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Evaluation of Antidermatophytic activity of *Piper betle, Allamanda cathertica* and their combination: an *in vitro* and *in vivo* study

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Abstract: The present study was conducted to evaluate and establish the claim of antidermatophytic activities of *Piper betle* Linn. and *Allamanda cathertica* Linn. *In vitro* and *in vivo* antifungal studies of the chloroform and methanol extracts of these plants and their mixture were conducted. Agar cup diffusion technique and two fold serial dilution methods were used to determine the antifungal activity against *Trichophyton mentagrophytes*, *Trichophyton rubrum, Trichophyton tonsurans* and *Microsporum gypseum in vitro.* Guinea Pigs were used in case of *in vivo* experiments. Chloroform extracts showed higher activity than methanol, while the activity of *Allamanda cathertica* was higher than *Piper betel*. The MIC of the extracts were found ranging between 0.156-1.25 mg/ml. 5% extract ointments were prepared and applied against induced ringworm in Guinea Pigs, with subsequent removal of infections in less than 60 days. Similarly methanol extracts of these two plants were also performed, the results of the *in vitro* experiments were found to be not up to the mark. Hence no *in vivo* experiments were undertaken. These results demonstrate the antifungal potential of the plants and hence lend support for the use of them in traditional medicine.
Key Words: Antifungal, Agar cup diffusion, Percent culture recovery.

Introduction

Skin ailment is a common health problem in most of the tropical countries, which requires major attention. Of the skin problems, dermatophytosis or Ringworm is the most prevalent. Dermatophytosis is a disease of the Keratinized parts of the body (skin, hair and nail) caused by a group of three genera (*Trichophyton, Microsporum* and *Epidermophyton*) of highly specialized fungi called the Dermatophytes. They cause a wide spectrum of diseases that range from guild scaling disorder to one that is generalized and highly inflammatory (1). Superficial fungal infections like Dermatophytosis are common in most tropical areas and in developing countries like India. The combination of hot climate and high humidity coupled with congregate living and malnutrition are the causes of prevalence of these infections (2). The incidence of fungal diseases predominated in North East India, accounting for almost 50% of the total infectious skin diseases (3). In a study, it was revealed that skin diseases constituted 6.3% of the total number of the patients, who attended medical care in Assam, India (4). The incidence of dermatomycoses has been severe in recent years because of the increase in number of immuno compromised hosts (5). Modern antifungal drugs which are in common use, cause considerable adverse effects such as gastrointestinal disturbances, cutaneous reaction, hepatotoxicity, leucopenia etc. (6-

9). Moreover, treatment of dermatophytosis has become quite challenging owing to the limited antimicrobial spectrum of most of the drugs and emergence of multi drug resistant strains (10). Limitations in the efficacy and intolerability of the existing drugs in persons with chronic illness like Diabetes Mellitus, AIDS, Cancer etc demands for alternative treatment options (11).

The demand for herbal medicines is increasing rapidly all over the country due to their lack of side effects and low cost (12). Treating skin disorders with traditional medicine is increasing in the developed countries also (13). The people of the North East region of India are rich in ethnomedical tradition and use many herbal preparations to cure skin infections and other diseases instead of conventional synthetic drugs. The region is considered as one of the ecological hot spots of the world (14) and has an abundance of medicinal plants known to the native people (15).

Allamanda cathertica Linn. (Apocyanaceae) is a widely growing perennial shrub of the region with smooth and thick leaves. The leaf extract can promote wound healing activity (16) and the roots are used against jaundice and malaria in traditional medicine (17). Plumieride from *Allamanda cathertica* acts as an antidermatophytic agent (18). Ethanolic extract of the roots is active against P-388 leukaemia *in vivo* in mice, and *in vitro* against human carcinoma of nasopharynx. *Piper betle* Linn. (Piperaceae) is a slender creeping plant which has alternate, heart shaped, smooth, shinning and long stalked leaves, with pointed apex, widely distributed in Assam. Leaves of the plant possess strong aromatic flavour and widely used as a masticatory with areca nuts. Decoction of leaves is used for healing wounds. Leaves yields an essential oil used in respiratory catarrh and diphtheria. Roots of the plant with black pepper are used to produce sterility in women (19). To the best of our knowledge no works on the *in vivo* antidermatophytic activity of the two plants grown in the North East Region of India and their combination in Guinea pig animal model have been studied. The present study attempts to determine the antifungal property of the two plants against the following dermatophytic fungi- *Trichophyton mentagrophytes*, *Trichophyton rubrum, Trichophyton tonsurans* and *Microsporum gypseum.* Many traditional practitioners/vaidya of this region, particularly in the rural and hilly areas use these two plants singly or as combinations for the treatment of fungal infections like dermatophytosis, which inspires us to take up this study. Chloroform and methanol extracts were tested *in vitro* where the activity of the chloroform extract was found to be significantly higher. Hence emphasis was given to test the activity of the chloroform extract only.

Experimental

Plant Materials

Plant materials (leaves and tender shoots) were collected from different parts of Kamrup district, Assam in the month of April 2008. Plants are authenticated by a Taxonomist from Gauhati University and herbariums were prepared and deposited as voucher specimen (No. IASST/MEP/H No. 27, & 19) in the Medicinal Plant and Biochemistry Laboratory of IASST for future reference.

Preparation of Plant extracts

Freshly collected plant materials were dried under shade and 500 gm of the fine dried powdered materials were then extracted separately with chloroform (Merck) and methanol in an extractor at room temperature. The extracts concentrated in a rotary evaporator (Buchi R124, German) at <40 ^{o}C were kept in refrigerator at 4 ^{o}C for future use. The yield of the Chloroform extract was 45.50 gm and 34.25 gm for *Piper betle* and *Allamanda cathertica* respectively. For the methanol extract the yield was 50.0 gm for *Piper betle* and 28.50 gm for *Allamanda cathertica.*

Experimental Animal

Healthy adult albino Guinea Pigs weighing 350-450 gm of both sex (with exception of pregnant and nulliparous animals) were taken from the animal house of IASST for the *in vivo* experiment. Animals were caged individually in Polypropylene cages for 2 weeks for acclimatization at 25±2 ^{o}C with food and water *ad libitum*. IAEC has cleared this work.

Microorganisms

Fungal cultures were procured from Institute of Microbial Technology (IMTECH), Chandigarh-160 036 (India). The organisms tested were *Trichophyton rubrum* (MTCC 8477), *Trichophyton mentagrophytes* (MTCC 8476), *Trichophyton tonsurans* (MTCC 8475) and *Microsporum gypseum* (MTCC 8469). The procured samples were sub-cultured and are maintained in Sabouraud Dextrose Agar (HIMEDIA) Slants at 4^{O}C.

In-vitro assay with Agar Cup diffusion Technique

The *in-vitro* antifungal screening is done by Agar cup diffusion methods (20). In brief, 200 µl of fungal suspension were uniformly spread over solidified Sabouraud Dextrose Agar (SDA) plates with the help of a sterilized spreader. Wells of 6 mm diameter were made in the centre of these agar plates with the help of a sterile cork borer. The wells were then filled with 200µl of the respective test extract at different concentration ranging from 10mg/ml-0.321mg/ml and allowed to diffuse at room temperature for an hour. Then the plates were incubated at 28±2^{O} for 72-96

hours to 2 weeks depending on the growth rate of the test pathogen. The antifungal activities of the extracts were determined by measuring the diameter of the inhibition zone around the well that was filled with the extracts.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

MIC and MFC were determined according to the method described by Irobi *et al.* (21). MIC was determined by incorporating various concentrations of extract (10 mg/ml to 0.312 mg/ml) in Sabouraud Dextrose broth. 100 µl of fungal inoculum was added to each tube and incubated at room temperature for 21 days. Clotrimazole was used as the positive control. The MIC was regarded as the lowest concentration of the extract that did not permit any visible growth after 7-21 days of inoculation.

The MFC was determined using the method of Rotimi *et al.* (22). The tube of extracts which showed no visible growth after 21 days of incubation when sub cultured on to extract free Sabouraud Dextrose Agar plated at using as inoculum size of 0.5 ml is considered to be the MFC.

Animal infection and Ointment application

In the *in vivo* experiment, Guinea Pigs were first anaesthetized under light ether and they are then infected with fungal inoculums of *T. mentagrophytes, T. rubrum, T tonsurans and M gypseum* separately following Kishore *et al.* (23) with slight modifications. In brief, 10cm^2 area is clearly shaved off hair in both the flanks of the animals with the help of a pair of sterilized scissors where 3-5 small circular incisions were created with the help of a sterilized scalpel and needle. Fungal inoculums were prepared from 10-15 day old cultures slants. 500mg of sterilized white sand powder and 2.5 ml of sterilized honey were macerated with fungal mycelial mat grown on SDA slants. Mycelia from one slant were used to infect two sites. The paste of inoculum was applied on the incisions and the whole areas are covered with cloth bandage and tied with non irritant leucoplast. After 24 hours, the bandage is removed, area is cleared with sterilized with 70% ethyl alcohol and water and the animals were cased individually for four days with adequate supply of food and water.

For preparing the ointments, 5 grams of the individual chloroform extracts of both the plants and their 1:1 (w/w) mixture were mixed separately with 100 gm of white petroleum jelly and are stored in glass vials till use. The treatment of the animals was started from the 5th day of inoculation, after the confirmation of the infection (24). The infected areas were applied with 0.2 g ointment twice daily in the morning and afternoon. In the positive control set 0.2 g of 1% Clotrimazol ointment was applied where as in the negative control set only petroleum jelly 0.2 g was applied. Treatment continued regularly at the same time till complete recovery was achieved. Effects of the extracts were evaluated every fifth day by culturing skin scrapings and hair samples from the infected areas in SDA plates.

Percent culture recovery was calculated using the formula (23)-

$$\% \text{ culture recovery} = \frac{\text{Total number of sites positive for culture in each set}}{\text{Total number of sites in each set}} \times 100$$

Results

The *in vitro* results of both the plants and their combination are given in Table 1 and 2. Chloroform extract of both the plants and their 1:1 (w/w) combination was found to be effective against the tested dermatophytes in the Agar cup diffusion technique evident from the zone of inhibition they have produced (Table-1). The maximum inhibition zone of 58mm was observed against *T. tonsurans* for the *A. cathertica* extract while *P. betle* and the 1:1 combination of both the plant showed maximum inhibition zone of 46 mm and 51mm against *T. mentagrophytes* and *T. tonsurans* respectively. Table 2 represents the MIC/MFC values of the extracts and the standard drug Clotrimazole. The MIC value for all the three extract ranged between 0.156 mg/ml-1.25 mg/ml (Table-2). The MIC values of the two plants and their combination was higher than the MIC values shown by the standard antifungal drug Clotrimazole which is expected as we have used the crude extract.

Table 1: Zone of Inhibition at different concentration.

Extract Concentration mg/ml	Zone of Inhibition (mm) *T. mentagrophytes*	*T. rubrum*	*T. tonsurans*	*M. gypseum*
A. cathertica				
10	57.6±0.16	53.0±0.20	58.3±0.36	47.0±0.23
5	49.6±0.22	41.6±0.15	50.0±0.14	39.3±0.15
2.5	38.4±0.11	37.3±0.04	39.6±0.11	31.0±0.14
1.25	31.5±0.08	27.0±0.18	28.0±0.14	22.7±0.11
0.625	24.0±0.15	18.5±0.70	19.5±0.15	10.0±0.15
0.312	10.5±0.70	9.3±0.11	12.3±0.18	-
P. betle + *A. cathertica* (1:1)				
10	45.6±0.21	40.8±0.21	51.4±0.16	40.0±0.04
5	33.3±0.11	35.6±0.18	41.4±0.18	30.2±0.14
2.5	28.6±0.07	24.3±0.07	33.0±0.07	22.5±0.11
1.25	21.3±0.11	20.5±0.14	25.5±0.14	16.0±0.18
0.625	15.2±0.15	14.5±0.11	17.4±0.11	9.3±0.040
0.312	-	-	11.7±0.07	-
P. betle				
10	46.5±0.15	39.3±0.08	40.6±0.11	36.0±0.21
5	36.0±0.11	33.0±0.11	28.5±0.15	25.2±0.07
2.5	29.6±0.11	24.5±0.15	21.0±0.14	18.6±0.11
1.25	20.0±0.19	18.5±0.15	17.0±0.15	14.0±0.16
0.625	13.4±0.19	10.0±0.90	12.0±0.19	-
0.312	-	-	-	-

Note: Petri Plate diameter is 90mm. (-) = no inhibition zone.

Table 2: Minimum inhibitory concentration and Minimum fungicidal concentration of the Extracts and Standard antifungal Clotrimazole. (µg/ml).

Test Fungi	*P. betle*		*A. cathertica*		Mix		Clotrimazole	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
T. mentagrophytes	312.5	625.0	156.2	312.5	312.5	625.0	62.5	62.5
T. rubrum	625.0	1250	156.2	312.5	312.5	625.0	31.2	62.5
T. tonsurans	312.5	625.0	156.2	312.5	156.2	312.5	31.2	62.5
M. gypseum	625.0	1250	312.5	625.0	312.5	625.0	31.2	62.5

The data presented in Table 3 to Table 6 indicate per cent decrease in dermatophytic infection following ointment application and hair culture. Individual extract ointment of the plants as well as their combination was found to be effective against induced dermatophytosis in Guinea pig in a concentration dependent manner. Zero percent culture recovery for *A. cathertica* against *T. rubrum, T. mentagrophytes, T. tonsurans* and *M. gypseum* infection was observed at day 40, 45, 40 and 50 respectively while for *P. betle* it was observed in 50, 55, 55 and 55 days respectively for the four fungus. The combined extract almost cured the infections of all the fungus by the 45th day with only 10.6% and 13.1% infection was found to occur for *T. mentagrophytes* and *M. gypseum* respectively at day 45th. The efficacy of the three prepared formulations can be stated as follows-

A. cathertica > *P. betle* + *A. cathertica* (1:1)> *P. betle*

In the control model, the hair culture was positive, which exhibited 100% culture recovery throughout the experiment.

Table 3: % culture recovery against *T. mentagrophytes*

Treatment Days	Control	*P. betle*	*A. cathertica*	Mix
5th	100	100	100	100
10th	100	100	92.33±1.24	100
15th	100	94.73±0.53	86.93±0.88	91.33±0.89
20th	100	86.13±0.55	73.83±1.74	79.13±0.75
25th	100	75.83±1.24	62.33±1.78	62.63±1.08
30th	100	66.20±1.12	45.83±2.35	47.83±1.08
35th	100	49.50±1.06	21.93±1.20	32.00±0.94
40th	100	30.83±1.02	7.50±1.77	21.83±0.41
45th	100	18.33±2.16	0.00	10.67±0.20
50th	100	9.67±1.08	0.00	0.00
55th	100	0.00	0.00	0.00
60th	100	0.00	0.00	0.00

Table 4: % culture recovery against *T. rubrum*

Treatment Days	Control	*P. betle*	*A. cathertica*	Mix
5th	100	100	100	100
10th	100	100	91.67±1.14	100
15th	100	92.73±1.67	83.90±1.42	93.17±0.54
20th	100	83.67±1.14	68.80±0.77	79.00±0.61
25th	100	70.17±0.54	55.60±1.62	58.50±0.71
30th	100	54.53±1.31	36.00±2.15	41.00±0.94
35th	100	40.00±0.61	17.23±0.70	25.80±0.65
40th	100	23.30±1.60	0.00	13.67±0.74
45th	100	11.63±1.00	0.00	0.00
50th	100	0.00	0.00	0.00
55th	100	0.00	0.00	0.00
60th	100	0.00	0.00	0.00

Table 5: % culture recovery against *T. tonsurans*

Treatment Days	Control	*P. betle*	*A. cathertica*	Mix
5th	100	100	100	100
10th	100	100	92.13±2.00	100
15th	100	96.87±0.84	71.37±2.54	98.87±0.39
20th	100	87.10±1.93	73.83±1.74	85.30±0.86
25th	100	76.57±1.18	57.60±1.07	61.17±2.16
30th	100	61.03±0.90	38.53±1.57	43.67±1.14
35th	100	55.60±1.19	18.83±1.63	24.03±1.27
40th	100	36.53±1.56	0.00	12.17±0.54
45th	100	24.45±2.12	0.00	0.00
50th	100	17.30±1.46	0.00	0.00
55th	100	0.00	0.00	0.00
60th	100	0.00	0.00	0.00

Table 6: % culture recovery against *M gypseum*

Treatment Days	Control	*P. betle*	*A. cathertica*	Mix
5th	100	100	100	100
10th	100	100	96.80±1.63	100
15th	100	100	89.67±1.24	100
20th	100	90.37±0.64	76.17±1.17	89.27±1.32
25th	100	70.45±0.67	66.37±1.00	69.80±0.75
30th	100	58.00±1.62	52.87±1.64	54.93±0.58
35th	100	43.20±0.93	41.23±1.94	34.37±1.25
40th	100	26.70±0.89	28.27±0.66	24.30±0.89
45th	100	17.00±0.71	7.60±1.17	13.10±0.32
50th	100	8.67±0.40	0.00	0.00
55th	100	0.00	0.00	0.00
60th	100	0.00	0.00	0.00

Discussion

Despite the stunning success of pharmaceutical industries in developing new antifungal drugs, finding novel and consumer friendly antifungal agents for treatment of wide range of fungal diseases, is still a top priority because of emerging multidrug resistant pathogens (25). Searches for substances with antimicrobial activity are frequent, and medicinal plants have been considered interesting by some researchers since they are frequently used in popular medicine as remedies for many infectious diseases (26). In the present study Chloroform extracts of both the plants were found to be effective against all the tested dermatophytes. However the activity of *A. cathertica* against the dermatophytes was slightly higher than that of *P. betle*. The susceptibility of all the tested fungus *in vitro* against *A. cathertica,* 1:1 combination of *A. cathertica* and *P. betle* was as follows-

T. tonsurans > *T. mentagrophytes*> *T. rubrum*> *M. gypseum*.

While the susceptibility against *P. betle* can be stated as-

T. mentagrophytes> *T. tonsurans* > *T. rubrum*> *M. gypseum*.

It is evident from our experiment that the mixing of *P. betle* with *A. cathertica* does not enhance the efficacy of the mixture as claimed by rural healers. The combination of the two plants was found to be more effective than individual effect of *P. betle* and less effective than individual effect of *A. cathertica.* The *in vivo* study was measured with decrease in skin redness, skin swelling, lesion severity in the animal models, with application of the extract ointments. The efficacy of the extracts was confirmed by recurrence of hair growth in the dermatophyte infected areas. Results were finally confirmed when skin scrapings and hair samples showed no growth of the tested fungus in SDA plates.

Nahar *et al.* (27) reported the antidermatophytic activity of dichloromethane and methanol extracts of *Allamanda cathertica* against *Trichophyton rubrum* and *Microsporum gypseum* where Trakranrungsie *et al.* (28) evaluated the efficacy of 10% *Piper betle* cream against zoonotic dermatophytes *in vitro*. The present study revealed significant efficacy of the Chloroform extract of *P. betle, A. cathertica* and their combination *in vitro* in agar cup diffusion test and also against induced superficial dermatophytosis in guinea pig in *in vivo* experiment. This study reveals a scientific base of the traditional use of *Piper betle* and *Allamanda cathertica* by traditional practitioner and does not support the use of *P. betle* and *A. cathertica* mixture against dermatophytosis.

Conclusion

The results of the present study suggest that *Piper betle* Linn and *Allamanda cathertica* possess antidermatophytic properties. The chloroform extract showed much better efficacy than the methanol extracts of the two plants, which suggests that the activity may be due to comparatively non polar compounds present in these plants. However the use of the mixture of the two plants by the traditional practitioner/ vaidya against fungal infection is not supported by our study as, the plant *A. cathertica* showed better activity than the mixture. Further studies are required to clearly elucidate the components responsible for antimicrobial activity as well as any pharmacological or toxicological properties that such extracts might have.

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