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Phytochemical Screening and Antifungal Activity of Solvent Extracts of *Averrhoa bilimbi* Leaves against *Aspergillus niger* and *Rhizopus stolonifer*

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ABSTRACT

Background: National Academy of Sciences (NAS) report on pesticide remains on food indicated that fungicides are more of a carcinogenic risk than insecticides and herbicides combined. Therefore, synthetic fungicides are odd in our food chain, and researches are on the way to find safer substitutes. Medicinal plants remain a rich source of therapeutic agents.
Aim: The present study was determined to study the antifungal activity and phytochemicals present in the leaf extracts of the plant *Averrhoa bilimbi* against selected fungal strains *Aspergillus niger* and *Rhizopus stolonifer*.
Methods: Acetone, chloroform and ethanol were the solvents used for soxhlet extraction. Agar well diffusion method was used for antifungal assay and zone of inhibition was measured in millimeters. Phytochemicals of leaf extracts were assessed using standard procedures.
Results: Results revealed that ethanol extracts of *A. bilimbi* exhibited maximum zone of inhibition of 14 mm and 20.4 mm against *A. niger* and *R. stolonifer* respectively. Moderate activity was by chloroform extract against *A. niger* and acetone extract against *R. stolonifer* producing a zone of inhibition of 13 mm and 15.2 mm at higher concentration respectively. The extracts of *A. bilimbi* when tested revealed the presence of various phytochemicals.
Conclusion: The objective of new antifungal strategies is to develop drugs that combine sustainability, high efficacy, restricted toxicity, safety for humans, animals, host plants and with low production cost. So it may be concluded that the solvent extracts of present study may be used as a botanical control agent for the control of selected fungal species.

Keywords: Antifungal, Phytochemical screening, *Averrhoa bilimbi*, *Aspergillus niger*, *Rhizopus stolonifer*

1. INTRODUCTION

Fungi are important destroyers of foodstuffs and grains during storage, making them inappropriate for human consumption by retarding their nutritive value and often produce mycotoxins. [1,2] Fungi are abundant in the environment, and infection due to fungal pathogens has become more common. [3,4] The large scale toxic effects are carcinogenicity, genotoxicity, teratogenicity, nephrotoxicity, hepatotoxicity, reproductive disorders and immune-suppression. [5,6]

The development of actions to limit fungal infections may be an effective means for therapeutic interventions. Fungicides based on synthetic chemicals induce serious and chronic environmental pollution, are highly and acutely toxic, and can even cause cancer in humans and wild animals. Pathogens may develop resistance to many of these chemicals. [7] Plant products or plant-derived compounds are likely to provide a valuable source of new medicinal agents [8,9] and the urgent need for alternative treatment has led to screen natural products for potential use in the therapy of fungal infections. Various plants are used as natural medicines without any scientific base. In recent years, several medicinal plants have been screened for the treatment of diseases. [10,11]

Traditional healers confirm that their medicine is cheaper and more effective than modern medicine. These communities have a reduced risk to get infectious diseases from pathogens than people from urban areas treated with traditional antibiotics. One method of prevention of antibiotic resistance of pathogenic species is by using new compounds that are not based on existing synthetic antimicrobial agents. Traditional people claim that medicinal plants are more efficient to treat infectious diseases than synthetic antibiotics. It is necessary to evaluate, the potential use of folk medicine for the treatment of infectious diseases produced by common pathogens. Medicinal plants might represent an alternative treatment in non-severe cases of infectious diseases. [12] Phytochemicals are important in the plant for normal growth development and defense against infection and injury. Flavonoids and other phenolics have been suggested to play a preventive role in the development of cancer and heart disease. [13,14]

In recent years, considerable focus has been given to identifying antioxidant properties of plant materials that may be used for human consumption. [15-18] The selected plant *Averrhoa bilimbi* belongs to the Family *Oxalidaceae*. It is attractive, long lived tropical tree, reaches 16-33 ft (5-10m) in height; has a short trunk soon isolating into a number of upright branches. *A. bilimbi* is used as traditional medicine for treating cough, cold, itches, rheumatism, syphilis, diabetes, whooping cough and hypertension in Asia. [19] The present study describes chemical composition and antifungal activities of different extracts from leaves of *A. bilimbi*.

2. MATERIALS AND METHODS

Collections of test materials

Leaves of *Averrhoa bilimbi* were collected from the Ganapathy area of Coimbatore locale and the specimens were identified; certified (BSI/SRC/5/23/2018/Tech./2477) respectively and the voucher specimen number were deposited at the Botanical Survey of India, Southern Circle, Coimbatore.

Preparation of leaf powder and extracts

Fresh leaves of *A. bilimbi* were air dried under shade. Dried leaves were powdered using an electric pulverizer. Fine powder was obtained by sieving. The powder was subjected to extraction. [20,21] Acetone extraction was followed by chloroform extraction and ethanol extraction so that the powders were subjected to extraction with solvents of increasing polarity. The leaf extracts thus obtained were concentrated by distillation and dried by evaporation in a water bath at 40°C. The residue obtained was stored in tightly closed glass vials in the refrigerator for further use. Antifungal activity was investigated.

Test microorganism

The fungal strains used were the clinical isolates obtained from laboratory culture in the College. The fungal strains used were *Aspergillus niger* and *Rhizopus stolonifer*.

Antifungal assay:

The activity of various solvent extracts of leaves of *A. bilimbi* on selected fungal strains was assayed by agar well diffusion method. For this, method of Murray *et al* [22] later modified by Olurinola [23] was used. Antifungal susceptibility was tested on solid media in petriplates. For fungus Rose Bengal agar was used for developing surface colony growth.

Reagents - Rose Bengal Agar Medium:

One litre of Rose Bengal agar was prepared by dissolving 32.15 g of commercially available Rose Bengal agar powder (Hi media) in 1L distilled water and boiled to dissolve the medium completely. The medium was prepared and poured on to the petriplates and was left on sterile surface until the agar has solidified. The plates were swabbed (sterile cotton swabs) with 24 h old culture of fungal strains. Wells (10 mm diameter and about 2 cm apart) were made in each of these plates using sterile cork borer. Stock solution of each solvent extract viz., acetone, chloroform and ethanol was

prepared at a concentration of 1 mg/ml. The concentrations viz., 10, 20 30 40 µl of different solvent extracts of the leaves of *A. bilimbi* were added into the wells and allowed to diffuse at room temperature for 2h. Forcan acted as positive antifungal control.

The plates were incubated at 37°C for 72 h for fungal pathogens. The antifungal activity was assayed by measuring the diameter of the inhibition zone formed around well. [24] Triplicates were maintained and the experiment was repeated thrice, for each replicates the readings were taken in three different fixed directions and the average values were recorded.

Statistical Analysis:

The antifungal data was interpreted by calculating standard deviation and mean of three replicates.

Phytochemical Analysis:

Qualitative phytochemical screening of leaf extract of selected plant was carried out using the standard procedures.

Test for Alkaloids

- **Mayer's test:** [25] 1 ml of extract was treated with a drop or two of Mayer's test reagent along the sides of test tube and observed for the formation of white or cream coloured precipitate.
- **Wagner's test:** [26] 1 ml of extract was treated with Wagner's reagent along the sides of the test tube and observed for the formation of reddish brown colour precipitate.
- **Hager's test:** [27] 1 ml of extract was treated with 1 or 2 ml of Hager's reagent and observed for the formation of prominent yellow precipitate.

Test for Tannins

- **Ferric chloride test:** [28] 0.5 g extract was stirred with about 10 ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 ml of the filtrate, and observed for the blue-black, green or blue-green precipitate.

Test for Phenols

- **Ferric chloride test:** [29] The extract (50 mg) was dissolved in 5 ml of distilled water and treated with few drops of 5% ferric chloride and observed for the formation of dark green colour
- **Lead acetate test:** [30,31] The extract (50 mg) was dissolved in 5 ml of distilled water and 3 ml of 10% lead acetate solution was added and observed for the formation of bulky white precipitate.

Test for Flavonoids

- **NaOH test:** [28] 1 ml the extract was dissolved in water and filtered; to this 2 ml of the 10% aqueous sodium hydroxide was later added to produce a yellow colouration. A change in colour from yellow to colourless on addition of dilute hydrochloric acid was an indication for the presence of flavonoids.
- **Lead acetate test:** [30,31] Fifty milligram of the extract was taken in a test tube and few drops of lead acetate solution was added to it and observed for yellow coloured precipitate.

Test for Sterols

- **Liebermann-Burchard test:** [32] The extract (50 mg) was dissolved in 2 ml of acetic anhydride. To this one or two drop of Conc. H_2SO_4 was added along the side of the test tube and observed for any colour change.

Test for Terpenoids

- **Liebermann-Burchard test:** [33] A little of extract (50 mg) was dissolved in ethanol. To it 1 ml of acetic anhydride was added followed by the addition of Conc. H_2SO_4. Change of colour from pink to violet indicates the presence of terpenoids.

Test for Saponins

- **Foam Test:** The extract (50 mg) or dry powder was diluted with distilled water and made up to 20 ml. The solution is vigorously shaken for 15 minutes and observed for the formation of 2 cm layer thick foam.

Test for Anthraquinones

- **Borntrager's test:** [34] Extract (0.2 g) to be tested was shaken with 10 ml of

benzene and then filtered. Five ml of the 10% ammonia solution was added to the filtrate, shaken and observed for the appearance of a pink, red or violet colour.

Test for Proteins

- **Ninhydrin test:** [35] Three drops of ninhydrin solution (10 mg of ninhydrin in 200 ml of acetone) was added to 2 ml of extract and observed for the present of characteristic purple colour.
- **Biuret test:** [35] Two ml of extract was treated with one drop of 2% copper sulphate solution. To this 1 ml of 95% ethanol was added followed by excess of potassium hydroxide pellets and observed for the formation of pink ethanolic layer.

Test for Quinones

- **H_2SO_4 test:** [31,20] To 1 ml of extract, 1 ml of Conc. H_2SO_4 was added and observed for the formation of red colour.
- **HCl test:** [36,37] To 1 ml of the extract, 5 ml of HCl was added and observed for the presence of yellow colour precipitate.

3. RESULT AND DISCUSSION

Antifungal activity of leaf extract of *A. bilimbi* against *A. niger*

Antifungal activity of the leaf extract of *A. bilimbi* leaf was studied against fungal strains *Aspergillus niger* and results are presented in Fig 1 & 2. Among the three solvents of *A. bilimbi* leaf extracts tested, ethanol extract showed the highest antifungal activity against *A. niger*. Greatest inhibition was shown at 40 µl with 14 mm inhibition zone, followed by 30 µl with 13.5 mm zone of inhibition. Antifungal activity was found to be concentration dependent and as concentration was decreased antifungal activity was found to decrease. Similar results were observed by Wilson *et al* [38] and reported that plant extracts that showed greatest antifungal activity were those from species of *Allium* and *Capsicum* among the various tested species and was dose dependent. Results parallel were observed by Abhishek *et al* [39] that Hydro-alcoholic extract of *Andrographis paniculata* possessed potent antifungal activity amongst all the hydroalcoholic extracts of other plants against *A. niger* while hydroalcoholic extracts of *Achyranthes aspera* showed similar antifungal activity against *Candida albicans* and *Aspergillus niger.*

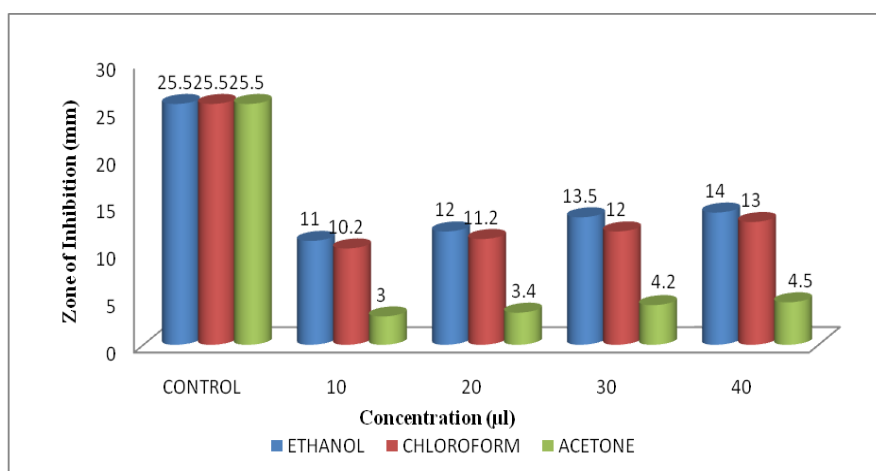

Fig 1: Graph showing antifungal activity of *A. bilimbi* leaf extracts against *A. Niger*

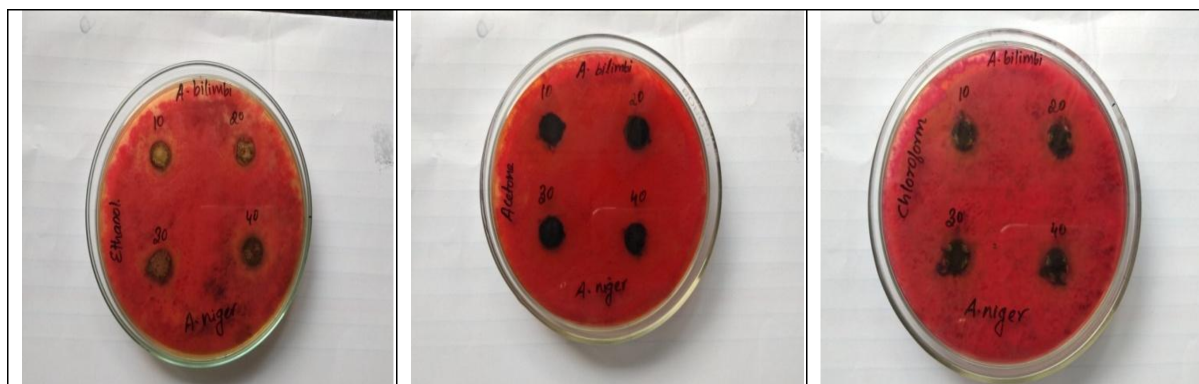

Fig 2: Plate showing antifungal activity of *A. bilimbi* leaf extracts against *A. niger*

Among the three solvents, moderate antifungal activity was shown by chloroform extract of *A. bilimbi* leaf showing highest inhibitory potential thereby producing a zone of inhibition of 13 mm at a concentration of 40 µl, followed by 30 µl concentration that displayed a zone of 12 mm. Similar results were recorded by Satish *et al* [40] who reported that the aqueous extract of *Acacia nilotica*, *Achras zapota*, *Datura stramonium*, *Emblica officinalis*, *Eucalyptus globules*, *Lawsonia inermis*, *Mimusops elengi*, *Peltophorum pterocarpum, Polyalthia longifolia*, *Prosopis juliflora*, *Punica granatum* and *Sygigium cumini* revealed significant antifungal activity against one or the other *Aspergillus* species tested.

Minimum antifungal activity among the three extracts was showed by acetone extract. The concentration of 40 µl marked an inhibitory zone of 4.5 mm, followed by 30 µl concentration which recorded an inhibitory zone of 4.2 mm. Similar results were observed by Mudasir *et al* [41] that acetone extract of *L. camara* leaves inhibited the fungal growth of *A. niger* by 76.74% at 20% concentration, followed by chloroform extract (68.99% at 20% concentration).

Antifungal activity of leaf extract of *A. bilimbi* against *R. stolonifer*

Antifungal activity of the leaf extract of *A. bilimbi* leaf was studied against fungal strain *Rhizopus stolonifer* and results are presented in Fig 3 & 4. Among the three solvents of *A. bilimbi* leaf extracts tested, against *R. stolonifer,* ethanol showed the highest antifungal activity. Greatest inhibition was shown at a concentration of 40 µl, which produced a zone of inhibition of 20.4 mm followed by 16.6 mm inhibition zone at 30 µl concentrations. The positive control Forcan exhibited an antifungal potential of 25.5 mm. Similar results were seen by Buch and Arya [42] who reported that methanol fractions exhibited more promising results than aqueous fractions in suppressing the fungal growth. The periodic data regarding fungal growth, exposed to various concentrations of plant extracts were of *Annona reticulata, Balanites roxburghii, Cochlospermum religiosa, Gliricidia sepium, Limonia acidissima, Sapindusem arginatus* and *Tephrosia jamnagarensis.*

Moderate antifungal activity among the three solvents of *A. bilimbi* leaf extracts against *R. stolonifer* was shown by acetone extract that showed an inhibition zone of diameter 15.2 mm at 40 µl concentration, followed by the concentration of 30 µl with 14.2 mm zone of inhibition. Similar study was carried out by Venkatasaichaitanya *et al* [43] in that the antifungal properties of more than 50 plant extracts against two important human and agricultural pathogens, *Aspergillus niger* and *Rhizopus stolonifer*. Multiple extracts exhibited promising antifungal values.

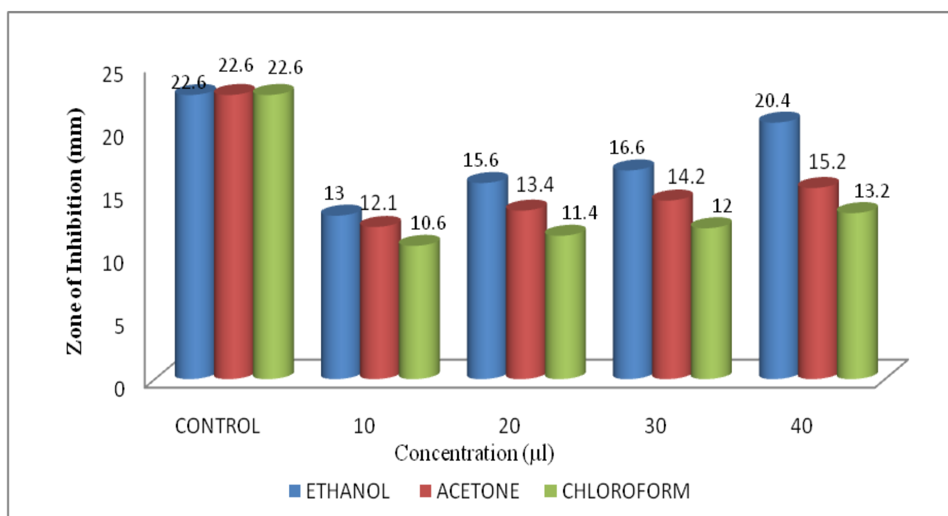

Fig 3: Graph showing antifungal activity of *A. bilimbi* leaf extracts against *R. Stolonifer*

Fig 4: Antifungal activity of *A. bilimbi* leaf extracts against *R. stolonifer*

Among the three extracts chloroform showed the minimum antifungal activity. The highest concentration of 40 µl showed an inhibitory zone of 13.2mm, followed by an inhibition zone of 10.6 mm at 30 µl concentration. From the results of antifungal activity, solvent extract of *A. bilimbi* showed highest inhibitory potency against *R. stolonifer* than *A. niger*. Similar results were observed by Swapna [44] screened *in vitro* antifungal activity of aqueous and solvent extracts of leaves of *Azadiracta indica*, *Callistemon citrinus*, *Eucalyptus lanceolatus* and *Pongamia pinnata* against some dominant fungal species viz. *Chaetomium spiralis*, *Alternaria alternata*, *A. flavus*, *A. niger*, *A. fumigates* and *R. stolonifer* were tested. Plant extracts in solvents exhibited potent inhibitory activity than aqueous extracts. However, these plant extracts exhibited moderate activity against *A. flavus*, *C. spiralis*, *R. stolonifer* and *A. alternata.*

Phytochemical analysis of *A. bilimbi* leaves

Phytochemical analysis is carried out in ethanol, chloroform and acetone extracts of *A. bilimbi* leaves. In the present study maximum antifungal activity was exhibited by ethanol extract of *A. bilimbi* against *A. niger* and *R. stolonifer.* In the present study the *A. bilimbi* leaf ethanol extract, tested for its phytochemicals revealed the presence of 7 phytochemicals viz., alkaloids, terpenoids, sterols, tannins, saponins, flavonoids and quinones. Similar observations were reported by Madhu *et al* [45] that reported that the ten selected plants species were subjected to quantitative analysis by standard methods. Various organic solvents from selected parts of the ten plants were analysed for alkaloids, flavonoids, phenols, steroids and saponins.

Table 1: Phytochemicals present in *A. bilimbi* leaf extracts

Sl. No.	Constituents	*A. bilimbi* leaf		
		Acetone extract	Chloroform extract	Ethanol extract
1	Alkaloids	+	+	+
2	Flavonoids	+	+	+
3	Sterols	+	+	+
4	Terpenoids	-	-	+
5	Anthroquinones	-	-	-
6	Phenols	+	-	-
7	Saponins	-	+	+
8	Tannins	+	-	+
9	Proteins	-	-	-
10	Quinones	-	-	+

"+" Presence "-" Absence

The acetone extract of the *A. bilimbi* leaf showed the moderate activity against *R. stolonifer* and minimum antifungal activity against *A. niger*, showed the presence of 5 phytochemicals viz., alkaloids, phenols, sterols, tannins and flavonoids. Chloroform extract of *A. bilimbi* leaf showed minimum antifungal potency against *R. stolonifer* and moderate activity against *A. niger* when tested for phytochemicals recorded the presence of 4 phytochemicals viz., alkaloids, sterols, saponins and flavonoids. Similar results were observed by Suman *et al* [46] and reported that in *Celestrus emarginata,* alkaloids, glycosides, resins and quinones showed maximum presence in acetone extracts, phenols are maximum in methanol extracts and quinones are maximum in aqueous, methanol and acetone extracts. Sunita *et al* [47] have reported that phytochemical tests of the plant *Combretum roxburghii* leaf and bark showed the presence of flavonoids, tannins and saponins.

4. CONCLUSION

The leaf extracts of the plant *A. bilimbi* showed prominent antifungal activity against *A. niger* and *R. stolonifer*. The use of these solvent extracts in the treatment of pathogenic diseases associated with the infection of these pathogens is validated, scientifically supported by the results obtained in this work. The present study justified the claimed uses of leaves in the traditional system of medicine to treat various infectious disease caused by the selected fungal species. However, further studies are indeed necessary to better evaluate the potential effectiveness of the crude extracts as the antibacterial agents. The results of the study will form the base for selection of plant species for further investigation in the potential discovery of new natural bioactive compounds. Further studies that aim at the isolation of antibacterial active constituents from the plant have been initiated.

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