Antidermatophytic Activity of *Mikania micrantha* Kunth: An Invasive Weed

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ABSTRACT

Context: The incidence of dermatophytosis has risen dramatically in recent years. Limited availability of side-effect free drugs has led to a search for new antidermatophytic agents. **Objective:** The objective was to investigate antidermatophytic activity and *in vitro* anti-inflammatory activity (protease inhibition assay) of whole plant (aerial parts only) of *Mikania micrantha.* **Materials and Methods:** The dried and powdered aerial parts of *M. micrantha* were extracted separately with petroleum ether, ethyl acetate and methanol. Antidermatophytic activity was determined by agar tube dilution method against *Epidermophyton floccosum* var. nigricans, *Microsporum canis, Microsporum gypseum* and *Trichophyton rubrum*. The activities of various parts of the plant – flowers, leaves and stem were separately analyzed using their ethyl acetate extract. Fungicidal efficacy and trypsin inhibiting activity of the whole plant, flowers and leaves were also analyzed using the ethyl acetate extracts. **Statistical Analysis Used:** For trypsin inhibition assay results are expressed as mean ± standard division. For antidermatophytic assay, the significance of the difference between control and test was analyzed statistically using Fisher's exact test. **Results:** Ethyl acetate extract of *M. micrantha* exhibited excellent antidermatophytic activity, followed by petroleum ether and methanolic extracts. Ethyl acetate extracts of whole plant, flowers, leaves and stem completely inhibited the growth of dermatophytes at the tested concentration of 2 mg/mL. Furthermore, ethyl acetate extracts of whole plant, leaves and flowers were fungicidal, and the percentages of trypsin inhibition exhibited were 33.73 ± 0.306, 39.0 ± 0.505 and 35.53 ± 0.503, respectively. **Conclusions:** Since *M. micrantha* possesses antidermatophytic as well as anti-inflammatory activities, the plant is an excellent candidate for the development of new medicaments against dermatophytoses in traditional as well as modern medicine.

Key words: Anti-inflammatory action, *Epidermophyton, Microsporum, Trichophyton,* Trypsin inhibiting assay

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INTRODUCTION

The incidence of dermatophytoses has dramatically increased during the past several years due to an increase in the number of patients with impaired immunity such as organ transplant recipients, cancer patients, AIDS patients, etc., When compared with antibiotics, the spectrum of antifungal agents is narrow and most of the currently used antifungals have drawbacks like lack of fungicidal efficacy and various side effects such as hepatotoxicity, gastrointestinal disturbances, anemia, etc., Plants have their own mechanisms, especially production of antifungal secondary metabolites to protect against fungal pathogens and are used traditionally for the treatment of mycoses. Hence, plants appear as an important source for the discovery of new antifungal agents.[1]

Mikania micrantha is a tropical vigorously growing perennial creeper, which belongs to the family *Asteraceae*. It is native to the tropical zones of Central and South America but is now widely distributed in India, Southeast Asia, Pacific Islands, South China, etc.[2] Though the plant is used in traditional medicine in many areas of the world, not much study has been conducted on its medicinal properties. In India, people are not aware of its medicinal properties and the plant is

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considered as a weed. However in recent years, some studies on the pharmacological properties of *M. micrantha* have been reported. The plant has been reported to possess anti-inflammatory activity (Perez-Amador *et al*., 2010[2]), antibacterial activity (Perez-Amador *et al*., 2010[2], Hajra *et al*., 2010[3], Haisya *et al*., 2013[4]), antistress activity (Ittiyavirah[5] and ajid, 2013), trypanocidal activity (Laurella[6] *et al*., 2012), antiviral activity (Laurella *et al*., 2012[6]), inhibitory effect against plant pathogens (Li *et al*., 2013[7]) and antispasmodic effect (Colares *et al*., 2013[8]).

This paper reports antidermatophytic activity of the plant *M. micrantha,* which was selected from a screening study conducted among seven local plants in Kerala for antidermatophytic activity. This study also reports trypsin inhibiting activity of *M. micrantha,* which indicates its possible anti-inflammatory effect.

MATERIALS AND METHODS

Plant materials

Hygrophila schulli (whole plant), *Mucuna bracteata* (leaves), *Cyclea peltata* (whole plant), *Ficus exasperata* (leaves), *Chromolaena odorata* (flowers), *Pongamia pinnata* (bark), and *M. micrantha* (aerial parts) were collected from their natural habitat between September and December 2012 from the district of Kottayam, Kerala state, India. Plant materials were washed thoroughly in running water, shade dried at room temperature and powdered in a kitchen blender.

The selected plant *M. micrantha* Kunth was authenticated by a plant taxonomist, and a voucher specimen has been maintained at the institute.

Solvent extraction

Initial screenings of plants for possible antimicrobial activities typically begin using crude alcohol or aqueous extractions.[9] Hence, each of the powdered plant materials (25 g) was extracted separately with methanol at room temperature in an orbital shaker for 7 days. The extracts were filtered using Whatman number 1 filter paper. The filtrates were evaporated to dryness under reduced pressure using a rotary evaporator, and the dried extracts were kept in sterile bottles at 4°C in a refrigerator until use. For a detailed study of *M. micrantha* petroleum ether (a nonpolar solvent) and ethyl acetate (a mid-polar solvent) extracts were also prepared in addition to methanol, which is a polar solvent.

In order to study and compare the activities of different parts of *M. micrantha*-leaves, flowers (inflorescence with seeds) and stem-the shade-dried plant parts were powdered and extracted (25 g) separately with ethyl acetate (which was found to be the best solvent).

Fungal strains

Epidermophyton floccosum var. nigricans (MTCC NO-613), *Microsporum gypseum* (MTCC NO-2819), *Microsporum canis* (MTCC NO-2820) and *Trichophyton rubrum* (MTCC NO-296) were used as test fungi. Cultures of *M. gypseum* and *M. canis* were maintained in Sabouraud dextrose agar and *E. floccosum* var. nigricans and *T. rubrum* in Emmons modification of Sabouraud dextrose agar (Himedia MO 33).

The purity of standard cultures was assured by examining the morphology of macroconidia and shape and disposition of microconidia by lactophenol cotton blue staining as well as by macroscopic appearance and pigmentation of the colony on Sabouraud dextrose agar.[10]

Antidermatophytic assay

Antidermatophytic activity of the extracts was tested by agar dilution method using Sabouraud dextrose agar. Extracts were dissolved in Dimethyl sulfoxide (DMSO) and incorporated into Sabouraud dextrose agar slants so that the final concentration of DMSO was 1% in all cases. In screening study, 5 mg of various dried methanolic extracts were incorporated per milliliter of medium. For further studies on *M. micrantha,* ethyl acetate and methanolic extracts were incorporated into Sabouraud dextrose agar slants at a concentration of 2 mg dry weight of extract/mL of medium. Due to low solubility, petroleum ether extract was incorporated at a concentration of 0.5 mg/mL of medium. Tubes containing medium with 1% (v/v) DMSO were included as controls. All slants were spot inoculated with the fungi, incubated at 30°C and the inhibition for growth was visually graded after 14 days. In order to check whether all plant parts (leaves, flowers and stem) exhibit the activity, their ethyl acetate extracts were tested at a concentration of 2 mg/mL of medium.

For comparing the activities of flowers and leaves, minimum dry weight of ethyl acetate extract of flowers and leaves, which inhibited the growth of dermatophytes was determined. The tested concentration range was 0.2–2 mg of extract/mL of medium. 1% DMSO was used as negative control and Griseofulvin (1 mg/mL of medium) as a positive control.

Fungicidal activity of the ethyl acetate extract of whole aerial parts, flowers and leaves were tested according to Evron *et al*., 1988 and Warnock 1989.[11,12] Inoculum preparation was according to Clinical and Laboratory Standards Institute method–M38-A2 with slight modification.[13] Sabouraud dextrose broth (Himedia MO 33) was the media used instead of RPMI 1640. Conidia were harvested from 21 days old culture on Sabouraud dextrose agar slants, counted in a hemocytometer and adjusted to appropriate density with

Sabouraud dextrose broth. The extracts were dissolved in DMSO and were added to the tubes containing sterile broth (total volume 900 μL) so as to get 2 mg dry weight of extract/mL of medium (final concentration). Hundred μl of Inoculum was added to each tube (final inoculum dilution $1 \times 10^3 - 3 \times 10^3$ CFU/mL) and incubated at 30°C for 72 h. After mixing the contents well, 100 μL samples were drawn from each tube, and sub-cultured on Sabouraud dextrose agar plates (in triplicates). DMSO instead of extract was used as the control.

In vitro anti-inflammatory activity

Trypsin inhibiting activity-The assay was performed according to the modified method of Alam *et al*., 2011.[14] Bovine serum albumin (BSA) was used as the substrate for trypsin. If the test sample is having trypsin inhibiting activity, hydrolysis of albumin will not take place in its presence. The reaction mixture contained 100 μg of trypsin and 1000 μg of ethyl acetate extract of different plant parts, made up to 1 mL with 50 mM Tris–HCl buffer (pH 7.8) containing 1 mM calcium chloride. The mixture was incubated at room temperature for 10 min. Then added 1 mL BSA (4%) to all tubes and digestion was carried out at 37°C in a water bath for 20 min. Three milliliter of 5% Trichloroacetic acid (TCA) was added to stop the reaction, centrifuged at 2500 rpm, and the absorbance of the supernatant was read at 280 nm. Appropriate controls and blanks were also run.

Trypsin cleaves peptides on the C-terminal side of lysine and arginine amino acid residues. On adding TCA, unhydrolyzed BSA gets precipitated, and the peptide fragments formed by cleavage remain in the supernatant whose absorbance is read at 280 nm. The percentage of inhibition was calculated as:

$$\frac{Absorbance of control - Absorbance of test}{Absorbance of control} \times 100$$

Control and tests were done in triplicate.

Phytochemical analysis

Preliminary phytochemical analysis of various extracts of *M. micrantha* was carried out as per standard methods.[15]

Statistical analysis

For trypsin inhibition assay results are expressed as mean ± standard division (SD), where $n = 3$ (Calculated using Microsoft Office Excel 2007). For antidermatophytic assay, the significance of the difference between control and test was analyzed statistically using Fisher's exact test (Calculated using GraphPad software). For the determination of minimum inhibitory concentration and fungicidal activity, experiments were done in duplicate and repeated independently 3 times.[16]

RESULTS

The effects of methanolic extracts of different plants on the growth of different dermatophytes are shown in Table 1. The highest inhibitory activity was exhibited by *M. micrantha*. Other plants exhibited lesser or no antidermatophytic activity. Of different solvent extracts of the whole plant of *M. micrantha,* ethyl acetate extract completely inhibited the growth of dermatophytes at the tested concentration of 2 mg/mL. Even though the amount of petroleum ether extract incorporated was only 0.5 mg/mL due to low solubility, significant inhibition of growth was observed at this concentration. Methanolic extract did not show inhibition at the concentration of 2 mg/mL [Table 2]. Ethyl acetate extract of leaves (yield 3.8%), stem (yield 0.5%) and inflorescence (yield 2.86%) completely inhibited the growth of all dermatophytes tested at 2 mg dry weight of extract/mL of medium [Figure 1], but due to low yield stem was not considered for further study. The inhibitory effect of ethyl acetate extract of whole aerial parts, as well as various plant parts on the growth of dermatophytes, was significant ($^*P = 0.001$).

Activities exhibited by leaves and flowers were similar. Ethyl acetate extract of both flowers and leaves inhibited the growth of *E. floccosum* at the concentration of 1 mg/mL of medium and *M. canis* at 1.2 mg/mL of medium. *M. gypseum* and *T. rubrum* were inhibited by ethyl acetate extract of flowers at 1.2–1.4 mg dry weight of extract/mL of medium and of leaves at 1.4 mg/mL of medium. Experiments were done in duplicate and repeated independently 3 times. The same results were obtained all the times, and a range of values is presented when different results are obtained.

From the phytochemical analysis, terpenoids were the major component present in the ethyl acetate extract. Petroleum ether extract contained mainly terpenoids and steroids. In addition to terpenoids and steroids, methanolic extract contained various classes of phytochemicals. Detailed results of phytochemical analysis of various extracts of *M. micrantha* are shown in Table 3. Ethyl acetate extracts of whole plant, leaves and flowers were fungicidal at the concentration of 2 mg/mL against all the tested dermatophytes and also exhibited significant trypsin inhibiting activity. Percentage inhibition of Trypsin by 1 mg of ethyl acetate extract of whole plant, leaves and flowers were 33.73 ± 0.306, 39.0 ± 0.505 and 35.53 ± 0.503, respectively. Results are expressed as mean ± SD where $n = 3$.

DISCUSSION

The study has revealed that the plant *M. micrantha* possesses significant antidermatophytic activity, fungicidal efficacy

Table 1: Screening of plants for antidermatophytic activity[a]

Organism	*Chromolaena odorata*	*Cyclea peltata*	*Ficus exasperata*	*Hygrophila schulli*	*Mikania micrantha*	*Mucuna bracteata*	*Pongamia pinnata*
Epidermophyton floccosum	2+	4+	3+	3+	−	2+	+
Microsporum canis	−	2+	2+	3+	−	3+	+
Microsporum gypseum	2+	4+	3+	4+	−	2+	+
Trichophyton rubrum	2+	3+	4+	4+	−	4+	2+

[a]4+=Growth as in control; 3+=Slight inhibition; 2+=Moderate inhibition; +=Strong inhibition; −=No growth

Table 2: Antidermatophytic activity of different solvent extracts of *Mikania micrantha*[b]

Organism	Petroleum ether	Ethyl acetate	Methanol
Epidermophyton floccosum	+	−	4+
Microsporum canis	+	−	3+
Microsporum gypseum	+	−	3+
Trichophyton rubrum	+	−	4+

[b]4+=Growth as in control; 3+=Slight inhibition; 2+=Moderate inhibition; +=Strong inhibition; −=No growth

and protease inhibiting activity. Highest antidermatophytic activity was observed with ethyl acetate extract of the plant, followed by petroleum ether and methanolic extracts. Since petroleum ether and ethyl acetate extracts, which are rich in terpenoids exhibited good inhibition, the antidermatophytic activity of the plant may be attributed to the terpenoids present in the plant. In the case of petroleum ether extract, plant sterols might also have contributed to the antidermatophytic activity. Since methanol is capable of extracting a wide variety of secondary metabolites, the relative concentration of antifungal principle (content of antifungal principle per milligram dry weight of crude methanolic extract) might be low in methanolic extract (percentage yield: Petroleum ether extract - 1.2%, ethyl acetate extract - 3.4% and methanolic extract - 9.8%). That is why higher quantity of methanolic extract is required to inhibit the growth of dermatophytes. Further studies of fungicidal efficacy and anti-inflammatory activity were carried out with the ethyl acetate extracts (of whole plant, leaves and flowers) based on the factors of solubility and activity. The extracts were fungicidal and exhibited significant trypsin inhibiting activity.

Fungicidal agents are more effective in the treatment of dermatophytoses than fungistatic agents as fungicidal agents completely kill the pathogens.[17] Innovation in the treatment of dermatophytic infections has involved the use of topical agents with anti-inflammatory as well as antifungal actions,[18] since recovery from infection depends not only on fungal growth restriction but also on resolution of inflammatory pathology. Trypsin inhibiting assay is a commonly used method to investigate the anti-inflammatory activity of test materials. Trypsin has been shown to induce *in vivo* epidermal proliferation, vasodilatation and inflammatory infiltration in the upper epidermis by the activation of proteinase activated receptor 2 (PAR-2) family.[14] PARs are a recently described G-protein coupled receptors. They are activated by cleavage of the N-terminus of the receptor by a serine protease. Four PARs have been identified up to date-PAR-1, PAR-2, PAR-3 and PAR-4.[19] PAR-2 plays an important role in inflammation and pain.[20] PAR-2 is activated by trypsin and tryptase. Thus agents who inhibit trypsin can act as anti-inflammatory agents. Furthermore, serine proteases secreted by dermatophytes causes itching through activation of PAR-2 receptors.[21] Agents with trypsin inhibiting activity may also be able to restrain the action of these serine proteases secreted by dermatophytes thereby rendering the treatment more beneficial and comfortable.

Since *M. micrantha* possesses antidermatophytic activity as well as anti-inflammatory activity it is an excellent medicament for dermatophytoses therapy, because antifungal preparations with inherent anti-inflammatory activity can provide rapid symptomatic relief in the treatment of dermatophytoses. From the phytochemical analysis, it can be concluded that the above said properties might be due to the terpenoids and sterols present in the plant. The present study is the first report on antidermatophytic and protease inhibiting activities of the plant.

The plant is available in plenty from its natural habitat, which makes the plant cheaply and easily available medicine in traditional treatment and ensures adequate supply of raw material for the pharmaceutical industry to develop modern medicines. However, phytochemical content of a plant may show geographical variation. Hence, the study using samples collected from other geographical region is required to confirm the universal medicinal application of the plant. In this study, we have evaluated *in vitro* anti-inflammatory activity by trypsin inhibiting assay. Anti-inflammatory activity can also be analyzed *in vitro* on cultured human monocytic or macrophage cell lines using assays like cyclooxygenase inhibition, lipoxygenase inhibition, estimation of myeloperoxidase activity, estimation of cellular nitrite level, etc., which will further substantiate the result. However Perez-Amador *et al*., 2010[2] have been established *in vivo* anti-inflammatory activity of

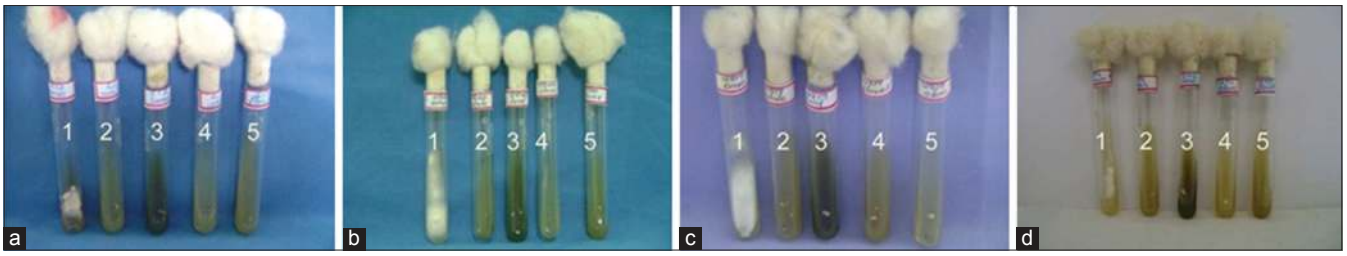

Figure 1: Antidermatophytic activity of ethyl acetate extract of (2 mg dry weight of extract/mL of medium) whole plant (2), leaf (3), stem (4) and inflorescence with seeds (5) of *Mikania micrantha* (control [1]). All the extracts completely inhibited the growth of the tested dermatophytes. (a) *Epidermophyton floccosum* var. nigricans, (b) *Microsporum canis*, (c) *Microsporum gypseum* and (d) *Trichophyton. rubrum* (reproduction – at full page width)

Table 3: Phytochemical analysisc

Phytochemical	Petroleum ether extract (whole plant)	Ethyl acetate extract (whole plant)	Methanol extract (whole plant)	Ethyl acetate extract	
				Flower	Leaves
Steroids	++	+	+	+	+
Terpenoids	+++	+++	++	+++	+++
Flavanoids	–	–	+	–	–
Phenolics	–	–	++	–	–
Alkaloids	+	+	++	+	+
Tannins	–	–	+	–	–
Saponins	–	–	–	–	–
Glycosides	–	+	+++	+	+

c+++=Present in abundance; ++=Moderately present; +=Present in trace amounts; –=Absent

petroleum ether and ethyl acetate extracts of leaves and inflorescence of *M. micrantha* by mouse ear edema test which is a supportive evidence to the present study.

People, especially in developing countries, usually take self-medication to treat skin ailments like ring worm infections and other inflammatory conditions. *M. micrantha* can act as excellent candidate for such uses. The plant can be used to develop herbal formulations and as raw material for the development of new drugs in modern medicine against dermatophytoses. Since the plant possesses protease inhibiting activity, it can also be used to develop medicaments for various allergic and inflammatory diseases in traditional as well as modern medicine. However through analysis is required to study toxicity of the plant material if any before developing oral medications from the plant. Future perspective includes toxicity study, testing of activity against other pathogenic fungi, study of the mechanism of action of antifungal activity, detailed study on anti-inflammatory activity and isolation and purification of active principles.

CONCLUSIONS

Dermatophytoses are refractory to treatment, and the spectrum of antifungals for treating dermatophytoses is narrow. Furthermore, most of them have toxic side effects on prolonged use and lack fungicidal activity. *M. micrantha* is a promising antifungal species because of its significant activity, fungicidal efficacy and anti-inflammatory activity. Further detailed studies on its effect on other pathogenic fungi and the elucidation of the molecular basis for the antidermatophytic and anti-inflammatory activities will be highly useful and are on the way.

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