

Bioassay-guided isolation of antidermatophytic compounds from *Piper longum* L.

ABSTRACT

Aims: *Piper longum*, a medicinal plant, grows wild in Northeast India. In our earlier study, the chloroform extract from leaves of *P. longum* L. (Piperaceae) exhibited promising *in vitro* antidermatophytic activity. The present investigation was undertaken to isolate and identify the antidermatophytic compounds present in *P. longum* (leaf).

Study Design: Air dried powdered leaves (100 g) of *P. longum* were extracted with petroleum ether. After removal of the petroleum ether soluble part, the residue left over was extracted with chloroform. The solvent was evaporated under reduced pressure at 40 °C using rotary evaporator, lyophilized and kept in glass vials till further use. Chloroform extract was subjected to bioassay-guided fractionation using column chromatography. Antidermatophytic activity of the isolated fractions was determined by broth microdilution assay. Chemical compounds were identified by GC-MS analysis.

Place and Duration of Study: Defence Research Laboratory, Tezpur, Assam, India and Department of Botany, Gauhati University, Guwahati, Assam, between Jan 2011 and Oct 2011.

Results: Repeated column chromatography afforded a yellowish semi solid mass. The fraction showed significant antidermatophytic activity with minimum inhibitory concentration (MIC) values of 1.25 mg mL⁻¹ for *T. mentagrophytes* (MTCC 8476) and 2.5 mg mL⁻¹ for *T. rubrum* (MTCC 8477). GC-MS analysis of the fraction revealed the presence of nine compounds namely Benzene (2-methyl-1-propenyl) (5.19%), 2,4-Bis (1,1-dimethylethyl)-phenol (8.11%), 1b-Bisabolene (12.48%), 2,8-decadiyne (13.79%), Methyl 14-methylpentadecanoate (15.41%), methyl linolenate (25.52%), octadecanoic acid, methyl ester (5.31%), 11-dodecen-1-ol (7.63%) and dicyclohexyl phthalate (6.54%).

Conclusion: The results highlighted the antidermatophytic potential of *P. longum*. Further investigation should be focused on the active constituents that could be used for development of antidermatophytic agent.

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Keywords: *Piper longum*, Bioassay-guided isolation, Antidermatophytic compound

1. INTRODUCTION

Superficial mycoses are the most frequent forms of infections predominantly caused by a group of closely related keratinophilic fungi, known as dermatophytes. The dermatophytic infections affect approximately 40% of the world's population. Among all mycotic skin infections dermatophytic infections represent 30% [1-5]. Predominance of dermatophyte species in Northeastern part of India has also been reported by many authors [6-9]. Effective treatment of these infections is difficult, as majority of the antifungal drugs have several drawbacks like emergence of resistance, toxicity, high cost etc. This necessitates to identifying and developing novel, safe and effective antifungal agents from alternative sources. Plants are the most important resources on the earth, which are of critical importance for health security of the

present and future generations. Effect of medicinal plants, either in the form of crude extracts or their active components, on pathogenic microorganisms has been studied for a long time. Greater importance is being attached to the ethno medicinal uses besides documented reports on medicinal plants [10-18]. The Northeast India, a part of the Indo-Burma biodiversity hotspot, is one of the two centres of species diversity of Indian *Piper* [10, 19]. *Piper* species are of great interest owing to their variety of biological properties and a number of bioactive molecules have been isolated from it [20-24]. The local people use different *Piper* species in various ailments [10]. Among these, *P. longum* a widely grown medicinal plant of this region is used in traditional as well as Ayurvedic system of medicine. *P. longum* is a flowering vine in the family Piperaceae cultivated for its fruit. Primary constituents isolated from various parts of *P. longum* are piperine, piperlongumine, sylvatin, sesamin, diaeudesmin piperlonguminine, pipermonaline, and piperundecalidine. It is most commonly used to treat chronic bronchitis, asthma, constipation, gonorrhea, paralysis of the tongue, diarrhea, cholera, chronic malaria, viral hepatitis, respiratory infections, stomachache, bronchitis, diseases of the spleen, cough, and tumors [25]. Medicinal uses of *P. longum* essential oils, roots, fruits and whole plants were reported earlier [18]. Prasad et. al., (2005) isolated several compounds from *Piper* species and studied the potentiality of chloroform extract and the pure compounds isolated from it for anti-inflammatory activity. From the body of literature it has been observed that majority of the compounds were isolated from the fruits and other parts of *P. longum*. We reported the antidermatophytic activity of *P. longum* leaves and subsequently three major compounds were detected in the chloroform extract [26]. However, apart from this, we have not come across any information regarding antidermatophytic compound from leaves of *P. longum*. Biological screening followed by activity-guided fractionation has been used successfully for the discovery of antifungal, antibacterial, antimalarial bioactive compounds [27]. This approach should be the basis of drug discovery of bioactive compounds from natural sources.

Encouraging with the findings of the previous works, the present investigation was undertaken to identify the antidermatophytic compounds present in the chloroform extract of *P. longum* leaf through activity guided fractionation and subsequently by GCMS analysis. The active compound present in the chloroform extract of *P. longum* leaves might be useful as lead molecule in designing novel, efficient and safe drug for treatment of dermatophytic infections.

2. MATERIAL AND METHODS

2.1 Dermatophyte culture

The stock cultures of dermatophyte species namely *Trichophyton mentagrophytes* (MTCC 8476) and *T. rubrum* (MTCC 8477) were maintained at 4°C on sabouraud dextrose agar (SDA, Himedia) slants. Fresh culture for experiments were prepared by transferring a loopful of stock culture to 50 mL of sabouraud dextrose broth (SDB, Himedia) and incubated at 28±2 °C.

2.2 Plant material

Fresh leaves of *P. longum* were collected from the foothills of the Himalayan range in Assam-Arunachal Pradesh border in May 2010. The plant was authenticated at Botanical Survey of India, Kolkata, India and deposited a voucher specimen at Gauhati University, Guwahati, Assam, India.

2.3 Extraction

The leaves were shade dried and crushed into coarse powder. The powdered sample (100g) was extracted sequentially with petroleum ether followed by chloroform following the protocol as shown in figure1 [28]. Each of the solvent extracts was filtered using whatman filter paper (No-1) and the solvents were evaporated under reduced pressure at 40°C and finally lyophilized to get the extracts. The extracts were kept in air tight glass bottles at -20°C till further use.

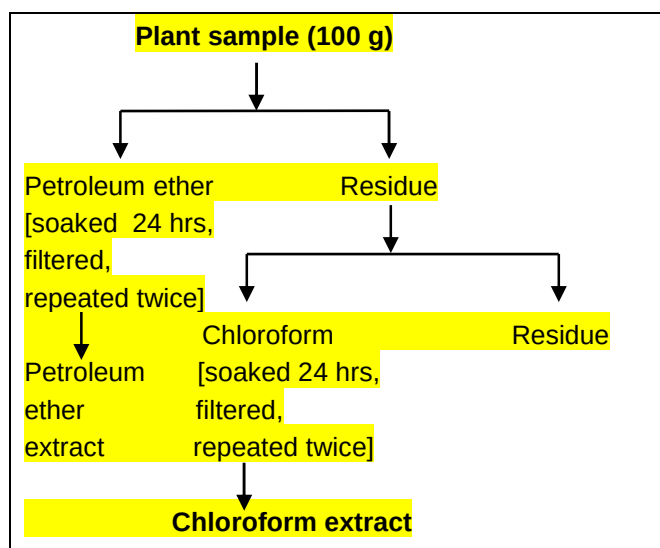


Figure 1. Sequential extraction of *Piper longum*

2.4 Fractionation of chloroform extract

The chloroform extract was fractionated into semi purified fractions through silica gel column chromatography. The extract (1.25 g) was dissolved in small amount of chloroform and adsorbed on pre activated silica gel (12.5 g) and loaded into glass column (400 x 40 mm), packed with pre activated silica gel (230-400 mesh, 125 g), slurried in petroleum ether. The fractions were eluted stepwise by gradient of petroleum ether, ethyl acetate and methanol (40%) with polarity increase of 10% in each step. Forty five fractions were collected and the fractions with similar TLC profile (Silica gel F₂₅₄ aluminium backed sheet, Merck) were pooled into nine major fractions (F₁ - F₉). The first fraction (F₁) was found to be active in our previous study (25) and hence this fraction was subjected to rechromatography using the mobile phase mentioned as above and isolated an active fraction of yellow coloured oily mass (F₁-SF₂) [23, 29].

2.5 Determination of minimum inhibitory concentration (MIC)

The MIC of the fraction (F₁-SF₂) was determined by broth microdilution assay as described earlier by Hammer et al., 2002 (30) with some modifications. Twofold serial dilutions (0.039 to 5 mg mL⁻¹) of the fraction were prepared in DMSO in 96-well microtiter plate with RPMI 1640 medium (Rosewell Park Memorial Institute, Himedia). Colony of the test organisms, *T. mentagrophytes* (MTCC 8476) and *T. rubrum* (MTCC 8477) were separately cultured on SDB. Inoculum concentration of approximately 2.5x10⁴ c.f.u. mL⁻¹ was adjusted in each well. The plates were incubated at 28 ±2°C for 15-20 days. Each experiment was performed in triplicate and repeated twice. The MIC was interpreted as the lowest concentration of the test sample showing no visible growth.

2.6 Gas chromatography mass spectroscopy (GC-MS) analysis of the fraction (F₁-SF₂)

The isolated fraction (F₁-SF₂) was analyzed by GC-MS to determine the active compounds present in the fraction. The sample was treated with 2% sulphuric acid in methanol (5 mL) for 2 hr at 55°C. The progress of methanolysis was monitored by TLC using the solvent system of hexane: ethyl acetate (9:1, v/v). The resultant fatty acid methyl ester was extracted with hexane, washed with water until neutral, dried over anhydrous Na₂SO₄ to get fatty acid methyl esters. GC-MS was performed on Agilent 6890 GS/5973MS apparatus using an HP-1 MS capillary column (I.D 0.25 mm, length 30 m and film thickness 0.25 µm) with an initial temperature of 80°C for 2 min and then temperature programming to 300°C at t he

rate of 10°C/min (Agilent Tech., USA). The compounds were characterized by comparing GC-MS data with NIST mass spectral library.

3. RESULTS AND DISCUSSION

Among the nine major fractions, obtained by first step column chromatography from the chloroform extract, four fractions namely F1, F4, F5 and F7 were observed to be highly active against *T. mentagrophytes*, which we reported earlier [26]. In this study, the first fraction (F1, 359 mg) was further purified by silica gel column chromatography to obtain a yellowish semi solid mass (F1-SF2) (11mg). MIC values of this fraction (F1-SF2) were recorded as 1.25 mg mL⁻¹ for *T. mentagrophytes* and 2.5 mg mL⁻¹ for *T. rubrum*. This isolated fraction was found to be more active against dermatophytes compared to activity of different solvent extracts from *P. longum* (leaf), which was reported by us earlier [25]. This may be mainly because the active compounds were concentrated in the fraction. This fraction was found to be soluble in hexane, ethyl acetate and chloroform but insoluble in methanol and water. The GC-MS analysis of the active fraction (F1-SF2) showed the presence of nine compounds (Table 1). It can be hypothesized that the activity of the fraction might be related to the presence of the major compounds, although it is difficult to attribute the activity of a complex mixture to a particular component. However, in most cases, the inhibitory effect has been attributed to the major compounds [31]. Some of the compounds detected in *P. longum* in the present study, were also isolated previously from other medicinal plants and their antimicrobial activity was reported. For example, the antibacterial activity of phenol, carboxylic acid and ester was observed earlier. The oils, containing phenol as a major constituent, possessed the highest activity against *T. mentagrophytes* [32-33]. Methyl 14- methylpentadecanoate was first reported from ethanolic extracts of *Azadirachta indica* pericarp [34]. Presence of Octadecanoic acid, methyl ester in *Plumbago zeylanica* (roots) and *Ocimum basilicum* (leaves) was reported earlier [35-36].

From different parts of *P. longum*, a large number of bioactive compounds have been isolated using silica gel column chromatography in the past [22-24, 37-38]. A piperidine alkaloid, piperine was isolated from the ethanol extract of *P. longum* fruits, which possessed antidepressant-like activity [23]. Another fungicidal compound, pipernonaline, a piperidine alkaloid, was isolated from the hexane fraction of *P. longum* fruits [38]. Pipernonaline, isolated from the hexane fraction of the methanol extract of *P. longum* fruit, was also found to be mosquito larvicidal in nature [22]. Three biologically active compounds namely (+)-Asarinin, Guineensine and Retrofractamide A were isolated from petrol extract of air-dried stems and leaves of *P. longum* [37]. It has been observed that the fruits and other parts of *P. longum* have been studied extensively. Earlier we reported the presence of three major compounds, namely, 2-benzenedicarboxylic acid, bis (2-ethylhexyl) ester, 2,2-dimethoxybutane and b-myrcene in the chloroform extract from leaf of *P. longum* [26]. In the present study, we identified nine compounds in an antidermatophytic fraction separated out from chloroform extract of *P. longum* leaves. Among the compound identified, dicyclohexyl phthalate may be impurity from solvent because phthalate is used as stabilizer. It can be inferred that the antidermatophytic effect of the fractions might be due to single compound or due to synergistic effect of the two or more compounds present in it. However in most cases, the inhibitory effect of the compound has been attributed to the most dominant components and not to the other minor compounds [39]. The results of our present work on the establishment of the efficacy of the fraction or the identified compounds seem to be the first report of this kind.

Table 1. Compounds identified in esterified fraction (F₁-SF₂) of *P. longum* leaf extract

RT	Area (%)	Compounds	Molecular formula
10.94	5.19	Benzene, (2-methyl-1-propenyl)	C ₁₀ H ₁₂
11.18	8.11	2,4-Bis (1,1-dimethylethyl)-phenol	C ₁₄ H ₂₂ O

11.33	12.48	lb-Bisabolene	C ₁₅ H ₂₄
12.18	13.79	2,8-Decadiyne	C ₁₀ H ₁₄
15.81	15.41	Methyl 14-methylpentadecanoate	C ₁₇ H ₃₄ O ₂
17.41	25.52	Methyl linolenate	C ₁₉ H ₃₂ O ₂
17.72	5.31	Octadecanoic acid, methyl ester	C ₁₉ H ₃₈ O ₂
18.55	7.63	11-Dodecen-1-ol	C ₁₂ H ₂₄ O
21.07	6.54	Dicyclohexyl phthalate	C ₂₀ H ₂₆ O ₄

RT: Retention time

4. CONCLUSION

The antidermatophytic potential of the compounds present in *P. longum* leaves is encouraging. Further isolation and characterization of the active compound (s) is under investigation, which would be useful as lead molecule in developing novel, efficient and safe product for treatment of dermatophytic infections.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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