus.[10] 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 oil thus obtained was subjected to various antimicrobial investigations.

Table 2. State-wise area, production and productivity of turmeric in NE Region.[8]

State	Area ('000ha)	Production ('000t)	Productivity ('000t/ha)
Arunachal Pradesh	0.40	1.50	3.75
Assam	12.00	8.00	0.67
Manipur	0.37	2.09	5.69
Meghalaya	1.60	8.70	5.44
Mizoram	0.30	2.97	**9.9**
Nagaland	0.60	3.10	5.17
Sikkim	0.50	1.70	3.40
Tripura	1.50	4.30	2.87
N.E. Regions	17.27	32.36	1.87
India	150.5	521.9	3.47

Table 3. Annual production in Mizoram and Aizawl (area 4000- 5000 ha).[9]

Year	Mizoram (mt)	Aizawl (mt)
2010-2011	23970	1284
2009-2010	22500	1100
2008- 2009	39862	6458

Procurement of test organism

The authentic cultures (MTCC - Microbial type culture collection) of *T. mentagrophytes* and *T. rubrum* were procured from the Institute of the Microbial Technology (IMTECH), Chandigarh, India. The strains of the cultures used in the present investigation were:

1. *T. mentagrophytes* (Robin) Blanchard (MTCC-8476)
2. *T. rubrum* (Castellani) Sabouraud (MTCC-3272)

The cultures thus collected were revived and multiplied on the Sabouraud Dextrose Agar (SDA) for the present investigations, and the routine sub-culturing was applied for purification and maintaining the pure culture throughout the study.

In vitro antimicrobial investigations

The minimum effective concentration (MEC) of the oil against the test pathogen *T. mentagrophytes* (MTCC-8476); *T. rubrum* (MTCC-3272), was determined by using the technique of Shahi *et al.*[11] 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/ MFC) action of the oil was tested by aseptically re-inoculating the fungi in culture tubes contain-

Table 4. Minimum effective concentration of the oil C. longa against test fungi.

Concentration (mg/ml)	Human Pathogenic Fungi	
	T. mentagrophytes	*T. rubrum*
2.5	100[c]	100[c]
2.4	100[c]	100[s]
2.3	100[s]	100[s]
2.2	100[s]	100[c]
2.1	100[s]	100[s]
2.0	100[s]	88
1.9	100[s]	82
1.8	94	60
1.7	88	44
1.6	72	--
1.5	58	--

[c] indicates cidal and [s] indicates static.

ing sabourad dextrose broth (Table 4). 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.

Effect of inoculums density

The effect of inoculums density on the minimum fungicidal concentration (MFCs) of the oil against the test fungi was determined using the method of Shukla *et al.*[12] Mycelial discs of 5 mm diam of 7-day oil cultures were inoculated in culture tubes containing oil at their respective MFCs. 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 5).

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 fungicidal concentration, was also determined. It was determined following the method of Shahi *et al.*;[11] Shukla *et al.*[12] Samples of oil in small vials, each contains 1 ml, were exposed at 40, 60 and 80°C in hot water bath, respectively. Further, the oil's efficacy was tested against the test fungi at their respective MFCs (Table 5).

Minimum killing time

The MKT of the pure oil and their respective MCCs of *C. longa* against the test fungi was determined by using the method of Shahi *et al.*[13] (Table 6).

Fungi-toxic spectrum

The fungi-toxic spectrum of the oil at lethal and hyper lethal concentration (i.e. 2.5 mg/ml and 5.0 mg/ml respectively) was determined against some common human pathogenic fungi

Table 5: Detailed *in-vitro* investigations of *C. longa* against the test fungi.

Properties studied	**Observations**	
	T. mentagrophytes	***T. rubrum***
Minimum Inhibitory Concentration (mg/ml)		
MEC	1.9	2.1
MFC	2.4	2.5
Minimum Killing Time		
Pure oil	45 sec	30 sec
MFC	7.00 hrs	5.30 hrs
Inoculum Density (up to 25 disc, 5mm diam)	No Growth	No Growth
Thermostability (up to 80 ^{0}C)	No Growth	No Growth
Effect of Storage (up to 36 months)	No Growth	No Growth

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

Table 6. Minimum killing time of the oil of *C. longa* against dermatophytes.

Minimum Killing Time (MKT)	**Mycelial Growth Inhibition (%)**			
	T. mentagrophytes		***T. rubrum***	
	P.O.	**MFC**	**P.O.**	**M.F.C.**
7.0	100	100	100	100
6.30	100	80	100	100
6.0	100	60	100	100
5.30	100	---	100	100
5.0	100	---	100	80
2.30	100	---	100	40
2.0	100	---	100	---
1.30	100	---	100	---
1.00	100	---	100	---
45 min	100	---	100	---
30 min	100	---	100	---
15 min	100	---	100	---
60 sec	100	---	100	---
45 sec	100	---	100	---
30 sec	96	---	100	---
15 sec	60	---	40	---

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

Table 8. Comparative MECs of the oil of *C. longa* with some synthetic antifungals.

Fungi Tested	Lethal Concentration (2.5 mg/ml)	Hyper Lethal Concentration (5.0 mg/ml)
Human Pathogens		
Epidermophyton floccosum	100[s]	100[c]
Microsporum gypseum	100[c]	100[c]
M. auddouinii	100[s]	100[c]
M. canis	100[s]	100[c]
M. nanum	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.

viz., *Epidermophyton floccosum* (Hartz) Langeron et Mitochevitch; *Microsporum gypseum* (Bodin) Guiart and Grigorakis, *M. auddouinii* Gruby, *M. canis* Bodin, *M. nanum* Fuentes, *T. tonsurans* Malmstem, and *T. violaceum* Bodin. This was done by using the method of Shahi *et al.*[11] (Table 7).

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.* [12] (Table 7).

Comparison with some synthetic fungicides

The comparative efficacy of oil of *C. longa* with some synthetic antifungal drugs was carried out by comparing MECs. This was done by using the method of Shahi *et al.*[13] (Table 8 & 9).

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 de-

Table 8. Comparative MECs of the oil of *C. longa* with some synthetic antifungals.

Test/drugs	Active Ingredients	Minimum Effective Concentration (mg/ml)	
		T. mentagrophytes	***T. rubrum***
Curcuma longa	Essential oil	1.9	2.1
Dactrine	Miconazole nitrate	6.0	6.0
Nizaral	Ketoconazole	6.0	0.5
Tenaderm	Tolnaftate	2.0	1.5

termined to be within the 95% confidence level for reproducibility. The ANOVA was computed using the SPSS version 16.0 software package.

RESULTS

The minimum effective concentration (MEC) of the oil of *C. longa* was 1.9 mg/ml against *T. mentagrophytes* and 2.1 mg/ml against *T. rubrum*; however, it was fungicidal at 2.4 mg/ml and 2.5 mg/ml against the same, respectively (Table 4). The oil's toxicity persists heavy doses of inoculums (i.e. up to 25 discs, each of 5 mm), thermo stable up to 80°C and remains constant even after autoclaving at 15 lb/ sq inch pressure for 30 min. (Table 5).

The pure oil kills the test fungi within 30-45 second; however, their MFCs required 7.00 hrs to 5.30 hrs to kill the same (Table 6). Fungi toxic spectrum of the oil at lethal and hyper lethal concentration (i.e. 2.5 mg/ml and 5.0 mg/ml), against some common pathogenic fungi reveals that the oil contains a broad fungicidal spectrum (Table 7). Furthermore, on comparing MFCs of the oil with some synthetic antifungal drugs, the oil shows an edge over the synthetics, such as Dactrine, Nizaral and Tenaderm (Table 8 & 9).

DISCUSSIONS

Although, in northeastern states, some researches on ethno medicinal plants have already been made like in the states of Assam,[14-16] Meghalaya,[17-18] Nagaland,[19] Arunachal Pradesh[20-21]and in Mizoram.[22-30] But, there were no such reports on ethno medicinal plants used against skin diseases, as considered in the present investigation.

Further, essential oils obtained from the leaves of *Cymbopogon martini* var. motia,[31] *Hyptis leucodendron,*[32] *Alpinia galangal*[33] was found to contain fungistatic activity. However, some essential oils, *Cymbopogon flexuosus*,[34] *Eucalyptus* oil,[35] *C. flexuosus*,[36] and *Curcuma dometica*[12] prove to have fungistatic action at lower concentration and fungicidal action at higher concentration. Similarly, in the present investigation the oil of *C. longa* showed fungistatic activity at the lower concentration 1.9 mg/ml against *T. mentagrophytes* and 2.1 mg/ml against *T. rubrum*; and fungicidal at the higher concentration 2.4 mg/ml and 2.5 mg/ml against the same, respectively (Table 4).

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.*[37] reported the oil of *Peperomia pellucida* was active up to 80°C; Shahi *et al*.[36] reported *C. flexuosus* activity up to 100°C, and Shukla *et al.*[12] reported the oil's efficacy of *Curcuma domestica* up to 80°C. Similarly, in the present investigation the oil of *C. longa* was not only thermostable up to 80°C but also autoclavable up to 15 lb/ sq inch pressure for 30 min (Table 5). Besides, 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 (Table 5 to 9).

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,[38-39] but in the present study oil of *C. longa* exhibited broad antifungal spectrum (Table 7).

A toxicant should be tested under both *in vitro* and *in vivo* conditions in order to prove its potential as promising antifungal for the control of disease. Since, detailed *in vitro* studies on the essential oil of *C. longa* indicate their potentiality to be as ideal antifungal agent against the test pathogens; hence, the same can also be subjected for detailed *in vivo* investigations as well as clinical trials in further investigations.

CONCLUSIONS

The preliminary *in vitro* investigations of the oil against the test pathogen *T. mentagrophytes* and *T. rubrum* reveals that after the detailed *in vivo* as well as multi central clinical trials, *C. longa* can be an effective antimicrobial agent against dermatophytes.

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