

Therapeutic Effects of Essential Oil from Waste Leaves of *Psidium guajava* L. against Cosmetic Embarrassment Using Phylogenetic Approach

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ABSTRACT

Medicinal plants are facing threats due to loss of habitat and overexploitation of known species. Therefore the present work shows utilization of essential oil from discarded leaves of *Psidium guajava* Linn. against human dermatophytic fungi *Trichophyton rubrum*, *T. mentagrophytes*, *Trichophyton violaceum*, *Trichophyton tonsurans*, *Epidermophyton floccosum*, *Microsporum gypseum* and *Microsporum canis*. The oil was found to be fungicidal at 3.0 µl/ml and it tolerated heavy inoculum of pathogens at fungicidal concentrations. The fungicidal activity of the oil was thermostable, up to 70°C and shelf life was found to be six months, which was maximum the time taken into consideration. The oil having cineole, caryophyllane, copaene, azulene and eucalyptol as main constituents, exhibited broad fungicidal activity. The oil did not show any adverse effect on mammalian skin upto 5% concentration. Phylogeny of the dermatophytes with respect to toxicity of the oil has also been discussed using molecular data. The cost effective formulation from waste leaves can be exploited after undergoing double blind successful multicentral topical testing.

Keywords: Essential Oil; Dermatophytes; Phylogeny; Discarded Leaves; *Psidium guajava* L.; *Trichophyton rubrum*; *T. mentagrophyte*

1. Introduction

Humid and unhygienic environment in tropical and subtropical countries are the main reason of a common cosmetic problem—tinea pedis (ring worm of feet), caused dominantly by *Trichophyton rubrum* followed by *T. mentagrophytes* and *Epidermophyton floccosum* [1-3]. Among these, ringworm infections are most prevalent and are named after the body parts affected. It is caused by a closely related group of fungi known as dermatophytes which have the ability to utilize keratin as a nutrient source by an enzyme keratinase. Tinea pedis caused by anthropophilic dermatophytes by shedding of skin scales which are containing viable infectious hyphal element of the fungus. The increased use of synthetic antifungal agents in recent years has resulted in the development of resistance against these drugs [4]. Moreover these antifungal drugs are largely non renewable petro products that are non biodegradable and have adverse effect and residual toxicity [5], apart from being fungistatic in nature. In recent years, plant secondary metabolites rapidly became alternate source of antifungal drugs [6], as they are mostly

biodegradable and are devoid of side effects [7]. Work on volatile plant products is gaining momentum [8,9] and worldwide spending on finding new anti-infective agents is expected to increase [10], as a result sales of botanical medicines has been increased in recent years [6].

Although too much emphasis has been given on the medicinal and aromatic plant species for discovering in them some new drugs and other compound of commercial interest, yet little or no concerted effort has been made for their conservation. Unfortunately medicinal plants are facing threats as well, due to loss of habitat and overexploitation of known species and efforts have been started to conserve them [11]. Keeping these views in mind, the present work was undertaken to explore the antifungal potentiality of essential oil of discarded plant leaves.

Psidium guajava Linn belonging to the family Myrtaceae is a native plant of tropical America. A survey of the literature shows *P. guajava* L. is mainly known for its antispasmodic and antimicrobial properties in the treatment of diarrhea [12] and dysentery [13], apart from being used an anti-inflammatory, for diabetes, hypertension, carries, wounds, pain relief and reducing fever [14]. Leaves

of this plant have been reported to contain several compounds such as various flavonoids [15], tannins [16] and terpenoids [17]. Quercetin is the best known flavonoid from guava leaves [15]. GC/MS analysis shows presence of cineole, caryophyllene, copaene, azulene and eucalyptol as the major constituents besides sixty others [18,19].

Allahabad city (25°28'N and 81°54'E) is famous for its so many varieties of delicious guavas [20]. During November to February, huge amount of guava are sold in the local market and also exported to neighboring cities and states. During the process leaves are also plucked to put as a cushion and packing of the fruits to avoid injury. After the guava fruits are sold, the sellers discard the leaves. In the present investigation, these discarded leaves were collected from the local market and the extracted essential oil was tested against the dominant dermatophytes- *Trichophyton rubrum* (Castellani) Sabouraud, *Trichophyton mentagrophytes* (Robin) Blanchard, *Trichophyton violaceum* Sabouraud, *Trichophyton tonsurans* Malmsten, *Microsporum canis* Bodin, *Microsporum gypseum* (Bodin) Guiart et Grigorakis and *Epidermophyton floccosum* (Harz) Langeron & Miloch.

2. Materials and Methods

2.1. Extraction of Essential Oil

The essential oil was extracted from the leaves of *Psidium guajava* L. by hydrodistillation using a Clevenger's apparatus [21]. Clear greenish yellow oil was obtained on the top of the aqueous distillate, which was separated from the latter and dried over anhydrous sodium sulphate. The physiochemical properties of the oil were determined by the technique described by Langenau [22].

2.2. GC-MS Analysis

Gas Chromatography analysis of the oil was performed on a Perkin-Elmer GC 8500, using a fused capillary column (25 m × 0.55 mm i.d., film thickness 0.25 µm), coated with dimethyl siloxane (BP-1). The oven temperature was programmed at 60°C to 220°C at 5°C/min. then held isothermal at 220°C for 15 min. injector temperature, 250°C, detector temperature, 300°C, carrier gas, nitrogen at a linear velocity of 10 psi:split, 1:80.

GC-MS data were obtained on a Shimadzu QP-2000 mass spectrometer at 70 eV and 250°C. GC column: Ulbon HR-1 equivalent to OV-1, fused silica capillary column 0.25 mm × 50 m, film thickness 0.25 µm. The initial temperature was 100°C for 7 min. and heated at 5°C/min to 250°C. Carrier gas helium at a flow rate of 2 ml/min.

2.3. Procurement of Test Dermatophytes

Seven selected dermatophytes viz. *Trichophyton rubrum*,

Trichophyton mentagrophytes, *Trichophyton violaceum*, *Trichophyton tonsurans*, *Microsporum canis*, *Microsporum gypseum* and *Epidermophyton floccosum* were obtained from Microbial Type Culture Collection (MTCC), IMTECH Chandigarh, India. The cultures of were maintained on Sabouraud Dextrose Agar (SDA) medium (pH 5.6) throughout the study.

2.4. Determination of Minimum Inhibitory Concentrations (MICs)

The test pathogens were obtained from Microbial Type Culture Collection, Institute of Microbial Technology Chandigarh. The minimum inhibitory concentrations (MICs) of the oil against test pathogens were determined *in-vitro* using poisoned food technique [23] with slight modification [24]. The requisite quantity of the oil samples were mixed in acetone (2% of the required quantity of the medium) and then added in pre-sterilized Sabouraud Dextrose Agar (SDA) medium, pH 5.6. In control sets, sterilized water (in place of the oil) and acetone were used in the medium in appropriate amounts. Mycelial discs of 5 mm diameter, cut out from the periphery of 7 days old cultures, were aseptically inoculated upside-down on the agar surface of the medium. Inoculated petri plates were incubated at 27°C ± 1°C and the observations were recorded on the seventh day. Percentage of fungal growth inhibition (FGI%) was calculated according to the formula:

$$\text{FGI}\% = \frac{dc - dt}{dc} \times 100$$

where dc = fungal colony diameter in control sets, dt = fungal colony diameter in treatment sets.

2.5. Determination of Minimum Fungistatic/Fungicidal Concentrations

The minimum fungistatic/fungicidal concentrations of the oil at minimum inhibitory concentrations (MICs) were ascertained by the method of Garber and Houston, [25]. This was done by re-inoculating the inhibited fungal discs at MICs on SDA medium. Fungal growth on the seventh day indicated a fungistatic nature, while the absence of fungal growth denoted fungicidal action of the oil.

2.6. Effect of Inoculum Density

The effect of inoculum density (increased progressively up to 30 discs in multiples of five, each of 5 mm diameter) of the test pathogens on MICs of the oil was determined following the procedure of Dikshit and Dixit [26]. The effect of physical factors viz., temperature and expiry of toxicity during storage of the oil, was evaluated according to Shahi, *et al.* [24]. Five lots of 5 ml oil were kept in small vials and exposed to 40°C, 60°C and 80°C

temperatures in an incubator for 1 h. Antidermatophytic activity was then tested at MICs by the poisoned food technique. Expiry of toxicity of the oil was determined by storing the oil at room temperature and testing antidermatophytic activity at MICs at regular intervals of 60 days up to 6 months.

2.7. Determination of Minimum Killing Time

The Minimum Killing Time (MKT) of the oil was determined by the mycelial disc killing technique (MDKT) [24]. Two treatment sets were maintained, one with pure oil and the other with the minimum fungicidal concentrations (MCCs) of the oil. The treatment set using MCCs of the oil was prepared by mixing the required quantity of the oil samples in acetone (5% of the total quantity of the treatment solution) and then adding this to the appropriate quantity of distilled water. Simultaneously, controls were maintained using sterilized water (in place of the oil) and acetone, adding into the distilled water in appropriate quantities. Mycelial discs of 5 mm diameter, cut out from the periphery of 7 days old cultures of the test pathogens, were aseptically placed in the culture tubes of different treatment and control sets. These mycelial discs were taken out of the tubes at different time intervals and washed immediately in the washing solution (containing acetone: sterilized distilled water, 1:2) to remove the treatment solution. These washed mycelial discs were aseptically transferred upside-down to the Sabouraud Dextrose Agar (SDA) medium (pH 5.6) in the petri plates. The same procedure was followed with the control sets. The inoculated petri plates were incubated at $27^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and the observations recorded as an average value of five replicates on the seventh day. The percentage of fungal growth inhibition (FGI %) was calculated according to the formula:

$$\text{FGI}(\%) = \frac{dc - dt}{dc} \times 100$$

The experiments were repeated twice, each containing five replicates, and the data presented here are based on their mean values.

2.8. Patch Test Method for Irritant Activity

Patch test method of Roxburgh and Borrie, [5] was followed to find out the maximum tolerable concentrations (MTCs) for irritant activity of the oil by their topical application. People of both sexes aged 15 - 45 years were selected randomly and a group of 30 individuals of each sex was constituted. Circular areas of 5 cm^2 on upper hairy and lower glabrous surface of the palms and 3 cm^2 of neck region of each individual were first washed with distilled water followed by 70% ethyl alcohol and then allowed to dry for 5 min. Five drops of the graded con-

centrations of testing solution were applied to each individual separately for 3 weeks. The volunteers were not allowed to wash the applied area. Qualitative observations were recorded at intervals of 24 h for 3 weeks.

2.9. Phylogenetic Study of Dermatophytes

To find out why the oil is more effective against certain dermatophytic fungi, phylogenetic relationship of these dermatophytes have been studied including the genera *Trichophyton*, *Microsporum* and *Epidermophyton*, and identified the species using the base pair sequences of ITS1 [27,28] **Figure 1**. The ITS1 sequences of the standard strains used in this study—*Trichophyton mentagrophytes*, accession no. AB011463; *T. rubrum*, AB011453; *T. violaceum*, AB017174; *T. tonsurans*, AB017172; *Microsporum gypseum*, AB017177; *M. canis*, AB017179 and *Epidermophyton floccosum*, AB017181, as reported by the authors [28,29] were aligned by using the Clustal W computer program [30] 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 [31] and the neighbor-joining (NJ) [32] method in the NJPLOT program [33]. Bootstrap [34] analysis with the Clustal W program was performed by taking 1000 random samples from the multiple alignments.

3. Results and Discussion

Dermatophytes causes cosmetic problem and treating them has always been a difficult job as most antifungal synthetic agents are static in nature. Moreover there is increasing development of resistance against them. Workers like Stock [35] emphasized the need to discover powerful and specific antifungal agents on an increasing scale to combat fungal infections. Essential oils exhibiting wide range of antifungal activity, as suggested in the present investigation, may prove useful in the development of cheaper and potent antifungal substances against dermatophytes.

The waste leaves of *Psidium guajava* L. upon hydro-distillation yielded 0.4 % essential oil. The constituents of essential oils from the leaves of *Psidium guajava* Linn on GC/MS analysis showed the presence of sixty compounds. The major components were Caryophyllene—18.81%, Copaene—11.80%, [1aR-(1a α -,4a α -,7a-,7a β -,7ba-)]-decahydro-1,1,7-trimethyl-4-methylene-1H-cyclo-prop[e]azulene—10.27% and Eucalyptol—7.36% [18].

Further studies showed the presence of Guajavolide (2 α -,3 β -,6 β -,23-tetrahydroxyurs-12-en-28,20 β -olide) and Guavenoic acid (2 α -,3 β -,6 β -,23-tetrahydroxyurs-12,20(30)-dien-28-oic acid) along with one known triterpene oleanolic acid as well [36]. The oil was characterized by various physicochemical properties (**Table 1**).

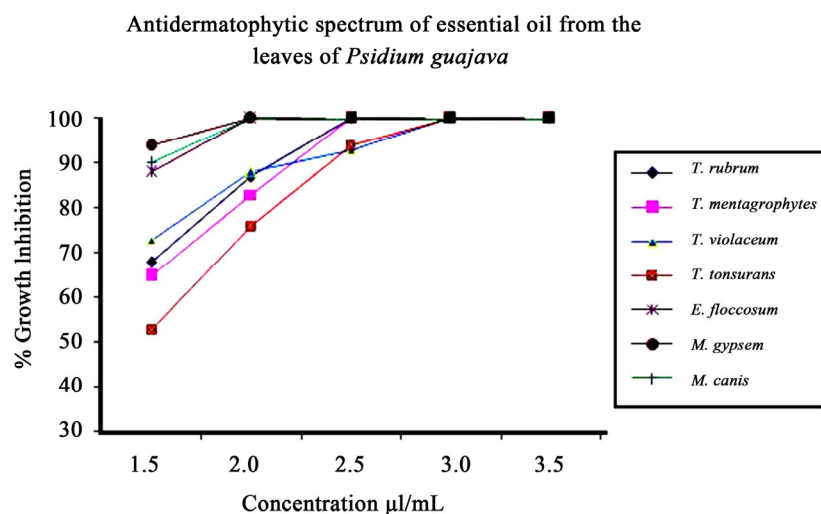


Figure 1. Graphical representation of antidermatophytic spectrum of *Psidium guajava* L. leaf essential oil.

3.1. In-Vitro Studies of Selected Dermatophytes against Waste Leaves' Essential Oil from *Psidium guajava* L.

Many workers in India have studied the antifungal activity of candidate substances employing qualitative assay techniques [37,38] but have not described the nature of the toxic action (fungistatic/fungicidal). In the present work the minimum static and minimum inhibitory concentration was 2.5 µl/ml and 3.0 µl/ml against *Trichophyton rubrum* and *Trichophyton mentagrophytes* respectively.

The oil could withstand heavy doses of inocula up to 30 discs at their respective minimum inhibitory concentration (Table 2). The activity of the oil persisted even up to 6 months of storage and the oil was thermostable up to 80°C. The pure oil killed the test pathogens *Trichophyton rubrum* and *Trichophyton mentagrophytes* in just 30 sec. while at its minimum inhibitory concentration it required 200 min (Table 2).

Work on controlling dermatophytosis using essential oils from various medicinal plants is going on worldwide. Recent works on essential oils e.g. *Ligusticum chuanxiong* essential oil exhibited strong antidermatophytic action against *Trichophyton* species such as, *T. erinacei*, *T. mentagrophytes*, *T. rubrum*, *T. schoenleinii*, *T. tonsurans* and *T. soudanense* [39]. Inouye, *et al.*, [40] also using a wide variety of plant (*Cinnamomum zeylanicum*, *Eugenia aromatica*, *Geranium maculatum*, *Lavandula angustifolia*, *Cymbopogon citratus*, *Origanum vulgare*, *Cymbopogon martinii*, *Mentha piperita*, *Melaleuca alternifolia* and *Thymus vulgaris*) essential oils against *T. mentagrophytes* and *T. rubrum*. *Allium sativum*, *A. cepa* and *A. fistulosum* demonstrated strong antidermatophytic activity against three species of *Trichophyton*. This was confirmed by Pyun and Shin, [41]. At the same time, a

Table 1. Physicochemical properties of essential oil from the leaves of *Psidium guajava* L.

S. No.	Properties studied	Observations
1	Plant height (m)	4 - 5 (approx.)
2	Oil yield (%)	0.4
3	Colour of the oil	Yellowish-green
4	Specific gravity at 26°C	0.9102
5	Refractive index at 23°C	1.487
6	Specific rotation	-13.1 ⁰
7	Ester value	4.7
8	Solubility	Acetone

Table 2. In-vitro antidermatophytic properties of essential oil from waste leaves of *P. guajava* L. against test pathogens *Trichophyton rubrum* and *T. mentagrophytes*.

Properties	Mycelial growth inhibition (MGI-%)			
	<i>Trichophyton rubrum</i>		<i>T. mentagrophytes</i>	
A. Conc. of oil (µl/ml)				
3.5	100 ^c	100 ^c	100 ^c	100 ^c
3.0	100 ^c	100 ^c	100 ^c	100 ^c
2.5	100 ^s	100 ^s	100 ^s	100 ^s
2.0	87	87	83	83
1.5	68	68	65	65
B. Effect of Inoculum density (No. of fungal discs)				
5	100	100	100	100
10	100	100	100	100
15	100	100	100	100
20	100	100	100	100
25	100	100	100	100
C. Minimum killing time				
	<i>Trichophyton rubrum</i>		<i>T. mentagrophytes</i>	
	PO	MCC	PO	MCC
200 Min.	100	100	100	100
100 Min.	100	96	100	91
50 Min.	100	89	100	82
30 Sec.	100	--	100	--

variety of medicinal and aromatic plants of commercial importance are currently facing extinction or severe genetic loss arising from increased demand of their products all over the world, and vastly increasing human population and extensive destruction of plant rich habitats. Therefore these findings are first, where the discarded guava leaves of no commercial value have been utilized to extract oil and their antidermatophytic use, thereby making it economical. The minimum static and minimum inhibitory concentration (MIC) of the oil was found to be 2.0 µl/ml and 2.5 µl/ml against test pathogens *Microsporum gypseum* and *Trichophyton mentagrophytes* respectively (**Table 3**).

Antimicrobial activity of *Leptospermum petersonii*, *Syzygium aromaticum* essential oils against a wide range of dermatophytes such as *Trichophyton mentagrophytes*, *T. rubrum*, *Epidermophyton floccosum* and *Microsporum gypseum* were also noted [42]. Likewise, different fractions, such as hexane, chloroform and essential oil of *Ocimum gratissimum* proved effective against *Microsporum canis*, *M. gypseum*, *Trichophyton rubrum* and *T. mentagrophytes*, *Trichophyton rubrum* [43]. Unlike the oil of *Adenocalymma allicea* where fungitoxicity expired after 21 days [44], fungitoxicity of *Psidium guajava* leaf essential oil persisted for 6 months, maximum time taken into consideration. In the present study, *Psidium guajava* L. leaf essential oil exhibited a broad spectrum antifungal activity against *Trichophyton rubrum*, *T. mentagrophytes*, *Trichophyton violaceum*, *Trichophyton tonsurans*, *Epidermophyton floccosum*, *Microsporum gypseum* and *Microsporum canis* at concentration in the range of 2.5 - 3.5 µl/ml (**Figure 1**).

The oil was compared with some prevalent synthetic antifungal drugs having Miconazole nitrate, Ketoconazole and Tonlaflat. The minimum static concentration of the oil was 2.5 µl/ml as compared to the MSC of these drugs, ranging from 5.0 to 6.0 µl/ml with exception of Tonlaflat (0.4 to 0.8 µl/ml) (**Table 4**).

Table 3. Antidermatophytic spectrum of essential oil from the leaves of *Psidium guajava* L.

Human pathogenic fungi	Mycelial growth inhibition at various concentrations (µl/ml)				
	1.5	2.0	2.5	3.0	3.5
<i>Trichophyton rubrum</i>	68	87	100 ^s	100 ^c	100 ^c
<i>T. mentagrophytes</i>	65	83	100 ^s	100 ^c	100 ^c
<i>Trichophyton violaceum</i>	73	88	93	100 ^s	100 ^c
<i>Trichophyton tonsurans</i>	53	76	94	100 ^s	100 ^c
<i>Epidermophyton floccosum</i>	88	100 ^s	100 ^c	100	100
<i>Microsporum gypseum</i>	94	100 ^s	100 ^c	100	100
<i>Microsporum canis</i>	90	100 ^s	100 ^c	100	100

s = static, c = cidal.

Table 4. Comparative efficacy of guava leaf essential oil with some synthetics.

Oil and trade names of antimycotic drugs	Active principles	Concentrations (µl/ml)			
		<i>T. rubrum</i>		<i>T. mentagrophytes</i>	
		MSC	MCC	MSC	MCC
Guava leaves	Essential oil	2.5	3.0	2.5	3.0
Dactarin	Miconazole nitrate	5.0	—	6.0	—
Nizral	Ketoconazole	5.0	—	5.0	—
Tenaderm	Tonlaflat	0.4	—	0.8	—

Synthetic drugs on one hand may give quick relief but on the other hand are only static in nature, having lots of side effects. Apart from being cidal in nature the essential oil is much cheaper and cost effective as compared to the synthetic formulation since it is obtained from waste leaves of *Psidium guajava* L. leaves. The oil did not show any irritation or adverse effect at 5% concentration up to three weeks.

3.2. Phylogenetic Relationship of Dermatophytes

The phylogenetic relationship of dermatophytic genera *Trichophyton*, *Microsporum* and *Epidermophyton* were demonstrated by using internal transcribed spacer 1 (ITS1) region ribosomal DNA sequences. The ITS1 sequences of the standard strains used in this study [28,29], were aligned by using the Clustal W computer program [30] 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 [31] and the neighbor-joining (NJ) [32], method in the NJPLOT program [33]. Bootstrap [34] analysis with the Clustal W program was performed by taking 1000 random samples from the multiple alignments. The result of phylogenetic relationship of dermatophytic genera was shown in unrooted Neighbour Joining Tree plot on the basis of their ITS1 sequences (**Figure 2**). The ITS1 sequences of 7 standard test strains 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 [30]. The Cladogram (Neighbour Joining Tree plot) of dermatophytes clearly indicate in **Figure 3** that the *Trichophyton mentagrophytes*, *T. rubrum*, *T. violaceum* and *T. tonsurans* belonging to same genetic stock were having close inhibitory concentrations as compared to *Microsporum gypsem*, *M. canis* and *Epidermatophyton floccosum*. Mishra, *et al.*, [45] has also discussed the phylogenetic relationships of the dental caries causing pathogens with respect to the toxicity of leaf essential oil of *Micromeria biflora* Benth.

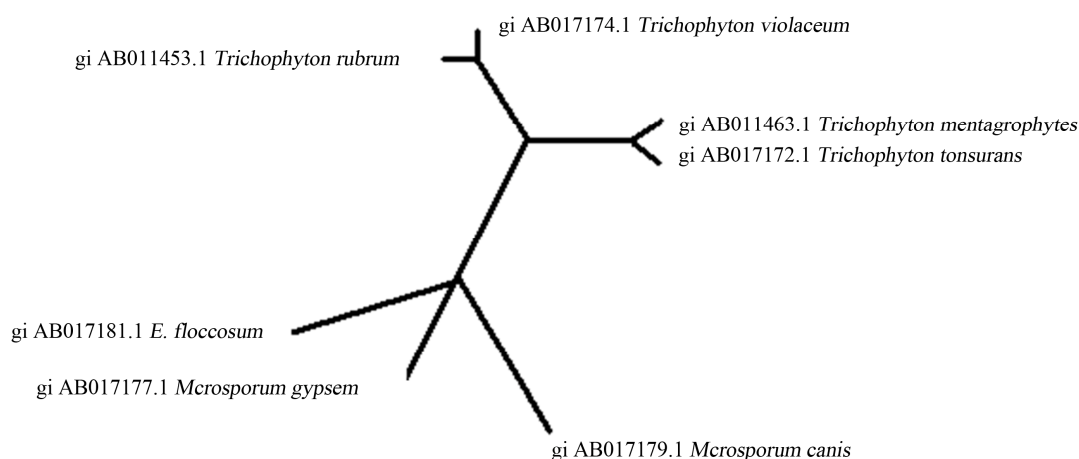


Figure 2. Unrooted Neighbour Joining Tree plot of dermatophytes on the basis of their ITS1 sequences.



Figure 3. Result of cladogram (Neighbour Joining Tree plot) of dermatophytes on the basis of their ITS1 sequences. The NJ tree was constructed with data for standard strains of dermatophytes.

The oil at 2.5 $\mu\text{l/ml}$ showed 100% inhibition against *Microsporum gypsem*, *M. canis* and *Epidermatophyton floccosum* which were closely placed in cladogram while 93% and 94% inhibition in *T. violaceum* and *T. tonsurans* and 100% static inhibition was found in *Trichophyton mentagrophytes*, *T. rubrum* which were clustered in cladogram as well (**Figure 3**). 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.

4. Conclusion

The findings of the present study shows that waste leaves oil of *Psidium guajava* L. is a promising antifungal agent that could be used as a therapeutic remedy against tinea pedis causing human dermatophytic fungi on account of its various *in-vitro* and *in-vivo* antifungal properties, viz., strong fungicidal action, long shelf-life, its tolerability of heavy inoculum density, thermostability, broad range of antidermatophytic activity and absence of any adverse

effects. The effectiveness of waste leaf oil was in accordance with relationship of those dermatophytes which are close in phylogenetic tree. As such, the oil can be used as a potential source of effective and cheap herbal formulation after undergoing double blind successful multicentral topical testing.

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