

Original Research Article

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

ABSTRACT

Aims: *Piper longum*, a medicinal plant, grows wildly 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 and evaporated the solvent. 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.

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

1. INTRODUCTION

The incidence of dermatophytic infections is most common, affecting approximately 40% of the world's population and represents 30% of all mycotic skin infections [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 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 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]. *P. longum*, a wild

grown medicinal plant of this region is essential ingredient in many ayurvedic formulations. Medicinal uses of *P. longum* essential oils, roots, fruits and whole plants were reported earlier [18]. In our earlier study, chloroform and methanol extracts from *P. longum* leaves displayed promising antidermatophytic activity [25]. The present study is an attempt to determine the possible active antidermatophytic compounds of the chloroform extract of *P. longum* leaves.

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2. MATERIAL AND METHODS

2.1 Dermatophyte culture

Trichophyton mentagrophytes (MTCC 8476) and *T. rubrum* (MTCC 8477) were maintained on sabouraud dextrose agar (SDA) and sabouraud dextrose broth (SDB).

2.2 Plant material

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 a voucher specimen deposited at Gauhati University, Guwahati, Assam, India.

2.3 Extraction

The leaves were shade dried, crushed into coarse powder and extracted with petroleum ether followed by chloroform as described earlier [26]. After filtration the solvent was evaporated under reduced pressure using rotary evaporator at 40°C and lyophilized and kept in air tight glass bottles at -20°C till further use.

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 little amount of chloroform and adsorbed on pre activated silica gel (12.5 g) and applied 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 was rechromatographed using the mobile phase mentioned as above and isolated an active fraction of yellow coloured oily mass (F₁-SF₂) [23, 27].

2.5 Determination of minimum inhibitory concentration (MIC)

The MIC of the fraction (F₁-SF₂) was determined by broth microdilution assay as described by earlier with some modifications [28]. 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 (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 samples 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,

Comment [y1]: What informed the choice of F1 for rechromatographing? For instance, did you subject the nine fractions to bioassay and F1 gave the highest activity? Or was it just a random choice?

Comment [y2]: There is something missing here.

v/v). The resultant fatty acid methyl ester is extracted with hexane, washed with water until neutral, dried over anhydrous Na₂SO₄ to get fatty acid methyl esters. GC-MS was performed on an 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 the 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 [25]. 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). 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*. The fraction isolated here 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 is 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 [29]. 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* [30-31]. Methyl 14-methylpentadecanoate was first reported from ethanolic extracts of *Azadirachta indica* pericarp [32]. Presence of Octadecanoic acid, methyl ester in *Plumbago zeylanica* (roots) and *Ocimum basilicum* (leaves) was reported earlier [33-34]. A large number of bioactive compounds have been isolated from different parts of *P. longum* [22-24, 35-36]. We reported earlier the presence of three major compounds, such as 2-benzenedicarboxylic acid, bis (2-ethylhexyl) ester, 2,2-dimethoxybutane and b-myrcene in an antidermatophytic fraction separated from *P. longum* chloroform extract [25].

Comment [y3]: What is the weight of this sub-fraction?

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

Comment [y4]: From Section 2.6 it can be deduced that these compounds may be derived products from the methylation of the parent Fraction (F-SF₂). This should be noted on the table.

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	1b-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 C₂₀H₂₆O₄

RT: Retention time

Comment [y5]: This may likely be an impurity as it has been found that phthalates are used in solvent packaging.

4. CONCLUSION

From the results it could be inferred that the antidermatophytic activity might be due to the major components of the fraction, isolated from chloroform extract of *P. longum* leaves. However in this study, these compounds in combined form, was tested and found effective against dermatophytes. Future study should be focused on isolation and characterization of the active compounds and subsequent efficacy and safety studies to determine the viable prospects of the compounds for development into antidermatophytic agents.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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