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Evaluation of Inhibitory Effect of the Plant *Phyllanthus amarus* Against Dermatophytic Fungi *Microsporum gypseum*

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Objective The antifungal activity of various solvent extracts (such as ether, chloroform, ethyl acetate and ethyl alcohol) of the plant *Phyllanthus amarus* against dermatophytic fungi *Microsporum gypseum* was observed. **Method** Antifungal bioassay in terms of reduction in weight, colony diameter and sporulation of the target fungal colony was carried out using Broth Dilution method. **Results** Root part of the plant, extracted in various organic solvents did not show any noticeable antifungal activity. The percentage inhibition observed in different solvent extracts of aerial part was found as reduction in weight: chloroform [50.3%], ethyl acetate [27.7%] and ethyl alcohol [12.1%], reduction in colony diameter: chloroform [53.4%], ethyl acetate [31.4%] and ethyl alcohol [15.0%] and reduction in sporulation: maximum inhibition in chloroform extract, at test concentration of 4000 ppm at incubation period of 8 days. **Conclusion** Chloroform fraction of the aerial part of the plant *P. amarus* shows significant inhibitory effect against dermatophytic fungi *M. gypseum* and requires chemical characterization for its bioactive principle.

Key words: *Phyllanthus amarus*; Antifungal activity; *Microsporum gypseum*; Skin infections

INTRODUCTION

Fungal infections particularly those involving the skin and mucosal surfaces constitute a serious problem, especially in tropical and subtropical developing countries[1]. Dermatophytes have been reported to be potentially pathogenic[2] and are directly connected with the skin fungal infections. Unlike other fungal infections, cutaneous mycosis has been considered important in which host immune responses are highly evoked resulting in severe pathologic changes, which are extended deeper into epidermis as well as hair and nails. Such fungal infections are mainly caused by *Microsporum* species[3]. Although several antimycotic drugs are available at present, its use is becoming limited by a number of factors, such as low potency, poor solubility, development of resistant strains and drug toxicity. Therefore, there is a distinct need for the discovery of new, safer and more effective antifungal agents. Recently, extracts and their biological active compounds isolated from plant species have been the centre of interest[4,5]. National Primary Health Care Program clearly indicates that

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vast majority of plant species remains untapped and there is a need to evaluate plant extract scientifically for their medicinal properties[6]. Therefore, it is important to make sincere efforts to identify the newer potentials of the plant kingdom and to characterize the chemical constituents responsible for important pharmacological properties.

The present study submits the record of the evaluation of different solvent extracts of aerial part of the plant *P. amarus*, exploring their inhibitory nature against dermatophytic fungi *M. gypseum*. The plant *P. amarus* has already been characterized for its medicinally important properties like liver ailment[7], hepatitis B[8], diabetes[9], urinary disorders[10] and is now being explored for the existence of antifungal activity. *P. amarus*, a herb, grows up to 60 cms in height and is widely distributed in tropics as a wild winter weed throughout the hotter parts, particularly on cultivated lands.

MATERIALS AND METHODS

Plants were collected from nearby places of Dayalbagh Educational Institute, Agra, during the month of Sept.-Oct., 2002, and characterized as *P. amarus* (Taxonomy Department of the Institute). Plants were partitioned into aerial and root parts and dried under shade in laboratory.

Extraction

The powdered aerial and chopped root parts were separately subjected for soxhlet extraction successively with solvents (such as ether, chloroform, ethyl acetate and ethyl alcohol). The residual portions obtained after evaporating respective solvent (Rota vapour vacuum distillation) were dried by purging nitrogen and finally weighed.

Antifungal Bioassay

Dermatophytic fungus (*M. gypseum*) were isolated from soil samples, collected from nearby area of Dayalbagh Dairy, Agra and cultured in SDA medium[11]. Desired fungal species (Commonwealth Mycological Institute, Kew. No. 276190) was obtained by repeated sub culturing and finally incubated at 28℃±2℃.

Antifungal bioassay in terms of reduction in weight[12], colony diameter[13] and sporulation[14] of the target fungi was carried out in triplicate experiments. Different solvent extracts of the plant (aerial and root part) were considered in the test concentrations (1000-4000 ppm) and respective inhibiting parameters were recorded as a function of time (4, 8, and 12 days) at 28℃±2℃.

For antifungal bioassay (reduction in weight of mycelium), different solvent extracts were taken in Erlenmeyer's flasks. Sabourauds Dextrose medium (40 gm dextrose and 10 gm peptone in one liter of solution in water) (Broth) was added in fixed amount (40 mL) in each Erlenmeyer's flask and autoclaved at 15 lb pressure for 15 minutes. The solutions were inoculated with an inoculum disc (6 mm dia) cut from 15 days old fungal cultures. All the solutions were filtered through pre-weighed filter papers (Whatman No. 42) and dried in a folded condition at 80℃ for 24 h in an oven. The dry mycelial weight of each fungus was obtained by subtracting the weight of each filter paper from the total weight.

For antifungal bioassay (reduction in diameter of fungal colony), different solvent extracts were taken in pre-sterilized petridishes. Sabourauds Dextrose Agar medium (40 gm dextrose, 20 gm agar and 10 gm peptone in one liter of solution in water) was added in each petridishis. The petridishes were incubated with an inoculum disc (6 mm dia) cut from 15

days old mycelium of test fungi previously grown on SDA medium. At the end of incubation period, the sporulation was also observed before preparing spormount in lacto phenol and cotton blue mixture.

Percentage inhibition of myclial growth in each case was calculated by using formula: Percentage inhibition=100×(C-T)/C, where, C=Dry mycelial weight/Diameter of mycelial colony in control and T=Dry mycelial weight/Diameter of mycelial colony in given extract concentration.

Statistical Analysis

Percentage inhibition in each case was analyzed using SPSS/Pc$^{+}{}_{TM}$, statistical package, SPSS[15]. Correlation coefficients were used to relate percentage inhibition to various test concentrations. Tests for non-normal data were computed by Mann Whitney (Independent U test) to compare effectiveness of each extract[16].

RESULTS

The effectiveness (antifungal activity) of different solvent extracts (chloroform, ethyl acetate and ethyl alcohol) of aerial and root parts of the plant were determined on the basis of evaluated percentage inhibition in mycelial weight and colony diameter of the target species *M. gypseum*. The validity of experimental results of percent inhibition obtained at different test concentrations were checked by various statistical parameters and tabulated (Tables 1 and 2). The plots of percentage inhibition versus concentration at different time exposure are presented graphically (Figs. 1 and 2). EC_{50} (Effective Concentration required for 50% inhibition) value for each solvent extracts was also calculated.

TABLE 1

Effect of Various Solvent Extracts of Plant *P. amarus* (aerial part) on the Weight of Mycelial Growth of Dermatophytic Fungi *M. gypseum*

Treatment Conc. (ppm)	Weight of Fungal Colony in (mg) at Incubation Period of					
	4 days		8 days		12 days	
	MW ± *s*	% Inh.	MW ± *s*	% Inh.	MW± *s*	% Inh.
Chloroform						
1000	289.0±7.50	17.4	338.6±7.36	23.0	434.3±7.47	16.5
2000	250.6±8.27	28.4	285.6±6.51	35.1	371.6±9.37	28.6
3000	198.3±5.38	43.3	229.6±5.38	47.8	289.3±5.33	44.4
4000	192.6±5.33	44.9	218.6±5.96	50.3	279.3±5.19	46.3
Control	350.0±7.50	00.0	440.3±8.27	00.0	520.6±9.60	00.0
Corr. Coeff.	-0.93		-0.97		-0.99	
Regression	y=9.74X+9.15		y=9.46X+15.4		y=9.93X+7.65	
EC_{50}	4680.565		3793.492		4309.719	
Ethyl acetate						
1000	328.6±8.27	6.1	402.0±8.30	8.6	478.6±7.50	8.0
2000	302.3±7.50	13.6	370.3±7.50	15.8	446.3±8.89	14.2
3000	281.6±5.33	19.5	327.6±7.10	25.5	406.3±8.27	21.9
4000	274.3±5.19	21.6	318.3±5.37	27.7	395.0±7.90	24.1
Control	350.0±7.50	00.0	440.3±8.27	00.0	520.6±9.60	00.0
Corr. Coeff.	-0.94		-0.95		-0.96	

(To be continued)

(continued)

Treatment Conc. (ppm)	Weight of Fungal Colony in (mg) at Incubation Period of					
	4 days		8 days		12 days	
	MW ± *s*	% Inh.	MW ± *s*	% Inh.	MW± *s*	% Inh.
Regression	y=5.13X+2.1		y=6.52X+2.65		y=5.49X+3.05	
EC_{50}	15613.060		10376.280		15164.940	
Ethyl alcohol						
1000	332.3±4.30	5.0	408.6±7.67	7.1	485.0±7.50	6.8
2000	326.6±5.33	6.6	401.6±6.62	8.7	480.3±8.61	7.7
3000	320.3±5.19	8.4	393.3±5.19	10.6	472.3±7.50	9.2
4000	315.3±5.38	9.9	387.0±7.50	12.1	461.6±8.67	11.3
Control	350.0±7.0	00.0	440.3±8.27	00.0	520.6±9.60	00.0
Corr. Coeff.	-0.99		-0.99		-0.96	
Regression	y=1.65X+3.35		y=1.69X+5.4		y=1.5X+5	
EC_{50}	1643763.000		1192206.000		929565.400	

Note. s: Standard Error of Mean; Inh.: Inhibition; MW: Mycelial Weight.

TABLE 2

Effect of Various Solvent Extracts of Plant *P. amarus* (aerial part) on the Diameter of Fungal Colony of Dermatophytic Fungi *M. gypseum*

Treatment Conc. (ppm)	Weight of Fungal Colony in (mg) at Incubation Period of					
	4 days		8 days		12 days	
	CD± *s*	% Inh.	CD± *s*	% Inh.	CD± *s*	% Inh.
Chloroform						
1000	13.0±0.57	10.9	22.0±0.99	22.0	36.0±0.99	16.8
2000	11.6±0.77	20.5	19.0±0.50	33.5	33.6±0.89	22.4
3000	9.6±0.19	34.2	14.3±0.77	50.0	26.6±0.77	38.5
4000	9.3±0.21	36.3	13.3±0.77	53.4	25.3±0.68	41.5
Control	14.6±0.68	00.0	28.6±0.57	00.0	43.3±0.77	00.0
Corr. Coeff.	-0.93		-0.94		-0.93	
Regression	y=8.99X+3		y=11.07X+12.05		y=9.02X+7.25	
EC_{50}	6969.568		3431.731		5848.513	
Ethyl acetate						
1000	13.6±0.19	6.8	25.6±0.78	10.4	40.0±0.73	7.6
2000	13.3±0.77	8.9	24.3±0.64	15.0	38.6±0.57	10.8
3000	12.0±0.30	17.8	20.0±0.57	30.0	33.3±0.77	23.9
4000	11.6±0.19	20.5	19.6±0.77	31.4	32.6±0.85	24.7
Control	14.6±0.68	00.0	28.6±0.57	00.0	43.3±0.77	00.0
Corr. Coeff.	-0.93		-0.90		-0.88	
Regression	y=5X +1		y=7.8X+2.2		y=6.44X+0.65	
EC_{50}	19648.180		8524.711		12423.480	
Ethyl alcohol						
1000	14.0±0.77	4.1	26.6±0.77	6.9	41.0±0.89	5.3
2000	13.3±0.29	8.9	25.6±0.68	10.4	40.0±0.74	7.6
3000	13.0±0.19	10.9	24.6±0.50	13.9	39.0±0.57	9.9
4000	12.6±0.57	13.6	24.3±0.79	15.0	38.3±0.77	11.5
Control	14.6±0.68	00.0	28.6±0.57	00.0	43.3±0.77	00.0
Corr. Coeff.	-0.96		-0.95		-0.96	
Regression	14.0±0.77	4.1	26.6±0.77	6.9	41.0±0.89	5.3
EC_{50}	13.3±0.29	8.9	25.6±0.68	10.4	40.0±0.74	7.6
Regression	y=3.05X+1.75		y=2.78X+4.6		y=1.95X+3.35	
EC_{50}	52166.660		60537.090		262705.600	

Note. s: Standard Error of Mean; Inh.: Inhibition; CD: Colony Diameter.

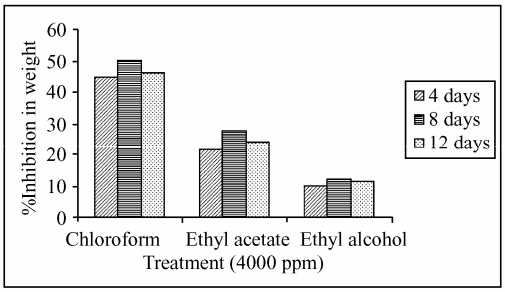

FIG. 1. Effect of various solvent extracts of the plant *P. amarus* (aerial part) on the mycelial weight of *M. gypseum*.

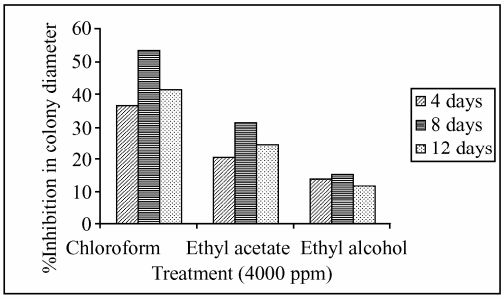

FIG. 2. Effect of various solvent extracts of the plant *P. amarus* (aerial part) on the colony diameter of *M. gypseum*.

DISCUSSION

No solvent extracts of root part of the plant showed any noticeable activity. All the three solvent extracts except (pt. ether) for aerial part exhibited antifungal activity against dermatophytic fungi *M. gypseum*, to the different extents. Maximum inhibition in the target fungal colony caused by various solvent extracts of aerial part of the plant was found to be, Reduction in weight: chloroform [50.3%], ethyl acetate [27.7%] and ethyl alcohol [12.1%], Reduction in colony diameter: chloroform [53.4%], ethyl acetate [31.4%] and ethyl alcohol [15.0%] at test concentration of 4000 ppm with time exposure of 8 days. The qualitative evaluation of the inhibition in sporulation observed in all the three solvents of the aerial part of the plant are in accordance with the trends of reduction in colony diameter and weight of mycelial growth.

Statistically significant increases in the percentage inhibition of the fungal species are observed with increasing test concentration [1000-4000 ppm] of each extract of the aerial part of the plant. On the time scale, in each case, rate of inhibition increases from four to eight days and then start decreasing (Figs. 1 and 2). These trends demonstrate that 8th day seems to be the optimum time for maximum phytotoxicity caused to the target fungi and after that the fungal colony appears to be acclimatized with the phytoextract. The observed increased in percentage inhibition (reduction in colony diameter, mycelial weight and sporulation) with increasing supplementation of extract indicates the presence of significant amount of some chemical moiety responsible for exhibited antifungal property in the aerial part. Bioassay (antifungal) directed toward to the evaluation of relative phytotoxicity of various extracts of aerial part of the plant is found to be in the folowing order: Chloroform

>Ethyl acetate> Ethyl alcohol (Mann Whitney test).

TABLE 3

Effect of Various Solvent Extracts of Plant *P. amarus* (aerial part) on the Sporulation of Dermatophytic Fungi *M. gypseum*

Treatment (ppm)	Chloroform	Ethyl acetate	Ethyl alcohol
1000	+++	+++	+++
2000	++	+++	+++
3000	+	++	+++
4000	+	++	+++
Control	+++	+++	+++

Note. Sporulation: + Poor, ++ Moderate, +++ Good.

Our detailed antifungal bioassay of the different solvent extracts (aerial and root parts) of the plant against dermatophytic fungi *M. gypseum* confirms the presence of significant inhibitory effect of chloroform fraction of the aerial part of the plant. Activity guided chromatographic separation of chloroform fraction of aerial part and its chemical characterization for bioactive principle has been initiated. Preliminary qualitative test of chloroform fraction indicates the presence of glycocidal flavanoids unit. The present communication establishes the antifungal activity of aerial part of the plant *P. amarus* against dermatophytic fungi *M. gypseum.*

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