

In Vitro Antifungal Activity of Ethanolic Extract of *Acacia leucophloea* (Roxb.) Willd. Against Selected Pathogenic Fungi Using Agar Disc Diffusion Method

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Abstract

Medicinal plant research provides important opportunities for identifying natural antifungal agents for managing fungal infections. However, the antifungal potential of *Acacia leucophloea* against selected pathogenic fungi remains insufficiently explored. This study aimed to evaluate the in vitro antifungal activity of the ethanolic aerial-part extract of *A. leucophloea* and compare its activity with clotrimazole. Aerial parts collected from Tirunelveli, Tamil Nadu, were authenticated, dried, powdered, macerated with ethanol, filtered, and concentrated by solvent evaporation. Phytochemical screening was performed to identify major bioactive constituents. Antifungal activity was evaluated against *Candida albicans* and *Aspergillus niger* using the agar disc diffusion method at 250, 500, and 1000 µg concentrations. Inhibition zones were recorded. The 1000 µg concentration generally produced greater inhibition than lower concentrations, although activity remained lower than clotrimazole. The findings demonstrate measurable antifungal potential and support further investigation of *A. leucophloea* as a source of bioactive antifungal compounds.

1. Introduction

Plants with medicinal properties are a source of therapeutic compounds and have been serving as a source of such compounds through time for therapeutic uses due to primary and secondary metabolites of these plants which exhibit different biological effects [1]. Numerous phytochemicals found in plant extracts, such as steroids, flavonoids, alkaloids, terpenoids, phenolics, and other specialized metabolites, are significant in terms of their biological characteristics and are presently being investigated for application in the development of antimicrobial medications [2]. Natural products are now gaining attention as potential sources of antifungal agents as they can target multiple cell targets and mechanisms [3]. Fungi, especially *Candida* spp., are an important therapeutic problem due to limited number of antifungal drugs and the risk of resistance reducing the effectiveness of the treatment [4]. As a result, extracts of plants have recently been explored as a source of antifungal drugs. Inhibitory activity of medicinal plant extracts against *Candida albicans* has been reported recently and interactions of medicinal plant extracts with conventional antifungal drugs like clotrimazole has also been reported [5].

Experimental studies also have shown that the antifungal activity may be different in various plant species, extraction techniques, extract composition and concentration [6]. Several candidates from the traditional use of medicinal plants have been tested against various *Candida* species, and exhibited activity in diffusion and broth microdilution assays [7]. Plant extracts have also demonstrated antifungal efficacy against *Candida albicans* in different experimental settings. Further research on natural antifungal chemicals in vitro and in vivo indicates that plant-based materials may be utilized to find novel antifungal medicines [8]. But the variability in phytochemical content, extraction methods, activity and toxicity, as well as standardization, can be significant in the assessment of plant-based antifungal preparations [9]. The study of plant extracts against drug resistant *C. albicans* also suggests the need for further research into natural sources of antifungal agents [10]. Hence, the present study aims at exploring the in vitro antifungal effect of aerial part ethanolic extract of *Acacia leucophloea* (Roxb.). Resistance to selected test fungi on the agar disc diffusion method: Willd. Phytochemical screening is also conducted to determine the major constituents of the extract and the antifungal activity is determined and compared with the reference antifungal drug clotrimazole.

1.1 Research Aim

The aim of the present study is to explore the in vitro anti-fungal activity of *Acacia leucophloea* (Roxb.). Selected pathogenic fungi (Wild.) are tested by agar disc diffusion method. This study also intends to compare the effect of the plant with the reference drug, an antifungal drug, clotrimazole.

1.2 Research Objectives

- To choose, gather, identify and analyze the plants.
- To choose appropriate solvent and extraction technique for the process.
- To perform preliminary phytochemical test for identification of phytochemicals.
- To check the antifungal activity of Clotrimazole induced fungi on Agar medium.
- To determine the importance of observed variations in antifungal activity of plant extract and various fungal strains at different concentrations.

2. Related Works

Yehia et al. [11] studied the phytochemical constituents and antifungal properties of extraction of ethanol from *Rorippa islandica* and *Carrichteria annua* against five fungi causing disease. Phytochemical characterization was done by gas chromatography mass spectroscopy and high-performance liquid chromatography while antifungal activity was evaluated by agar diffusion. The chemical profiling combined with biological evaluation was a strength of the study. It however concentrated primarily on phytopathogenic fungi, with little exploration of the evaluation of fungi associated with humans.

Bhattacharya et al. [12] studied the activity of α -phellandrene against antibiotic resistant *Candida albicans* using well diffusion and combination method with conventional antifungal drugs. The compound was inhibitory and exhibited synergistic interactions with some of the antifungals. The most significant contribution of the study was to the area of resistant *C. albicans* and combination therapy. It classified, however, an isolated compound, not a crude medicinal plant extract and thus whole plant antifungal screening is left as an area of research.

Hajji-Hedfi et al. [13] investigated the phytochemical makeup and antifungal characteristics of extracts from *Juniperus phoenicea*, *Pistacia lentiscus*, and *Eucalyptus globulus* against *Alternaria alternata* and *Colletotrichum gloeosporioides*. A strong point was the incorporation of the phytochemical analysis and antifungal testing. The study had a focus on postharvest plant pathogens but did not include the selected human associated fungi which resulted in the antifungal activity of other medicinal plants not being sufficiently explored.

Al-Otibi et al. [14] studied the antifungal activity of ethanolic extract of *Rhazya stricta* in comparison to the silver nanoparticles synthesized biologically against pathogenic fungi. The comparative design showed the potential of both plant extract and the formulation of the plant extract as nanoparticles. It was its ability to assess conventional and enhanced preparations that was its strength. The nanoparticle approach, however, is more complex in the experiments, and doesn't explicitly study the effects of *Acacia leucophloea* extract against the selected test fungi with clotrimazole as a reference.

Mwinga et al. [15] identified five botanicals by phytochemical profiling and tested their antifungal activity against *Pythium ultimum* and *Botrytis cinerea*. Various phenolic, flavonoid and other compounds were identified and antifungal activity was measured. A great plus point was the holistic treatment of the phytochemicals. The work was however restricted to fungal pathogens of agriculture and the in vitro results need to be confirmed and the evaluation of medicinal plants against selected pathogenic fungi was not explored enough.

2.1 Research Gap

The medicinal plant and the compounds derived from them are found to exhibit good antifungal activity in recent studies, but there are some research gaps. The present investigation was carried out to compare the selected human associated fungi with the crude medicinal plant extracts as well as existing investigations which are mostly phytopathogenic fungi, bioactive compounds or sophisticated formulations like silver nanoparticles. Moreover, there is very little research on the antifungal activity of the aerial part extract of *Acacia leucophloea*, especially its ethanolic extract. The current literature also lacks adequate data regarding the concentration-dependent inhibition of *Candida albicans* and *Aspergillus niger* using a standardized method. A direct comparison of the activity of the plant extract with an appropriate antifungal drug (clotrimazole) is also poorly discussed. Furthermore, screening with phytochemical and antifungal activity together with statistical assessment may give a more comprehensive picture of the activity of extracts. Hence, the present study focussed on filling the above mentioned gaps and

investigated ethanolic extract of *Acacia leucophloea* against selected test fungi and compared inhibition with clotrimazole.

3. Materials and Methods

The antifungal activity of aerial parts of *Acacia leucophloea* against selected pathogenic yeasts was assessed. The collected plant material from Tirunelveli in January 2025 was authenticated, washed, air dried, powdered, sieved and stored. The powder was macerated with ethanol and streamed and solvent dispersed to concentrate the powder. Crude isolate was stored in the appropriate manner. The following equipment was used: incubator, laminar air-flow cabinet, autoclave, digital balance and glassware. Clotrimazole was used as the reference antifungal agent.

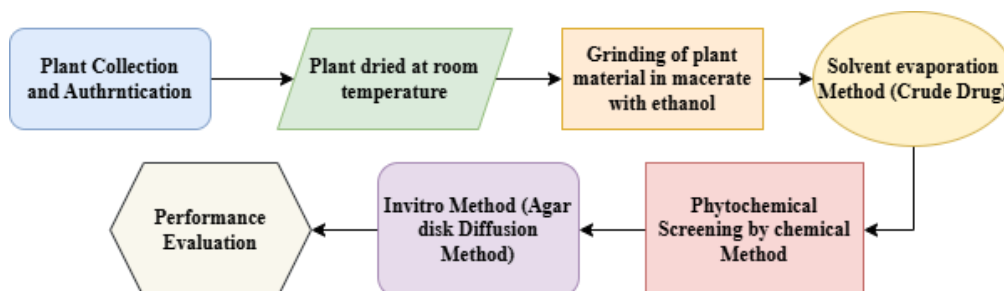


Fig. 1. Overall workflow of the experimental study for evaluating the in vitro antifungal activity of *Acacia leucophloea* using the agar disc diffusion method

Fig. 1. shows the workflow of the plant collection and authentication, room temperature drying, grinding and ethanol maceration, solvent evaporation, phytochemical screening, in vitro antifungal evaluation by agar disc diffusion and performance evaluation of prepared plant extract.

3.1 Plant Collection, Authentication and Preparation

The leaves, stem and branches of *Acacia leucophloea* (Roxb.) The specimens of Wild. were collected from Tirunelveli, Tamilnadu, India in the month of January, 2025. The recognition and validation of the plant material was done by Dr.S.Dr.Ruchi Sharma, Botanist, ACME Research Solutions, Pharmaceutical Lab, Delhi, India-110094. The fresh aerial parts were authenticated and washed and dried in air at room temperature. The dried material was sorted and ground with mechanical grinding machine. The powdered material was then sieved through 40-mesh and kept in an airtight container for further extraction.

3.2 Ethanolic Extraction

Powdered aerial plant material was extracted using maceration. The powdered material was put into a stoppered container and ethanol was added. The container was tightly closed and left for the duration of the extraction and shaken or stirred occasionally to ensure complete extraction. The extract obtained after maceration was filtered to remove the marc from it. The filtrate obtained was collected for evaporation of solvent.



Fig 2. Maceration

Fig. 2. shows a maceration apparatus with the powdered plant material in the solvent in a closed container. The dark extract is visible in the bottom part, and the container is tightly closed and kept upright while extracting.

3.3 Solvent Evaporation and Extract Storage

The filtered extract was then dried up by heating it on a heating mantle and hot plate. The temperature was checked using the control panel and the solvent was continuously stirred to achieve uniform temperature distribution. Evaporation was carried on until the crude ethanolic extract was obtained. The extract was then placed in an air tight waterproof container and kept in a cool place or a desiccator filled with calcium chloride until further studies.

3.4 Materials and Laboratory Equipment

The experimental study was carried out using incubator, laminar air-flow cabinet, autoclave and digital balance. All the experiments were carried out using borosilicate glassware from Borosilicate Crystal Scientific Ltd., Tirunelveli. The reference drug Clotrimazole was obtained from Shiv Scientific, Tirunelveli, India.

4. Results and Discussion

The findings from this study were found to be assessed by organoleptic properties, extractive value, phytochemical screening and antifungal activity. The inhibition zones formed by the extract of *Acacia leucophloea* plant were compared with the clotrimazole against the selected pathogenic yeasts.

4.1 Organoleptic Characteristics and Water-Soluble Extractive Value

The organoleptic characteristics and water-soluble extractive value were determined. The organoleptic properties of the extract were determined by its colour, odour, flavour and appearance. The extractive values of water-soluble components were calculated by macerating with distilled water, filtering, evaporating, drying and weighing to determine the amount of water-soluble components.

Table 1. Determination of Water-Soluble Extractive Value

Parameter	Value
Weight of powdered drug	4.00 g
Empty china dish	83.52 g
China dish + dried extract	84.95 g
Weight of dried extract	1.43 g
Equivalent extract for 100 mL	2.86 g
Water-soluble extractive value	71.5% w/w

Table 1 shows the water-soluble extractive value was determined using 4.00 g of powdered drug. The dried extract yielded was 1.43 g which is equivalent to 2.86 g per 100 mL of extract solution. The water soluble extractive obtained was 71.5% w/w, which is the percentage of water soluble constituents in the crude drug.

4.2 Phytochemical Screening

The ethanolic extract was exposed to phytochemical screening to determine the occurrence of the major bioactive constituents of *Acacia leucophloea* by using standard chemical methods. The screening involved testing various groups of phytochemicals and the observations made on the basis of the various qualitative reactions. All the identified constituents were considered in discussion of the observed antifungal activity.

Table 2. Phytochemical Screening of Ethanolic Extract

Phytochemical Constituent	Test Performed	Observation	Inference
Carbohydrates	Molisch's, Fehling's, Benedict's	Purple/reddish-violet ring; brick-red/red precipitate	Present
Glycosides	Borntrager's, Keller-Killiani	Pink/violet colour; reddish-brown to blue colour	Present
Sterols and Steroids	Salkowski	Cherry-red/purple colour and green fluorescence	Present

Flavonoids	Shinoda, Alkaline reagent	Magenta and yellow colour	Present
Fixed oils and fats	Spot, Saponification	Permanent oil appearance; soap formation	Absent
Phenolic compounds	Ferric chloride, Lead acetate	Bluish-black colour; yellow precipitate	Present
Tannins	Potassium dichromate, Potassium ferricyanide	Yellow and red colour	Absent
Proteins and Amino acids	Biuret, Ninhydrin, Xanthoprotein, Millon's, Tannic acid	Violet, blue, orange, red and white colours	Absent
Saponins	Frothing test	Frothing observed	Absent
Alkaloids	Wagner's, Dragendorff's, Mayer's	Reddish-brown precipitate; orange colour; cream precipitate	Present
Coumarins	Ferric chloride	Green colour	Absent

Table 2 indicates the phytochemical screening of the ethanolic extract of *Acacia leucophloea* was done with various qualitative chemical tests. Carbohydrates, glycosides, sterols and steroids, flavonoids, phenolic compounds and alkaloids were detected in the extract. The test reactions gave the results of the absence of fixed oils and fats, tannins, proteins and amino acids, saponins and coumarins.



Fig. 3. Results of Phytochemical Screening

Fig. 3. shows a phytochemical screening experiment using several test tubes arranged in a blue test-tube rack. The various plant extract samples are placed in the tubes and after qualitative chemical testing, they are seen to have varying colours and precipitates. Benedict's, Fehling's, Salkowski's, Shinoda's, Saponification's and the Keller-Killiani test can be seen.

4.3 In-Vitro Antifungal Activity

The selected pathogenic yeasts were tested for antifungal activity of ethanolic extract of *Acacia leucophloea* by agar disc diffusion method. The diameter of the reserve zone was verified and compared with the standard antifungal drug clotrimazole.

Table 3. Phytochemical Screening of Ethanolic Extract of *Acacia leucophloea*

Organism	Sample code	Zone of inhibition I	Zone of inhibition II	Zone of inhibition III
Aspergillus niger	Clotrimazole (100µg)	19	17	19
	Sample (250µg)	11	11	Nil
	Sample (500µg)	12	12	11
	Sample (1000µg)	13	13	13

Table 3 represents the antifungal activity against *Aspergillus niger* showed inhibition zones of 19, 17, and 19 mm for clotrimazole. The maximum zones of 11, 12 and 13 mm respectively were obtained with the samples of 250, 500 and 1000 μ g respectively, suggesting that the activity increased with the concentration of the samples.

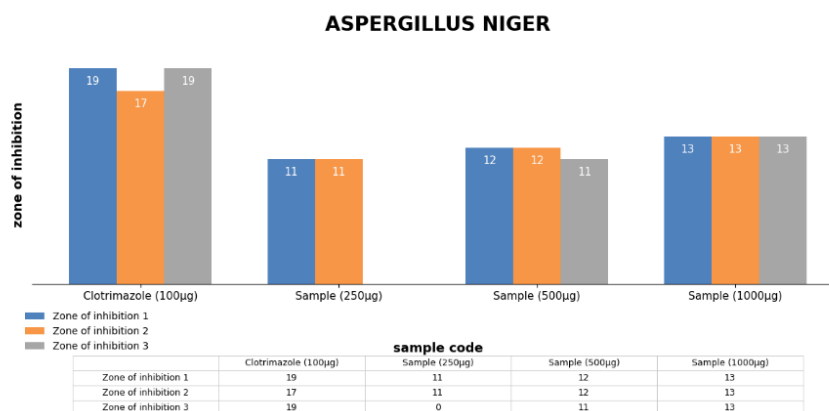


Fig. 4. Results of Invitro Antifungal activity study of *A.niger*

Fig. 4. shows the antifungal activity of ethanolic extract of *Acacia leucophloea* against *Aspergillus niger*. Clotrimazole (100 μ g) inhibited 19, 17 and 19 mm. The extract produced 11, 11, and 0 mm at 250 μ g, 12, 12, and 11 mm at 500 μ g, and 13, 13, and 13 mm at 1000 μ g. The results show that as the concentration of the extract rises the inhibition increases.



Fig. 5. Results of Agar-Disc Diffusion Method

Fig. 5. shows three agar plates used for the agar disc diffusion assay to evaluate the antifungal activity of the *Acacia leucophloea* extract. There are four paper discs that have been put on the surface of the agar plates containing fungi. The discs are used to represent different concentrations of samples and visible clear zones around the discs are the result of inhibition of fungal growth.

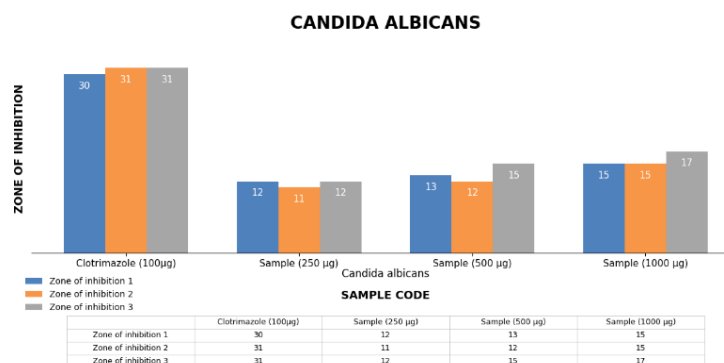


Fig. 6. Result of the Invitro Antifungal Activity Status of *C.Albicans*

Fig. 6. presents the antifungal activity of the ethanolic isolate of *Acacia leucophloea* has been studied against *Candida albicans*. Clotrimazole (100 μ g) prevented the growth of the fungi with inhibition zones of 30, 31 and 31 mm. The extract produced 12, 11, and 12 mm at 250 μ g, 13, 12, and 15 mm at 500 μ g, and 15, 15, and 17 mm at 1000 μ g. The results demonstrate that there is greater inhibition with the higher extract concentration.



Fig. 7. Result of the Invitro Antifungal Activity Status of C.Albicans

Fig. 7. shows three agar plates used for the agar disc diffusion assay of *Acacia leucophloea* against *Candida albicans*. Growth of fungi on each plate with four marked discs of tested sample or concentrations. There is indication of inhibition of fungal growth from the clear zones surrounding the discs, showing the antifungal effect of the extract tested.

4.4 Discussion

Antifungal activity of the ethanolic isolate of *Acacia leucophloea* (Roxb.) was tested against two test fungi, *Candida albicans* and *Aspergillus niger*. Wild. The activity was determined by measuring the inhibition zones from three observations on different concentrations of the activity. In the case of *A. niger*, the 250 μ g concentration yielded zones of 11, 11 and 0 mm, and the 500 μ g concentration yielded 12, 12 and 11 mm, while 1000 μ g concentration yielded 15, 15 and 17 mm. Inhibition zones of 13 mm were obtained for the 1000 μ g concentration every time. In general, 1000 μ g was more active than the low concentrations. The ethanolic extract, though, showed lower activity than the standard drug, clotrimazole.

5. Conclusion and Future Work

Present study aimed to investigate in vitro antifungal activity of Ethanolic Aerial-part extract of *Acacia leucophloea* (Roxb.). Resistance to *Candida albicans* and *Aspergillus niger* was determined by agar disc diffusion method. The phytochemical screening revealed that there were several constituents present such as carbohydrates, glycosides, sterols and steroids, flavonoids, phenolic compounds and alkaloids. The antifungal results indicated concentration-dependent inhibition with generally more inhibition shown with 1000 μ g as compared to the lower concentrations. The activity of the plant extract, however, was still less than that of clotrimazole. The results showed that *A. leucophloea* exhibits antifungal activity and can be a source of bioactive phytochemicals. More work is needed to identify the compounds that are active, and how they relate to treatment. The study also gives preliminary indication for further pharmacological studies, such as isolation of active constituents, determination of minimal inhibitory concentration and safety & antifungal mechanism studies in the future.

Further studies need to focus on isolating the specific bioactive compounds which contribute to the antifungal effect in *A. leucophloea*. Various extraction solvents and methods may be explored to assess their effect on the antifungal activity. Assessment of the activity could also be done using the minimum inhibitory concentration, minimum fungicidal concentration and time-kill study. It could also be determined how effective is the plant extract in vivo, toxicological studies, resistant fungal species and mode of action to determine further information regarding its safety and effectiveness.

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