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ANTIFUNGAL ACTIVITY OF *Phyllanthus niruri L.*

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ABSTRACT

Integrated disease management is a non-separable part of all the eco-friendly stable agricultural programs. Plant extracts have played significant role in the inhibition of pathogens and in the improvement of crop production. Phyllanthus niruri L. Roxb. is a parasitic plant. Agriculturalists consider Phyllanthus niruri L. spp is only a destructive weed and attempt to eradicate it. The main purpose of this study was to assess its significance in developing plant based formulations for fungal disease management. In vitro, antifungal potential of C. reflexa (parasitizing Bougainvillea glabra L.) extract was evaluated against two different pathogenic fungi namely, Candida albicans, Aspergillus fumigates Different aqueous concentrations (100, 150, and 200) of Phyllanthus niruri L.were prepared. Aqueous concentrations of Phyllanthus niruri L.were evaluated against fungal isolate by well diffusion method. The fungitoxicity of extract in terms of inhibition zone diameter was calculated. Results indicated that fungal growth inhibition was directly proportional to the concentration of Phyllanthus niruri L.extract. Phyllanthus niruri L. extract exhibited significant antifungal activity against all test fungal isolates. However, extract was highly effective Candida albicans, Aspergillus fumigates. It was also found that 200 (mg/ml) concentration was significantly effective in reducing the Flucanazoles growth of fungal isolates after 6 days of incubation. Further investigations however are required to analyze nature of antifungal compounds in Phyllanthus niruri L.and their stability.

Key words: *Phyllanthus niruri L., parasitic plants, antifungal extract, pathogenic fungi, parasitic weeds*

Introduction

Phyllanthus niruri L. Roxb. is an angiospermic hustorial advance, obligate parasite belonging to family convolvulaceae. Members of this family are holoparasitic plants subsisting on other dicotyledonous plants (Alberta, et al., 2006). These plants can parasitize on very wide variety of plants including a number of agricultural and horticultural crop species such as alfalfa, lespedeza, flax, clover and potatoes. These can grow on common ornamental plants through the plans of Pakistan (Bhattacharya, 1976; Malik et al., 1980). Agriculturalists consider Phyllanthus niruri L. species a destructive weed and attempt to eradicate it.

However on biological aspects, strong antifungal and antibacterial compounds have been extracted from Phyllanthus niruri L.(Loffler et al., 1997; Qin et al., 2000). Its therapeutic properties such as anticancer, antidiabetic, antiviral, and anti-inflammatory are also well documented (Awasthi, 1981; Poudel, 2002). Studies have also proved that the presences of some stable phytochemicals in Phyllanthus niruri L. species are irrespective of host plants and locations (Loffler et al., 1997).

Plant metabolites and plant-based pesticides appear to be one of the better alternatives as they are known to have minimal environmental impact and in the improvement of crop production (Varma and Dubey, 1999; Nwachukwu and Umechuruba, 2001). Conventional breeding of disease-resistant cultivars and plant protection based on extensive use of agrochemicals represent still common strategies in the contest between human approaches to combat microbial food competitors and the evolutionary adaptability of bacterial and fungal phytopathogens. These are responsible for enormous crop losses worldwide and therefore threaten human nutrition (Osusky et al., 2000). Integrated disease management is a non-separable part of all the eco-friendly stable agricultural programs. Biological control of plant diseases and plant pathogens is of great significance in forestry and agriculture (Inderjit, 2006). Therefore, new strategies to fight phytopathogens have to be explored (Moffat, 2001). Keeping in view the strong phytochemical potential of Phyllanthus niruri L., this study has been carried out to evaluate antifungal activity against important pathogenic fungal species with the ultimate aim of developing plant based formulations for plant disease management.

MATERIAL AND METHODS

Procedure:- The method was carried out according to the NCCLS guidelines.

Anti-fungal Screening by Cup Plate method.

This method is based on diffusion of antifungal component from reservoir hole to the surrounding inoculated Saboraud dextrose Agar medium, so that the growth of fungus is inhibited as zone around the hole. Two fungi were selected viz. s, Aspergillus fumigates and *Candida albicans.*

A. Preparation of inoculum

The suspension of fungus was prepared as per Mac-Farland nephelometer standard(Havsteen et. al., 1983). A 24 hr. old culture was used for the preparation of fungus suspension.A suspension of fungus was made in a sterile isotonic solution of sodium chloride and the turbidity was adjusted such that it contained approximately 1.5 X 10^6 cells / ml. It was

obtained by adjusting the optical density (650 nm) equal to 1.175% barium chloride in 100 ml of 1% sulphuric acid.

B.Sample preparation

The twenty extracts of Phyllanthus niruri L. were dissolved in DMSO to get a concentration of 200 mg/ ml.

C. Culture medium

Saboraud dextrose Agar medium (Hi Media) was used for preliminary antifungal activity. The medium was prepared by dissolving in water and autoclaving at 121^0 C for 15 minutes.

D. Standard Preparation

Fluconazole standard was prepared at a concentration of 10 µg / ml in sterile distilled water.

Nutrient Medium

Table 5 Sabouraud's Agar Medium*

Sl.No	Ingredients	Weight in g
1	Dextrose	40
2	Peptone	10
3	Agar	20
4	Distilled water	q. s. 1000 mL

******pH 5.6 was maintained for nutrient media.*

This medium was used for both sub culturing and also for estimating the antifungal activity. The pH of the medium plays an important role for the growth of fungi. Acidic medium favours

the growth but excess of acid will not allow agar to solidify. Hence, the pH of medium was adjusted using 0.1% lactic acid.

E. Antifungal screening of extracts

The extracts (pet ether, chloroform, ethanol, methanol and 40% methanolic) of propolis were subjected to standardized antifungal screening procedure by agar gradient method.

F. Preparation of assay medium

The above mentioned quantities of different ingredients were accurately weighed and dissolved water. The medium so prepared was sterilized by autoclaving at 121 ^{0}C for 15 minutes.

Working procedure

An inoculum was prepared by suspending a single isolated colony in about 5 ml of normal saline. This is mixed slowly to achieve a smooth suspension. Later, one drop of tween 20 was added for filamentous fungi and the mould was broken by shaking. A sterile cotton swab was moistened in the inoculum suspension and excess of moisture was removed by rolling the cotton swab on the inside of the tube, above fluid level 30 ml of sterile hot Sabouraud's agar medium was poured in each plate and allowed to harden on a level surface. The surface of Sabouraud's agar medium plate was streaked with the help of moistened cotton swab in all the direction ions. The surface of Sabouraud's agar plate was dried out 28^{0}C. Later, 4 bores per plate were made using sterile cork borer. The above procedure was carried out in aseptic condition and 0.1 ml test solution was added to the respective bore and 0.1 ml fluconazole was taken as standard reference. A control using DMSO was maintained in each plate. The plates were incubated at 28^{0}C for 48 hr. Later the values of zones of inhibition were recorded in triplicate and reported in Standard Error Mean (± SEM).

Plant material and extraction: Fresh plant material of Phyllanthus niruri L. was collected from Muzaffarnagar (U.P.) area. Plant material was washed thoroughly under running tap water, dried with blotting paper and

cut into small pieces. A 50% (w/v) aqueous extract was prepared by homogenizing plant material (50 g) in a blender with 100mL sterile distilled water for 5 min and allowed to stand for 24 hours. The mixture was filtered through muslin cloth and then centrifuged at 4000 g for 30 min; the supernatant was filtered through Whatman No.1 filter paper to remove the debris. Three concentrations (10, 20 and 30 %) were prepared by dilution of stock solution. Each concentration was simmered at 40^0 C for 15 minutes, cooled and stored at 4° C. To avoid contamination and prospective chemical change, the extracts were used within 2-3 days. Microorganism Pathogenic fungal isolates namely, Candida albicans, Aspergillus fumigates were obtained from the Microbial Institute of Technology (IMTECH) Chandigarh. Fungal cultures were maintained on 2% Malt extract agar (MEA) medium at 25.2^0 C for six days.

Antifungal Assay- Well Diffusion Technique: Three concentrations (100,150 and 200 mg/ml) were evaluated against five different fungal pathogens. Antifungal activity of Phyllanthus niruri L.extract was assayed by well diffusion method (Rani et al., 2008) with some modification. For antifungal assay, inoculum disc (10mm) was prepared aseptically from seven days old culture. Wells (10mm in diameter) were made on 2% MEA plates using a sterile glass tube. Each plate contained three wells, evenly distributed around the inoculum disc of test fungi that was placed aseptically at the center. For each test concentration, 100µL extract was poured into each well. Each treatment was replicated thrice. For control, sterile distilled water was filled in wells. The plates were incubated at 25.2^0 C for 6 days.

The clear zone and graph surrounding each well indicated inhibition activity of Phyllanthus niruri L.. The diameter of colony inhibition was determined after subtracting the colony diameter of control from treated. All the data was analyzed statistically.

RESULTS AND DISCUSSION

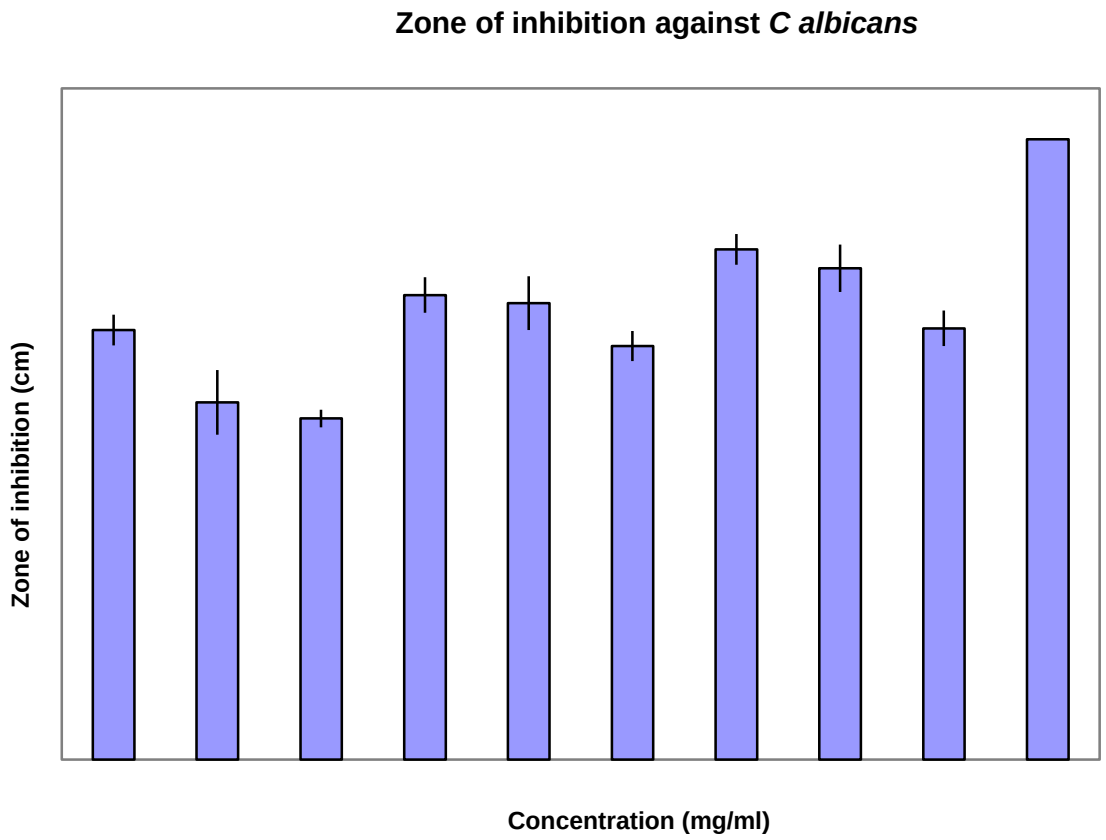
Antifungal activity of Phyllanthus niruri L.was analyzed by well diffusion assay against two pathogenic fungi. The results are shown in Fig1-6 and Graphs1, 2. The diameter of colony inhibition is actually the level of antimicrobial activity present in the extract, a greater diameter of inhibition means that antimicrobial is more potent. Results showed that Phyllanthus niruri L.extract was significantly effective against all test fungi. The Phyllanthus niruri L.extract exhibited variable degree of inhibition with respect to each test fungi. Phyllanthus niruri L.extract was more active against Candida albicans, Aspergillus fumigates (Fig 1 - 6),(Graphs 1,2). It had been observed that at 100mg/ml, Phyllanthus niruri L.extract showed least anifungal activity while 150mg/ml and 200mg/ml, showed significant inhibition potential against Candida albicans, Aspergillus fumigates . Results also indicated that size of inhibition diameter was directly proportional to the

concentration of Phyllanthus niruri L.extract. Studies have reported growing interest in antimicrobial potential of Phyllanthus niruri L. Diameter of colony inhibition (mm) Concentrations of Phyllanthus niruri L. extract (% w/v).

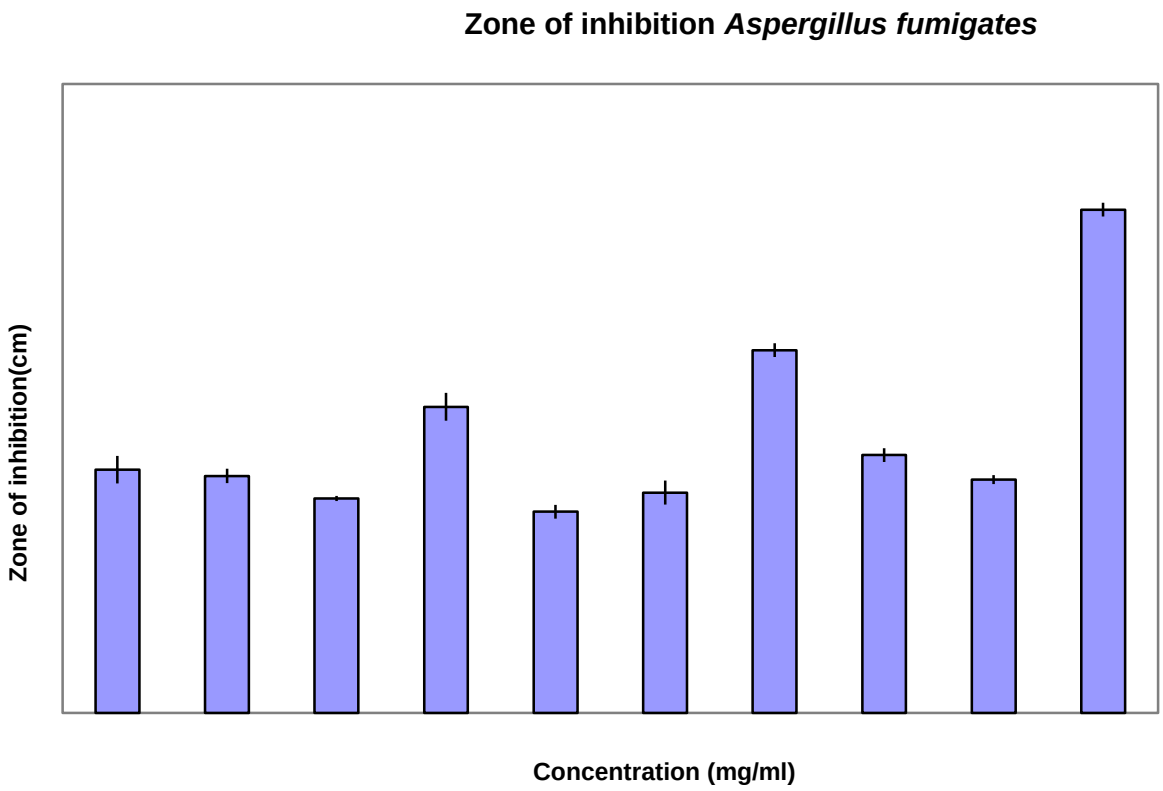
Plant	**Treatment**	**Dose (mg/ml)**	**Zone of inhibition (mm) (Mean±SEM)**	
			Candida albicans	***Aspergillus fumigates***
Phyllanthus niruri L.	Ethyl acetate extract	100	16±0.57**	11.6±0.66*
		150	17.3±0.66*	14.6±0.66*
		200	19±0.57**	17.3±0.33**
	Ethanol extract	100	13.3±1.20**	11.3±0.34*
		150	17±1.00**	9.6±0.33**
		200	18.3±0.88**	12.3±0.33*
	Aqueous extract	100	12.7±0.33*	10.23±0.12**
		150	15.42±0.56**	10.51±0.57*
		200	16.06±0.66**	11.13±0.21**
	Fluconazole	2ml/10ml	23.1± 0.00**	24±0.33*

Each value represent Mean±SEM, n=5. One-way ANOVA followed by Dunnet test through Instat software, compare all vs. standard applied. Statistically significant at **P<0.01, *P<0.05.

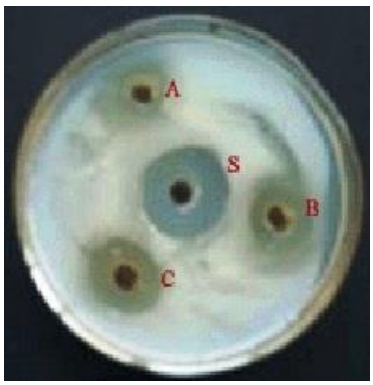
Phyllanthus niruri L. showed significant results (P<0.01) by applying one way ANOVA (Dunnet test). Ethyl acetate extract of *Phyllanthus niruri L.* was more effective than ethanolic extract of *Phyllanthus niruri L.* against *E. coli* (Gram –ve). Least active extract was ethanolic extract of *Phyllanthus niruri L.* against *E. coli* (Gram –ve). In case of antibacterial activity against (Gram –ve) *Pseudomonas aeruginosa*, ethyl acetate extract of *Phyllanthus niruri L.* more active followed by ethyl acetate extract and least active was ethanolic extract.

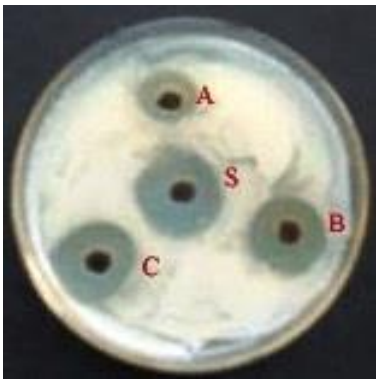


Graph 1 – Graphical representation of Antifungal activity against *C. albicans*



Graph 2 – Graphical representation of Antifungal activity against *A.fumigates*





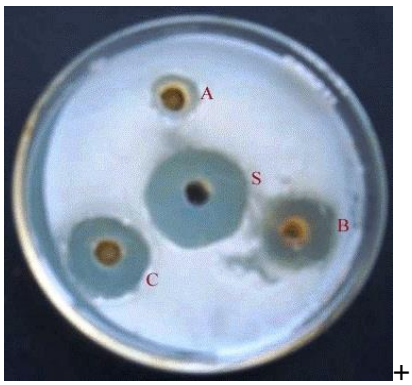


Fig. 1: Aqueous extract **Fig. 2**: E.A extract **Fig. 3**: Ethanol extract

Fig. 1-3 : Zone of inhibition of *Phyllanthus niruri L.* extracts against *C. albicans*

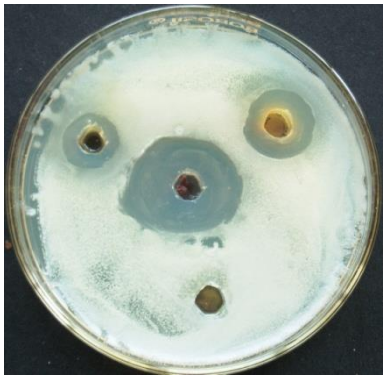


Fig. 4: Aqueous extract

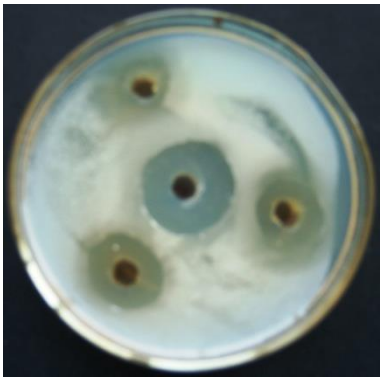


Fig. 5: E.A extract

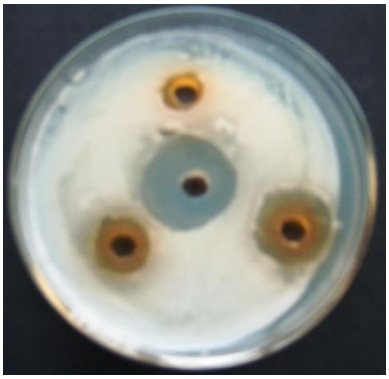


Fig. 6: Ethanol extract

Fig. 4-5: Zone of inhibition of *Phyllanthus niruri L.* extracts against *A. fumigates*

The significant results were also shown against (Gram +ve) *Streptococcus aureus* by ethanolic extract followed by ethyl acetate extract. The least activity was shown by ethanolic extract of *Phyllanthus niruri L.* . From above discussion, it was concluded that ethyl acetate extract of aerial parts of *Phyllanthus niruri L.* bear best antibacterial activity against Gram-ve bacterias.

Antifungal activity was also performed on different extracts of *Phyllanthus niruri L.* against *Candida albicans* and *Aspergillus fumigates*. Ethyl acetate extract of *Phyllanthus niruri L.* showed significant results against *Candida albicans* which was further followed by ethanolic extract of *Phyllanthus niruri L.* . In case of antifungal activity of different extracts against *Aspergillus fumigatus*, ethyl acetate extract of *Phyllanthus niruri L.* was more effective followed by ethanolic extract of *Phyllanthus niruri L.*. From the above, it was concluded that the ethyl acetate extract of *Phyllanthus niruri L.* has best antifungal activity against *Candida albicans* and *Aspergillus fumigatus*

It has been reported that phenol, flavonoid, diterpenoid, phytosterol are responsible for antimicrobial activity of many plants. So, on the basis of constituents present in *Phyllanthus niruri L.* species, it can be concluded that antimicrobial activity of both the plants is due to these constituents present in them.

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