e - ISSN – 2393-9672

European Journal of Environmental Ecology

Journal homepage: www.mcmed.us/journal/ejee

HOMALOMENA AROMATICA: AN ETHNOMEDICINAL PLANT, CAN BE A POTENTIAL SOURCE OF ANTIMICROBIAL DRUG DEVELOPMENT

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Article Info

Received 13/03/2015; Revised 29/03/2015
Accepted 25/04/2015

ABSTRACT

The genera *Homalomena* belongs to the family Araceae of the group Monocotyledon. There are about 140 spices in tropical Asia and South America; two species in India; one in Mizoram, i.e., *Homalomena aromatica* Schott. The plant is very popular among the Mizo-tribal communities. The boiled petiole is used as vegetable, rhizome as aromatic stimulant, powdered rhizome as gun-powder, burnt smoke of rhizome as mosquito repellent and infusion of the plant for easy labor. The juice of whole plant is used as lotion in skin diseases. Besides these, the plant contains strong antimicrobial activity. The minimum cidal concentration (MCC) of the oil against some common human pathogenic fungi was found to be 1.2 to 1.8 µl/ml, which contains heavy inoculums density. The oils toxicity persists up to 80°C and also autoclavable, with a broad fungi toxic spectrum. The pure oil kills the test pathogenic fungi just within a minute; however, its MCC takes 5.30 to 6.30 hrs to kill all the test fungi. Besides this, while comparing the MECs of the oil with some synthetic antifungal drugs, the toxicity of the oil shows an edge over the synthetics- Dactrine, Nizaral, Tenaderm and Batrafine. 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, during pre-clinical investigations, the efficacy of oil contains 60-80% cure of the skin diseases. Based on these findings as well as after detailed *in vitro, in vivo*, clinical as well as multi-central clinical investigations; formulations can be transferred to the pharmaceutical companies.

Keywords: Ethno medicinal plant(s), pathogenic fungi, essential oil, antimicrobial activity.

INTRODUCTION

In India, out of two species of *Homalomena;* the one *Homalomena pendula* (Bl.) Hakh. f. syn. *H. rubescens* (Roxb.) Kunth have been reported to occur in Sikkim and Meghalaya [1]; and the other *Homalomena aromatica* Schott is very frequent to occur in tropical evergreen forests (Kanhmun – Bairabi ranges) of Mizoram, especially in Kolasib, Kawrthah and Mamit forest divisions [2]. Besides this, *H. aromatica* is also reported from Assam and Chittagong hill tracks of Bangladesh [3-7]. Ethno botanical studies, with an aim in search for new drugs, food and other economic plants, have gained worldwide interest in recent years. The conservation and sustainable utilization of the bio-

resources are the main foci of the ethno botanical studies. Keeping these views in mind, in the present investigation, an attempt have been made to explore various scientific investigations of *Homalomena aromatica* - a potential ethno medicinal plant of Mizoram; so that, it can not only become an effective source of herbal medicine for pharmaceutical companies but also by contributing to the preservation and enrichment of the gene bank of such economically important species before they are lost forever.

METHODOLOGY

Ethno botanical investigations

Ethno botanical survey

One of the authors has made frequent field visits in different seasons, during the year 1995-2000 in Kawrthah and Kolasib forest divisions of Mizoram [2]. The local knowledgeable persons of Zamuang, Zawlnuam and Kanhmun villages in Kawrthah forest division, and Kolasib, Medium and Bairabi villages in Kolasib Forest Division were taken to the forests to collect ‘Anchiri’ plant. The collected plant materials were given necessary treatment in the herbarium of the Environment & Forest Department, Aizawl (Mizoram), and the voucher specimens were deposited in the said herbarium. A few living plants were also planted near Forest Training School, Aizawl as germplasm conversion. Further, their identification was also confirmed with the experts available at the Botanical Survey of India, Allahabad; before the current investigations.

Botanical description

H. aromatica is a perennial, aromatic and rhizomatous herb. The rhizome is covered with dark-brown leafy scales. The mature rhizome is sometimes curved and running over the ground. Numerous white roots were borne on every part of the rhizome. The stem is short and slow growing. The leaves are radical, lucid, 26-27 cm x 10-12 cm, acuminate; base cordate or sagittate; lobes rounded; petiole up to 100 cm long; spadix sub-cylindric; spathe pale green-yellow, erect or pendant; flower arising form the axils and centre of the leaves on the green scape; berries white, oblong, 1-seeded.

Flowering / Fruiting time : June – August.

Habitat

Anchiri is usually found in moist tropical evergreen forests. They grow on humus sandy-loam soil and / or brown loamy-clay soil in moist shady places on river banks and valleys in association with other plants like ferns, *Hedychium* sp. etc. as an undergrowth and shade demander. Usually, they are distributed in moist and warm climates below 1000 m asl.

Status

Locally, *Homalomena aromatica* is a conservation dependant (cd) in the lower risk of the IUCN category [2].

Threats

The greatest threat to ‘Anchiri’ plant is surface clearance of forests for slash-and burn method of agriculture. The unsustainable harvest and massive collection of the rhizomes for trade is equally dangerous to the local extinction and over exploitation of the genetic resources. Hunting for food and medicine also affect the degree of population reduction.

Conservation measures

The plant being a shade demander can be best conserved in natural habitats, such as protected forests and riverine reserved forests where they grow. Translocation of scattered growing stocks in suitable places through re-introduction programmes by the involvement of local people would be fruitful.

Uses

The rhizome is aromatic and stimulant and the powdered rhizome is used in the preparation of snuff and tobacco [3-5]. The essential oil and the whole plant parts are being used in skin diseases [5-6]. The essential oil (0.5 %) yielded on hydro/ steam distillation is used in perfumery ingredients [5]. The aroma of burnt smoke of dried rhizome is inhaled against influenza and the roots are used for jaundice [7]. The ethno-medical uses included powdered rhizome as gun-powder, boiled petiole as vegetable, burnt smoke of rhizome as mosquito repellant and infusion of the plant for easy labour.

In vitro Investigations

Extraction and Isolation of Essential oil

The essential oil was extracted from the rhizome of *H. aromatica* (1 Kg.) by hydro distillation using Clevenger's apparatus [8]. 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 for GC-MS analysis as well as for various fungi-toxic investigations.

Antimicrobial Investigation of the Essential oil

The minimum effective concentration (MEC) of the oil against some 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., [9]. 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 potato dextrose agar medium. 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 potato dextrose broth [10] (Table-1).

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 set.

Effect of Inoculums Density

The effect of inoculums density on the minimum cidal concentration (MCCs) of the oil against the test fungi was also determined [11]. 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-1).

Effect of some Physical Factors

Effect of some physical factors viz., temperature (40, 60 and 80^0C respectively) and autoclaving on efficacy of the oil, at minimum cidal concentration, was also determined [12]. Samples of oil in small vials, each contains 1ml, were exposed at 40, 60 and 80^0C in hot water bath, respectively. Further, the oil's efficacy was tested against the test fungi at their respective MCCs (Table-1).

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, M. canis, M. nanum, Trichophyton mentagrophytes, T. tonsurans* and *T. violaceum* [9 & 13] (Table-3).

Besides these, it was also determined against some common plant pathogenic fungi viz., *Aspergillus parasiticus* Speare, *Cladosporium cladosporioides* Boedijin, *Colletotrichum capsici* (Syd.) Butler & Bisby, *C. falcatum* Went, *Fusarium oxysporum* Schlecht, *F.* (Fresenius) de Vries, *Curvularia lunata* (Wakker) *udum* de vries, *Helminthosporium maydis* Nisikado & Miyakel, *H. oryzae* Breda de Haan, *Penicillium implicatum* Biourge and *P. minio-luteum* Dierckx [12], (Table-3).

Minimum Killing Time

The MKT of the pure oil and their respective MCCs of *H. aromatica* against the test fungi was determined by using the method of Shahi et.al. [9], (Table-3).

Comparison with some Synthetic Fungicides / Drugs

The comparative efficacy of the oil of *H. aromatica* with some synthetic antifungal fungicides/ drugs was carried out by comparing MECs [9] (Table-4 and 5).

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.

Phylogenetic study of dermatophytes

To find out the reason why the *H. 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 [14] (Fig. 1). The ITS1 sequences of the standard strains used in this study and of members of the *Trichophyton* spp complex (*Trichophyton mentagrophytes* isolates from humans, accession no. AB011463; *T. rubrum*, accession no. AB011453; *T. violaceum*, accession no. AB017174; *Microsporum gypsem*, accession no. AB017177 and *Epidermatophyton floccosum*, accession no. AB017181), as reported by Makimura *et al*. [15] were aligned by using the Clustal W computer program [16] 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 [17], and the neighbor-joining (NJ) [18] method in the NJPLOT program [19]. Bootstrap [17] analysis with the Clustal W program was performed by taking 1,000 random samples from the multiple alignments.

RESULTS

The GC-MS analysis of the essential oil of *H. aromatica* shows 'linalool (32.45%), followed by lerpinen-4-ol (14.10%) as major active constituents (Fig-1).

The minimum effective concentration (MEC) of the oil was found to be 1.6 µl/ml, 1.2 µl/ml and 1.4 µl/ml conc against the test fungi *Epidermophyton floccosum, Microsporum gypseum* and *Trichophyton rubrum* respectively; however it was cidal at 1.8 µl/ml against *E. floccosum* and 1.6 µl/ml against *M. gypseum* and *T. rubrum* (Table-1).

The efficacy of the oil contains heavy doses of inoculums (i.e. up to 25 discs, each of 5mm), and it still persisted even up to 80^0 C as well as after autoclaving, the maximum units taken into consideration. Furthermore, the fungi toxic spectrum of the oil at lethal and hyper lethal concentration (i.e. 1.8 µl/ml and 3.6 µl/ml respectively), against some common pathogenic fungi reveals that the oil contains a broad fungicidal spectrum (Table- 3).

The pure oil kills the tested human pathogenic fungi, pure oil kills the tested fungi just within a minute; but, its MCC takes 6.30 to 5.30 hrs to kill all the test fungi (Table- 2). On comparing the MECs of the oil with some synthetic antifungal drugs, the MECs of the oil's toxicity showed edges than the tested synthetic fungicides- Dactrine, Nizaral, Tenaderm and Batrafine (Table- 4).

The phylogenetic relationship of dermatophytic genera *Trichophyton*, *Microsporum*, and *Epidermophyton* was determined on the basis of their ITS1 sequences. The NJ tree was constructed with data for standard strains of dermatophytes [14] 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 floccosum* as an out-group, and within this cluster, all *Trichophyton* spp. except *Trichophyton terrestre* form a nested cluster (100 % bootstrap support). Dermatophytes in the cluster of *Trichophyton* spp. were classified into three groups with ITS1 homologies, each being a monophyletic cluster (100 % bootstrap support). The ITS1 sequences of 6 clinical isolates (Fig- 2) were also determined to identify the species. All strains were successfully identified by comparison of their base sequences with those in the ITS1 DNA sequence database [14]. NJ tree of dermatophytes is shown in Fig- 3.

The relationship of the toxicity of the essential oil vis-à-vis phylogeny was analyzed using molecular data. The effectiveness of the oil was equal in dermatophytes that are close in phylogenetic tree (Fig-3).

Table 1. Antimicrobial activity of the oil of *Homalomena aromatica* against pathogenic fungi

Properties Studied	Human Pathogenic Fungi		
	Epidermophyton floccosum	***Microsporum gypseum***	***Trichophyton rubrum***
MEC	1.6 µl/ml	1.2 µl/ml	1.4 µl/ml
MCC	1.8 µl/ml	1.6 µl/ml	1.6 µl/ml
Inoculum Density at MCC (25discs, 5mm each)	No growth	No growth	No growth
Thermostability at MCC (up to 80^0C)	No growth	No growth	No growth
Effect of Autoclaving (120lbs for 30 min)	No growth	No growth	No growth

MEC = Minimum Effective Concentration; MCC = Minimum Cidal Concentration

Table 2. Minimum killing time of the oil of *H. aromatica* against test fungi

Minimum Killing Time (MKT)	Mycelial Growth Inhibition (%)					
	Epidermophyton floccosum		***Microsporum gypseum***		***Trichophyton rubrum***	
	P.O.	MCC	P.O.	MCC	P.O.	MCC
7.00	100	100	100	100	100	100
6.30	100	100	100	100	100	100
6.00	100	60	100	80	100	100
5.30	100	---	100	---	100	100
5.00	100		100		100	80
2.30	100		100		100	---
2.00	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; MCC indicates Minimum Cidal Concentration.

Table 3. Fungi toxic spectrum of the oil of *H. aromatica* against some pathogenic fungi

Fungi Tested	**Lethal Concentration** (1.8 µl/ml)	**Hyper Lethal Concentration** (3.6 µl/ml
Human Pathogens		
Microsporum auddouinii (E. Bodin) Guiart & Grigoraki		
M. canis Bodin ex Guég.	100^{s}	100^{c}
M. nanum C.A. Fuentes	100^{c}	100^{c}
Trichophyton mentagrophytes Priestley	100^{c}	100^{c}
T. tonsurans Malmsten	100^{c}	100^{c}
T. violaceum Sabour.	100^{c}	100^{c}
Plant Pathogens		
Aspergillus parasiticus Speare	100^{s}	100^{c}
Cladosporium cladosporioides (Fresenius) de Vries	100^{c}	100^{c}
Curvularia lunata (Wakker) Boedijin	100^{c}	100^{c}
Colletotrichum capsici (Syd)Butler& Bisby	100^{c}	100^{c}
C. falcatum Went	100^{c}	100^{c}
Fusarium oxysporum Schlecht	100^{c}	100^{c}
F. udum de vries	100^{c}	100^{c}
Helminthosporium maydis Nisikado & Miyakel	100^{c}	100^{c}
H. oryzae Breda de Haan,	100^{c}	100^{c}
Penicillium implicatum Biourge	100^{c}	100^{c}
P. minio-luteum Dierckx	100^{c}	100^{c}

^{s} indicates static; ^{c} indicates cidal in nature

Table 4. Comparative MECs of the oil of *H. aromatica* with some Synthetic Antifungal Drugs

Oil & Trade Name of Antifungal Drugs	Active Ingredients	Minimum Effective Concentration (µl/ml)		
		Epidermophyton floccosum	***Microsporum gypseum***	***Trichophyton rubrum***
H. aromatica	Essential oil	1.6	1.2	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 5. Comparative Efficacy of the oil of *H. aromatica* with some Synthetic Antifungal Drugs

Antimycotic Drugs	Drugs %	Cost (Rs.)		Adverse Effects	Expiry Duration (months)	Environmental impact
		Ointment/g	lotion/ml			
H. aromatica	1% v/v	0.90	0.70	No adverse effects	24-60	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 -----do-----

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	

Figure 1. GC-MS analysis of *H. aromatica*: 1.α-pinene + α -thujene (0.60), 2. Sabinene (8.35), 3. (1.25), 4. ρ-cymene (4.75), 5. cis-linalool oxide (0.65), 6. Sabinene hydrat (0.25), 7. trans-linalool oxide (0.65), 8. Linalool (32.45), 9. Lerpinen-4-ol (14.10), 10. α -terpineol (1.85), 11. Piperitone (1.90), 12. Geraniol (0.65), 13. ρ -cymene-8-ol (0.30), 14. Caryophyllene oxide (1.85), 15. Epi-globulol (0.35), 16. Humulene oxide (0.60), 17. Spathulenol (1.85), 18. T-cadinol (0.60), 19. T-muurolol (1.70), 20. α-cadinol (4.00).

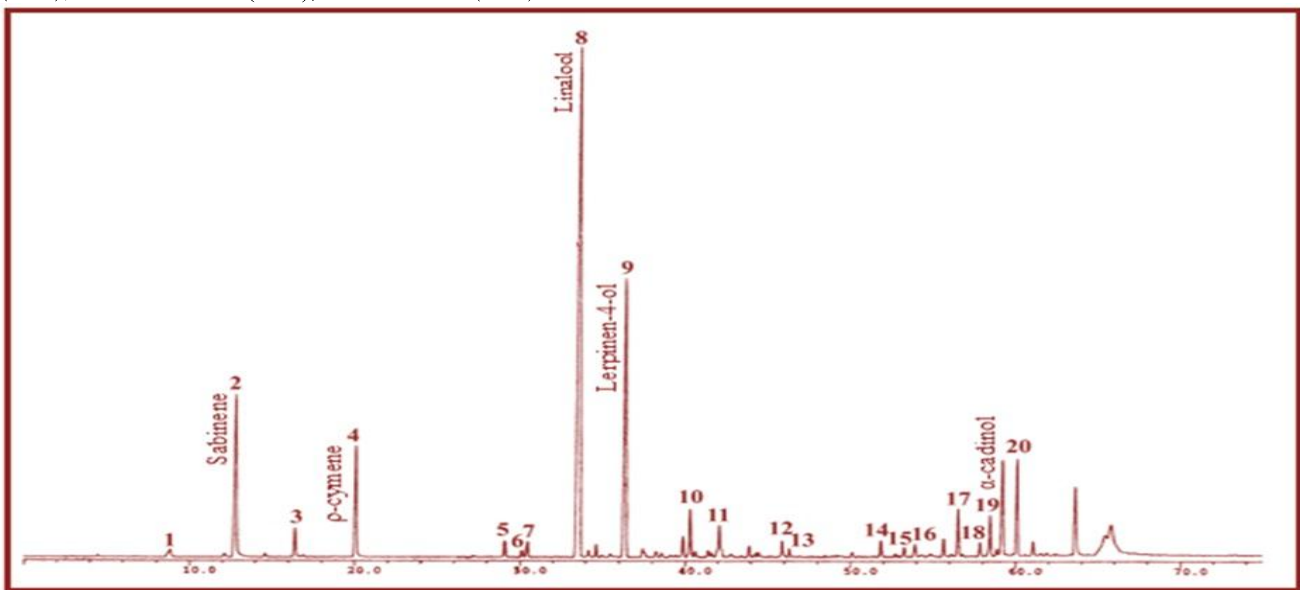


Figure 2. Alignment of ITS1 sequences of standard strains of dermatophytes

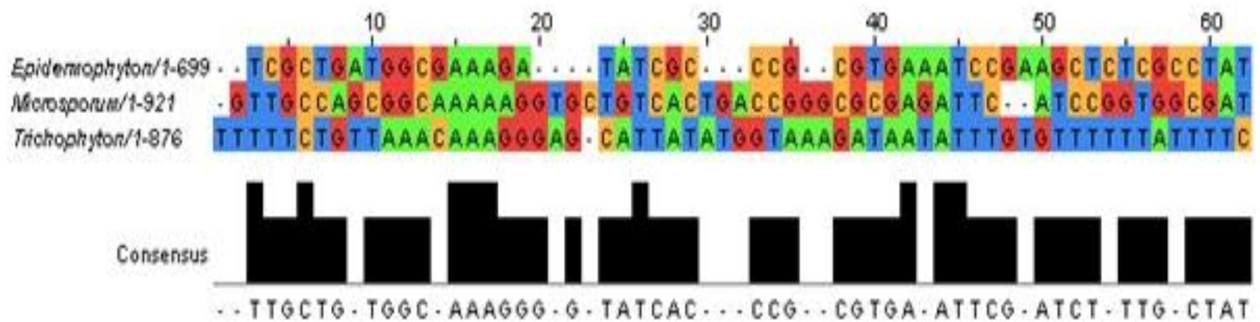
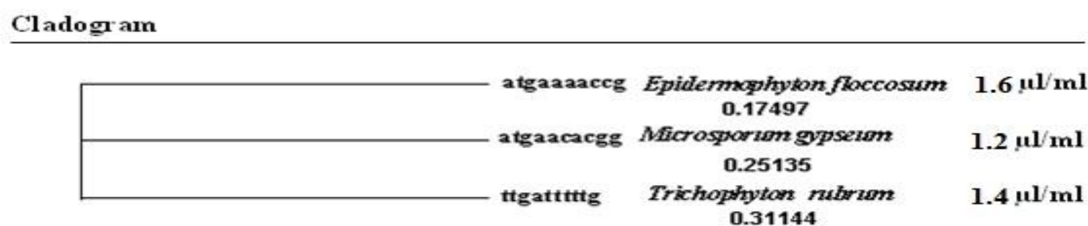


Figure 3. Result of Cladogram (Neigbour Joining Tree plot) standard strains of dermatophytes on the basis of their ITS1 sequences.



DISCUSSION

Essential oils obtained from the leaves of *Cymbopogon martini* var. motia [20], *Hyptis leucodendron* [21]; *Alpinia galangal* [22] was found to contain fungistatic activity. However, some essential oils, *Cymbopogon pendulus* [23]; *Eucalyptus* oil [24]; *Mentha arvensis* [24]; and *Curcuma* spp [25] prove to have fungistatic action at lower concentration and fungicidal action at higher concentration. Similarly, in the present investigation the oil of *H. aromatica* showed fungistatic activity at the lower concentration 1.2 µl/ml against *T. rubrum*, 1.4 µl/ml against *M. gypseum, and* 1.6 µl/ml against *E. floccosum;* and fungicidal at the higher concentration 1.6 µl/ml against *M. gypseum* & *T. rubrum*, and 1.8 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, Nizaral, Tenaderm and Batrafine. 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. The oil of *Pepromia pellucida* was active up to 80^0C [26]; *Cymbopogon flexuosus* activity up to 100^0C [24], and *Curcuma longa* up to 80^0C [25]. Similarly, in the present investigation the oil of *H. aromatica* was not only thermostable up to 80^0C 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 [21,27-29], but in the present study the oil of *H. 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 rhizome of *H. 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 *H. 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.

CONCLUSION

Homalomena aromatica is an important 'hidden economy' in the rural Mizoram. In-situ conservation should be enhanced and the micro-environments or ecological niches in which they grow should be protected. Sustainable harvesting techniques need to be adopted and ensure planting of the growing tips on the spot.

Beside this, the preliminary *in vitro* investigations of the oil against some pathogenic fungi (*Epidermophyton floccosum, Microsporum gypseum* and *Trichophyton rubrum*) reveals that after the detailed *in vitro, in vivo* as well as clinical trials, *H. aromatica* can also be an effective antimicrobial agent against the human pathogenic fungi.

ACKNOWLEDGEMENTS

The authors are thankful to the peoples of Mizoram especially the Mizo-tribal communities, who have shared their information during the course of study. The authors are also thankful to Prof. A.K. Bajaj, Former-Head Department of Dermatology, MLN Medical College, Allahabad; Dr. Uma Banerjee, Division of Microbiology, All India Institute of Medical Sciences, New Delhi, and Dr. Ashok Sharma, Deputy Director & Head, Department of Bioinformatics, Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow; for providing their microbial expertise. Besides this, the authors are highly thankful to the authorities of the Botany Dept, University of Allahabad as well as HAMP Dept, Mizoram University, Aizawl; for providing various kinds of support during the course of research.

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