

Antidermatophytic Activity of Some Medicinal Plants, Phytochemical Analysis and Isolation of Active Fractions from *Piper longum*

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The present study was executed to screen methanolic leaf extracts from 19 plant species for antidermatophytic activity. Based on highest activity, *Piper longum* was further fractionated to identify the fractions of better activity profile. The extracts and fractions were tested against five dermatophyte species by agar well diffusion and agar dilution methods. Extracts from *Aloe vera*, *Azadirachta indica*, *Camelia sinensis*, *Cassia sophera*, *Clitoria ternatea*, *Leucas plukentii*, *Lawsonia inermis*, *Ocimum basillicum*, *O. sanctum*, *O. gratissimum*, *P. betleoides*, *P. longum*, *Solanum melongena* and *Vitex negundo* showed antidermatophytic activity (zone of inhibition: 9-42 mm, MIC: 1.25 - $10 \times 10^4 \mu\text{g mL}^{-1}$). Furthermore bioassay of *P. longum* exhibited better activity of sequentially extracted chloroform and methanol extracts (MIC: $5 \times 10^3 \mu\text{g mL}^{-1}$). Two potential column fractions isolated from the methanol extract of *P. longum* showed MIC of 1.25×10^3 - $2.5 \times 10^3 \mu\text{g mL}^{-1}$ in broth microdilution assay. To the best of our knowledge, there is no scientific report on antidermatophytic activity of *P. longum* so far. The results highlighted future research on these potential plants especially *P. longum* to characterize the active constituents.

Key words: Antidermatophytic activity, Medicinal Plants, Isolation, Active Fractions, *Piper longum*.

The incidence of dermatophyte infections has increased worldwide. Immunocompromised persons have higher risk of such infections^{1,2}. Such infections predominated in Northeast India also³⁻⁵. In most cases, *Trichophyton rubrum* was the commonest followed by *T. mentagrophytes*⁶⁻¹¹. Antifungal drugs, presently used in treatment of such infections have several adverse effects, like toxicity, emergence of resistant strains, cost etc. Consequently, in recent years, research focuses on alternative source antifungals drugs^{12,13}. The search for novel antifungal agents relies in great part on ethnobotanical information and ethnopharmacologic exploration¹⁴⁻¹⁷. The present

investigation was undertaken to evaluate antidermatophytic activity of methanolic extracts from nineteen plant species, collected from the foothills of Assam-Arunachal Pradesh border, an excellent reservoir of medicinal plants, which are not scientifically explored yet. Subsequently the active plant extract was fractionated to purify and isolate the active fractions.

MATERIALS AND METHODS

Plant material and extraction

Fresh leaves of *Aloe vera*, *Alstonia scholaris*, *Azadirachta indica*, *Camelia sinensis*, *Cassia sophera*, *Clitoria ternatea*, *Leucas plukentii*, *Lawsonia inermis*, *Mimosa pudica*, *Ocimum basillicum*, *O. gratissimum*, *O. sanctum*, *Piper betleoides*, *P. brachystachium*, *P. griffitii*, *P. longum*, *P. nigrum*, *Solanum melongena* and *Vitex negundo* were collected during March - April 2010

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and identified on the basis of their morphological characters. The identity of the most active plant was confirmed at Botanical Survey of India, Kolkata, India. The powder of shade dried plant samples (1000 g) was extracted with methanol¹⁸. The extracts were filtered (Whatman filter paper No-1), concentrated at 40°C under reduced pressure using rotary vacuum evaporator (Heidolph Instruments GmbH & Co. KG, Germany) and were finally lyophilized. Further the potential plant, *P. longum* (100 g powdered leaves), was sequentially extracted with petroleum ether, chloroform, methanol and water¹⁹. Solvents were evaporated at 40°C under reduced pressure and then lyophilized. Test samples were prepared in dimethyl sulphoxide (DMSO) and filtered (miliopore filter, 0.22 µm).

Dermatophyte culture

The cultures of dermatophyte species namely *Trichophyton mentagrophytes* (MTCC 8476), *T. rubrum* (MTCC 8477), *T. tonsurans* (MTCC 8475), *Microsporum fulvum* (MTCC 8478) and *M. gypseum* (MTCC 8469) were maintained on sabouraud dextrose agar (SDA, Himedia) slants and sabouraud dextrose broth (SDB, Himedia).

Preliminary evaluation for antidermatophytic activity

The antidermatophytic activity of the crude methanol extracts was tested by agar well diffusion²⁰ and agar dilution method²¹. In agar well diffusion assay, SDA plate (80 mm dia) was swabbed with 150 µl of the inoculum (2.5×10^4 c.f.u. mL⁻¹). A well of 8 mm diameter was made in the agar plate, loaded with 150 µl of the test sample and incubated at 28±2°C for 15-20 days. The activity was determined by measuring the diameter of zone of inhibition caused by the test samples. In agar dilution assay, each test extract was incorporated in sterilized molten medium at concentrations from 10×10^4 to 0.625×10^4 µg mL⁻¹ and inoculated with dermatophytes and incubated at 28±2°C for 15-20 days. Visual observation of the growth of the mycelia was done and the percentage of mycelial inhibition (Inhibition%) was calculated²¹. DMSO as negative control and clotrimazole as standard were used. Each treatment was performed with five replications.

Determination of MIC

The MICs of the active extracts were determined by agar dilution method. The MICs of

the active fractions, isolated from methanol extract of *P. longum* obtained by sequential extraction were determined by broth microdilution method²² described earlier with some modifications. The test sample was diluted in 96-well microtiter plate with RPMI 1640 (Rosewell Park Memorial Institute, Himedia) to obtain a concentration ranging from 39 to 5000 µg mL⁻¹. Inoculum concentration of 2.5×10^4 c.f.u. mL⁻¹ (approx.) was adjusted in each well and incubated at 28±2°C for 15-20 days. The MIC was interpreted as the lowest concentration of the test samples that inhibited visible mycelial growth. Each experiment was performed in triplicate and repeated twice.

Determination of longevity of the active extracts

The longevity of the methanol extract from *P. longum* leaf derived from sequential extraction was recorded by storing the extract at 4°C and at room temperature (25-34°C) for 180 days. The efficacy of the extract (0.5×10^4 µg mL⁻¹) was tested at one month interval by determining the radial growth of *T. mentagrophytes*.

Phytochemical analysis

Methanol extract of *P. longum* leaf was analyzed for the presence of alkaloids, phenolic compounds, tannins and saponins. Test solution of 50 mg/mL was prepared in acetone. For alkaloids, 1 mL test solution was treated with a few drops of Dragendorfs reagent and Mayer's reagent separately²³. For phenolic compounds, 1 mL test solution was treated with 1% ethanolic ferric chloride²⁴. The test for tannins was carried out with 0.1% FeCl₃²⁵. For saponins, Test solution (1 mL) was mixed with 5 mL water and shaken to observe stable froth, further mixed with olive oil (3 drops) and shaken to observe formation of emulsion²³.

Isolation of active fractions

Methanol extract (1g) was eluted through a column of silica gel (100 g, 60-120 mesh, column size: 300 × 40 mm) using mobile phases, hexane-chloroform and hexane-ethyl acetate-methanol separately as a gradient system with 10% increase in their combinations in each subsequent step. The fractions with similar TLC profiles were combined and concentrated to dryness at 40°C under reduced pressure. Each fraction was evaluated against *T. mentagrophytes* and *T. rubrum* by agar well diffusion assay. The MICs of the active fractions were determined by broth microdilution method.

RESULTS AND DISCUSSION

Recently scientific studies focus on the effect of medicinal plants, either crude form or their active components on pathogenic microorganisms. Since the probability of detecting antifungal plants was more among the traditional medicinal plants, than the randomly selected plants¹³, the plants were chosen based on ethno pharmacological importance. In traditional medicines, water extract is mostly used, but organic solvents are better to extract maximum bioactive compounds. Therefore, for the preliminary evaluation, methanol extracts were used. In our preliminary evaluation, the methanol extracts from *A. vera*, *A. indica*, *C. sinensis*, *C. sophera*, *C. ternatea*, *L. inermis*, *L. plukentii*, *M. pudica*, *O. basilicum*, *O. gratissimum*, *O. sanctum*, *P. betleoides*, *P. longum*, *S. melongena* and *V. negundo* exhibited broad spectrum activity against all dermatophytes (zone of inhibition: 9-42 mm) (Fig. 1). In agar dilution assay, except *M. pudica*, the remaining 14 extracts caused complete inhibition of the mycelial growth at concentration range from 10×10^4 to 1.25×10^4 $\mu\text{g mL}^{-1}$. Extract from *P. longum* showed the highest activity (zone of inhibition: 37-42 mm, MIC- 1.25×10^4 $\mu\text{g mL}^{-1}$) (Fig. 1 and 2). In many ayurvedic formulations, *P. longum* is one of the essential ingredients and its different parts were reported as medicinal²⁶. But there is no scientific report on antidermatophytic activity of *P. longum* leaves. Activity of clotrimazole at very low concentration

(zone of inhibition 8-20 mm at 1×10^2 $\mu\text{g mL}^{-1}$, MIC- 0.313×10^3 $\mu\text{g mL}^{-1}$), compared to the plant extracts may be attributed to its pure nature. Conversely, higher MICs of the crude extracts might be due to presence of both active and non active compounds in crude form.

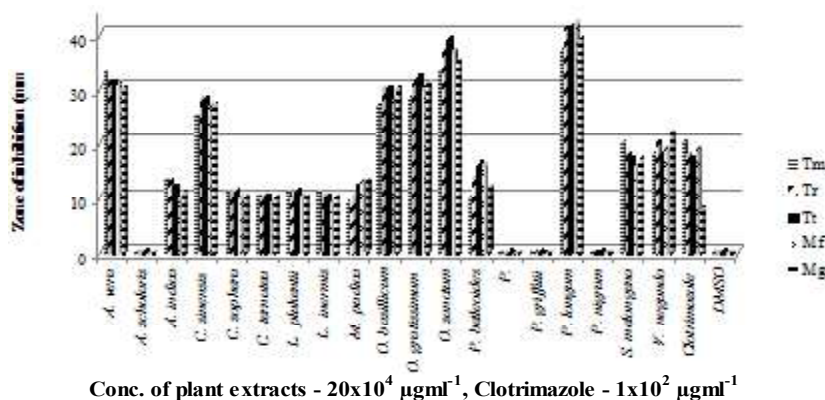
The plants found active in this study, have been used traditionally in various ailments including skin diseases. For example, *C. sophera* (seeds), *L. aspera* (Synonym- *L. plukentii*) (leaves) and *O. sanctum* were used in treatment of skin diseases in veterinary practices²⁷. Traditional uses of *A. indica*, *C. ternatea*, *L. plukentii*, *O. sanctum*, *S. melongena* and *V. negundo* were reported^{28, 29}. Essential oils and extracts of different *Ocimum* species were reported as antidermatophytic^{30, 31}. Antifungal activity was reported for *A. indica* and *S. melongena*^{19,32}. Various pharmacological effects of *Camelia sinensis*³³, *A. cathartica*^{34, 35} and *Vitex negundo*³⁶ were observed. The indigenous people of northeast India use many *Piper* species in traditional medicines³⁷. Despite the vast information of these traditional medicinal plants, their antidermatophytic activity has not been explored so far.

The most promising plant, *P. longum* (leaf) was subjected to sequential extraction to separate the fractions with better activity profile. The polarity of the solvent played an important role in the extraction of active components as shown by the higher yields, obtained in water (13.0%), followed by methanol (4.5%), chloroform (3.2%)

Table. 1. Antidermatophytic activity of fractions isolated from *P. longum* methanol extract

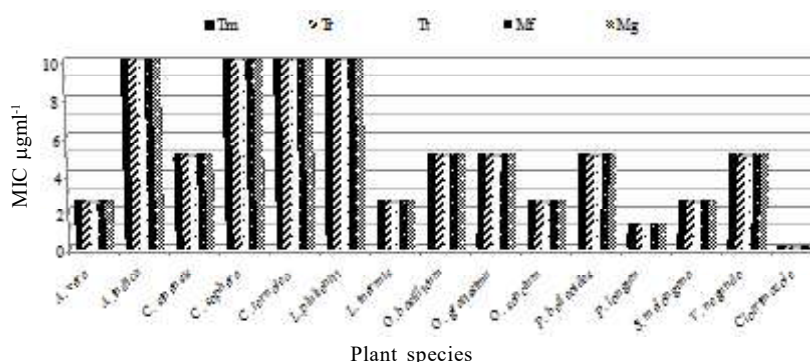
Mobile phase	Proportion	Fraction (5×10^3 $\mu\text{g mL}^{-1}$) [Extractive value (%)]	Zone of inhibition (mm)	
			<i>T. mentagrophytes</i>	<i>T. rubrum</i>
1	Hex : Chl (3:2)	F ₁ (15)	13	13
	Hex : Chl (1:1)	F ₂ (8)	13	13
	Hex : Chl (2:3)	F ₃ (2)	41	41
	MIC of Active fraction (F ₃): 1.25×10^3 $\mu\text{g mL}^{-1}$			
2	Hex - Hex:EA (1:0 - 4:1)	F ₁ (22)	20	16
	Hex:EA (7:3 - 3:2)	F ₂ (16)	26	27
	Hex:EA (1:1 - 3:7)	F ₃ (18)	13	13
	Hex:EA - EA (1:4 - 0:1)	F ₄ (10)	10	10
	EA:MeOH (9:1 - 4:1)	F ₅ (10)	10	12
	EA:MeOH (7:3 - 2:3)	F ₆ (12)	12	10
	MIC of Active fraction (F ₂): 2.5×10^3 $\mu\text{g mL}^{-1}$			

F: Fraction, Hex-Hexane, EA-Ethyl acetate, MeOH-Methanol, Chl-Chloroform



Tm- *T. mentagrophytes*, Tr- *T. rubrum*, Tt- *T. tonsurans*, Mf- *M. fulvum*, Mg- *M. gypseum*

Fig. 1. Zone of inhibition produced by methanol extracts from different plants against dermatophytes



Tm- *T. mentagrophytes*, Tr- *T. rubrum*, Tt- *T. tonsurans*, Mf- *M. fulvum*, Mg- *M. gypseum*

Fig. 2. MIC of different plant extracts against dermatophytes

and petroleum ether (1.8%). Among all solvent extracts, the chloroform and methanol extracts revealed better activity. Differential activity among different extracts from *P. longum* might be due to either difference in plant constituents in each extract or tolerance of the pathogens to different plant constituents. Similar observations were made by other workers^{16, 38, 39}. Owing higher yield, the methanol extract was tested for MIC and found to be $5 \times 10^3 \mu\text{g mL}^{-1}$ against all the test dermatophytes.

Methanol extract was found to be soluble in DMSO, water and methanol. The extract retained its activity upto 150 days at 4°C and 90 days at room temperature (25-34°C). The findings would be useful during experiments for bioactivity. Tannins, phenolic compounds and saponins were detected in the methanol extract.

Further fractionation and purification of

the sequentially extracted methanol extract of *P. longum* leaf, using silica gel column chromatography yielded more active fractions. Among three major fractions, eluted with hexane-chloroform, fraction-3 (F_3) eluted with hexane-chloroform (2:3) exhibited the highest activity (zone of inhibition: 41 mm against *T. mentagrophytes* and *T. rubrum* and MIC: $1.25 \times 10^3 \text{ g mL}^{-1}$ (Table 1). Another six fractions (F_1 - F_6) were eluted with hexane-ethyl acetate-methanol. Among these, fraction-2 (F_2), eluted with hexane and ethyl acetate combination (7:3 - 3:2) displayed maximum zone of inhibition (*T. mentagrophytes* - 26 mm and *T. rubrum* - 27 mm, MIC: $2.5 \times 10^3 \text{ g mL}^{-1}$) (Table 1). Higher antidermatophytic activity, observed in isolated fractions could be due to undesirable compounds present in the crude extract that got removed during fractionation. Fractionation leading to isolation of active compounds should result higher activity

than the original extract. This approach forms the basis of discovery of bioactive compounds from the naturally occurring sources and has led to discovery of many important drugs⁴⁰.

The results of the present study evidenced promising antidermatophytic activity of 15 tested plants. The highest activity of *P. longum* highlighted the possibility of finding novel compounds and hence it should be targeted for isolation and characterization of the active compounds for use as antifungal particularly in treatment of dermatophytes infections.

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