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# Evaluation of *In-Vitro* Anti-Oxidant, Anti- Microbial Activities and Screening of Medically Active Chemical Components of *Acacia Leucophloea* Leaf Extracts

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## ABSTRACT

The plant *Acacia leucophloea* has been traditionally used for several medicinal purposes. The present study is an attempt to explore antimicrobial activity, antioxidant activity and to analyse medically active compounds both qualitatively and quantitatively from leaves of plant *Acacia leucophloea* by using hexane, acetone, methanol and water as solvents. The extracts were evaluated for their antibacterial and antifungal activities on human pathogens and *in-vitro* antioxidant activity of *Acacia leucophloea* was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging and reducing power assay. Ascorbic acid is kept as standard reference and positive control for both the analysis.The total phenol content was determined by the Folin Ciocalteu procedure. Our investigations revealed that only methanol extract showed significant antimicrobial activity against all pathogen, whereas acetone and aqueous extracts showed better inhibitory action and hexane extract showed low antimicrobial activity against tested pathogens. The aqueous extract of *Acacia leucophloea* showed good antioxidant activity and methanolic extract has comparatively high phenolic content. It can be concluded that the extracts of *Acacia leucophloea* has good anti-microbial, antioxidant activities and finally we suggest that *Acacia leucophloea* leaf extracts are rich in bioactive compounds and have good biological activities. However, further detailed investigations are needed to ascertain the mechanisms and constituents behind its biological actions.

**Keywords:** *Acacia leucophloea*, anti-oxidant, Anti- microbial, Phytochemicals.

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## INTRODUCTION

*Acacia leucophloea* is a wild medicinal plant distributed throughout south & Southeast Asia[1, 2, 3]. The acacia name is derived from the Greek word 'akis' means point or barb. It is a moderate sized dry season fodder and pasture tree attains a height of about 20 to 30 feet and a girth of 2 to 3 feet. It belongs to the Fabaceae family, under the sub family Mimosoideae[4]. The common names for this plant are White bark acacia in English. It is most commonly found in countries like India, Bangladesh, Indonesia, Myanmar, Nepal, Pakistan, SriLanka, Thailand, Vietnam. Trunk is stout with several branches. Open grown specimens have wide umbrella like crown. Bark is white to yellowish grey, smooth; exfoliating long strips, on aging tree becomes black and rough. Leaves are bipinnate compound having 4-13 pairs of pinnae, each with 5-30 pairs of leaflets. The feathery green foliage offers a strong contrast to the light coloured bark.

Spines are 2-5mm long and flowers are conspicuous, light yellow to cream in colour, pods yellow, green or brown in colour, flat and fairly straight, 10-20cm long. The plant has therapeutic properties against cough, inflammation, skin diseases, diarrhoea, stomatitis, cancer, haemorrhages, pharyngitis, diabetes and fever.

USES**:**

1) *Acacia leucophloea* germinated seeds are cooked and eaten as a vegetable. The seeds are a rich source of minerals and nutrients
2) It is also used as fodder, firewood, fiber and timber.
3) A water soluble gum of fair quality can be extracted from the stem and root bark which can be used as emulsifying agent.
4) A Reddish brown substance is obtained from bark and the leaves yield a black dye used to manufacture dyes and tannins.
5) The bark is used in liquor distillation in India.
6) *Acacia leucophloea* is used for Reclamation, Nitrogen fixing and intercropping as it is a good reforestation species for poor soils in low rainfall areas.
7) The vivid colors of its leaves, flowers and bark make it a beautiful ornamental tree.
8) The brick-red heartwood is beautiful and used in making decorative furniture, cartwheels, posts and beams
9) The bark and gum are used to treat bronchitis in traditional medicine.

## MATERIALS AND METHODS

**Plant collection**

The whole plant was collected from the herbal garden of the Acharya Nagarjuna University, Guntur. The plant was identified accordingly to various literatures, including other pertinent taxonomic literature.

**Collection of microorganisms**

The microorganisms were collected from the Department of Biotechnology, Acharya Nagarjuna University, Guntur and they were reconfirmed by gram staining and sub cultured in appropriate selective media.

**Extraction procedure**

The leaves of *Acacia leucophloea* were carefully separated, cleaned, shade dried, mechanically grinded and coarsely powdered. The powder was subjected to solvent extraction with Hexane, Acetone, Methanol and Water. The Extracts were concentrated by using the Rotary Evaporator and the yield of the Extract was noted with respect to the dried plant material.

**Qualitative Phytochemical Analysis** [6, 7, 8, 9]

**Preliminary Phytochemical screening**

Phytochemical analysis were conducted for all extracts as per the standard procedures.

**Detection of Alkaloids**

Extract is dissolved in dilute Hydrochloric acid and solution was clarified by filtration.

**Mayer's Test:** The Filtrate obtained was treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow colour precipitate indicates the alkaloids presence.

**Wagner's Test:** The Filtrate obtained is treated with Wagner's reagent (Iodine in Potassium Iodide). Brown / reddish precipitate formation confirms the alkaloids presence.

**Dragendroff's Test:** Filtrate was treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the alkaloids presence.

**Hager's Test:** Filtrate was treated with Hager's reagent (saturated picric acid solution). Formation of yellow coloured precipitate confirmed the Presence of alkaloids.

**Phenols Detection**

**Ferric Chloride Test:** The filtered solution of extract was treated with three drops of freshly prepared 1% $FeCl_3$ and Potassium Ferro cyanide. Bluish- green colour formation indicates the presence of phenols. The methanol extract was dissolved in water and few crystals of ferric sulphate are added to mixture. Formation of dark violet colour indicated phenolic compounds presence in the mixture

**Detection of Flavonoids**

**Alkaline Reagent Test:** The extract when treated with few drops of sodium hydroxide intense yellow colour formation which later becomes colour less on addition of dilute Hydrochloric acid, confirms the presence of flavonoids.

**Lead Acetate Test:** Few drops of lead acetate solution were added to the extract and checked for yellow colour precipitate indicates the presence of flavonoids.

**Detection of Anthraquinones**

**Free Anthraquinones Test: (Borntrager's test)** 100 mg of the plant extract was shaken vigorously in 10 ml of benzene followed by filtration, and addition of 5 ml of 10% ammonia solution. Shake the mixture and the occurence of a pink, red, or violet colour in the ammonia (lower) phase confirms the presence of free Anthraquinones.

**Modified Borntrager's Test:** The solvent extract was treated with $FeCl_3$ solution and suspended in boiling water for about 5 minutes. The mixture was cooled and extracted with same volume of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonia layer indicates the presence of anthranol glycosides.

**Detection of Phyto sterols**

**Salkowski's Test:** Solvent extract is dissolved in 2 ml of chloroform. Conc. Sulphuric acid was added to the walls of the test tube. A reddish brown colour formation at the interface indicates the presence of a steroid ring (i.e., the aglycone portion of the glycoside).

**Libermann Burchard's Test:** Chloroform is added to plant extract and filtered. A few drops of acetic anhydride were added to the filtrate boiled and cooled. Conc. Sulphuric acid was added to it and observed for brown ring formation at the junction which indicates the presence of phyto sterols.

**Detection of Terpenoids:** 2 ml of acetic anhydride and Conc. $H_2SO_4$ was added to plant extract and formation of blue, green rings indicate the presence of terpenoids.

**Detection of Fatty acids:** The extracts were mixed with 5 ml of ether and allowed for evaporation on filter paper and filter paper was dried. The appearance of transparency on filter paper confirms fatty acids presence.

**Detection of Tannins**

**Ferric Chloride Test**. The solvent extract was dissolved in water. The solution was filtered and 10% ferric chloride solution was added to filtrate. This was observed for a change in colour to bluish black.

**Lead Acetate Test.** Solvent extract obtained was dissolved in water and 10% Lead acetate was added and yellow precipitate formation confirms the tannins presence.

**Potassium Dichromate Test:** The extract was dissolved in water and strong potassium dichromate solution is added, a yellow colour precipitate formation indicates the presence of phenolic compounds and tannins.

**Detection of Saponins**

**Froth Test:** The Solvent Extract is diluted with distilled water to 20 ml and is shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of "honey comb" froth indicates saponins presence.

**Anthocyanins:** 2 ml of 2N HCl and ammonia was added to the solvent extract. Initial formation of pink-red colour turning into blue-violet indicates the presence of Anthocyanins

**Leucoanthocyanins**

5 ml of isoamyl alcohol was added to the extract. Appearance of red colour in upper layer indicates leucoanthocyanins presence.

**Coumarins:** To the solvent extract 3 ml of 10% NaOH, yellow colour formation indicates the presence of coumarins

**Emodins:** 2 ml of $NH_4OH$ and 3 ml of Benzene was added to the extract. Appearance of red colour confirms the presence of emodins.

**Detection of Reducing Sugars:** Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

**Fehling's Test:** Filtrates is hydrolysed with dil. Hydrochloric acid, neutralized with alkali and heated with Fehling's A & B solutions. Red precipitate formation indicates the presence of reducing sugars.

**Keller - Kiliani test (for de-oxy sugars in cardiac glycosides):** Methanol extract obtained was dried. 50 mg of the extract was dissolved in 2 ml chloroform. Sulphuric acid is added and brown ring formation at interphase is characteristic of de-oxy sugars in cardenolides.

The crude extracts of all the four solvents were analysed for the presence of secondary metabolites using above mentioned standard protocols.

**Antibacterial activity**

Antibacterial activity was carried out by agar well diffusion method[10]. The extracts were checked for antimicrobial activity against both Gram-positive and Gram-negative bacteria. Four extracts (HEAL, ACAL, MEAL, AQAL) were prepared separately at 500µg/ml conc. by using dimethyl sulphoxide as solvent (DMSO). DMSO was used as negative control. Nine bacterial strains of late log phase culture were spread on different nutrient agar plates each having 4 holes (punches done using borer for loading the sample). 50 µl of each solvent extract and control were added in

the respective marked holes and incubated for 24 hours at 37 °C. The zone of inhibition exhibited by each extract is measured by diameter scale and recorded.

**Antifungal activity: (Agar well diffusion method[10])**

The extracts were tested for antifungal activities against four fungal strains that includes Candida *albicans, Aspergillus niger, Rhizopus oryzae, Candida rogas*. Four solvent extracts (HEAL, ACAL, MEAL, AQAL) were prepared at 500µg/ml in Dimethyl Sulphoxide (DMSO) and 50µl of DMSO without any extract is used as negative control. Four different fungal strains mentioned above were spread on 4 potato dextrose agar Petri plates. 4 punches were done using borer for loading the sample. 50µl of each solvent extract were added to respective marked holes and incubated for 72 hours at 37° C. The zone of inhibition is calculated and recorded by diameter scale.

**Determination of total phenols [11]**

Leaf extract was dissolved with solvent to prepare 100, 200, 300, 400 and 500µg/ml aliquots. Each aliquot of samples were added with 3 ml of water. 0.5 ml Folin Ciocalteu reagent was added to each tube and left for 3 minutes, to each tube 2 ml of 20 % sodium carbonate solution was added and mixed thoroughly. The test tubes were kept in boiling for 1 min. and cooled down. The absorbance was measured at 650 nm against blank and standard graph was plotted.

**Antioxidant Properties [12]**

**1, 1- Diphenyl-2-Picrylhydrazyl radical (DPPH) scavenging activity**

The free radical scavenging activity of methanol, acetone, hexane and water extracts was measured by 1, 1- Diphenyl-2-Picrylhydrazyl radical (DPPH) method[12]. For analyzing the DPPH activity, stock solutions are prepared for each solvent extracts by dissolving the crude extracts in DMSO at 1mg/ml concentration. From the above stock solution, working standard solution was prepared as required. Five aliquots are prepared for each solvent extract with 100-500µg/ml concentrations as working solutions. Later to each aliquot 0.004% (W/V) of DPPH solution was added. The mixture was shaken and incubated for 15 min in dark at room temperature. Finally absorbance was measured at 517 nm in spectrophotometer and the values obtained were noted. Control was prepared without sample.

The DPPH scavenging activity (I %) was calculated as follows:

$$I\% = \left[\frac{(Ao - As)}{Ao}\right] \times 100$$

Where **Ao** is the absorbance of the DPPH solution without sample extract and **As** is the absorbance of sample with DPPH solution. $Ic_{50}$ values are calculated for each solvent extract by plotting the values in a graph.

## RESULTS AND DISCUSSION

Research on Ethno botany (the study of traditional uses of plants) is a very old practice. But the emerging research is to isolate and evaluate new compounds from medicinal plants that are capable of treating traditional uses. Now a day's new unidentified diseases are evoking day by day. Medicinal plants have diverse pharmacological actions attributed due to the presence of secondary plant metabolites. Some medicinal plants are important sources of natural antioxidants that reduce the risk and progression of certain chronic and acute diseases. In the present study we aim to investigate the medicinal values of *Acacia leucophloea* by extracting its secondary metabolites in to different solvents followed by antimicrobial and anti-oxidant studies. The dry plant powder is subjected to solvent extraction with 4 solvents i.e., hexane, methanol, acetone, and aqueous. These mixtures of solvent and plant material was incubated and kept under shaking for overnight. The solvent mixture is filtered and solvent is condensed through Rotary Evaporator. After condensing the crude extract obtained from four different solvent extracts are collected, weighed and stored in refrigerator for future use

### Yield of Extract

The yield of crude extract is obtained by measuring its dry weight obtained after condensing through Rotary evaporator. Yield was found to be low in hexane extract due to its low polarity and the yield was found to be more in methanol (33.26%). The color of the extract was found to be dark brown in hexane extract, dark green in acetone and methanol extracts and dark red in aqueous extract. The yield of extract is represented in Figure 1.

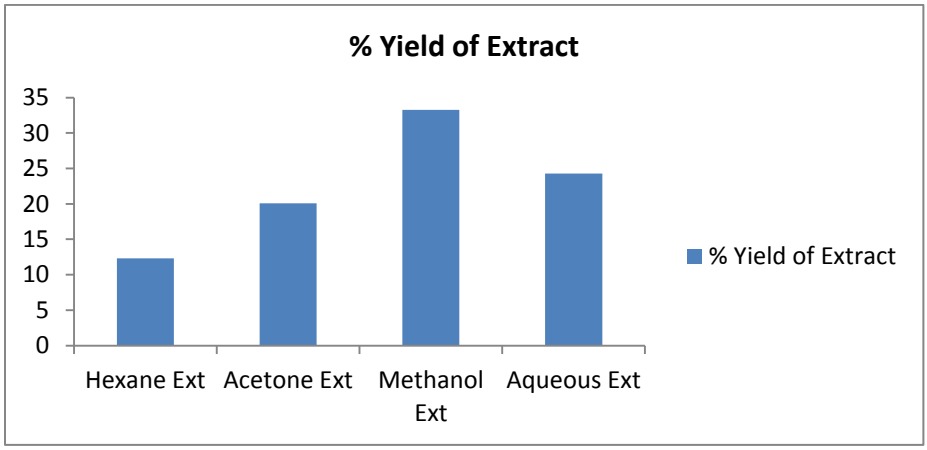


**Figure 1: Graphical representation of Yield of Extracts**

**Phytochemical Analysis**

The preliminary phytochemical screening results showed the presence of alkaloids, phenols, flavonoids, steroids, tannins, saponins and reducing sugars. Reducing sugars, alkaloids, phenols, tannins and flavonoids were present in methanol, acetone and aqueous extracts except hexane extract. (Table 1)

**Table 1: Phytochemical Analysis**

**Test for Alkaloids**

| S.NO | TESTS | Hexane Extract | Acetone Extract | Methanol extract | Water extract |
|---|---|---|---|---|---|
| **1** | Mayers | Negative | Positive | Positive | Positive |
| **2** | Dragon | Negative | Positive | Positive | Positive |
| **3** | Wagners | Negative | Positive | Positive | Positive |
| **4** | Hagers | Negative | Positive | Positive | Positive |

**Test For Phenolics**

| TEST | Hexane Extract | Acetone Extract | Methanol extract | Water extract |
|---|---|---|---|---|
| $Fecl_2$ Test | Negative | Positive | Positive | Positive |

**Test for Flavanoids**

| S.NO | TESTS | Hexane Extract | Acetone Extract | Methanol extract | Water extract |
|---|---|---|---|---|---|
| **1** | Lead Acetate Test | Negative | Positive | Negative | Positive |
| **2** | NaOH Test | Negative | Positive | Positive | Positive |
| **3** | Ethyle acetate Test | Negative | Negative | Negative | Positive |

**Test for Anthroquinones**

| TESTS | Hexane Extract | Acetone Extract | Methanol extract | Water extract |
|---|---|---|---|---|
| Borntrager's Test | Negative | Negative | Positive | Positive |

**Test for Steroids**

| S.NO | TESTS | Hexane Extract | Acetone Extract | Methanol extract | Water extract |
|---|---|---|---|---|---|
| **1** | Salkowski's Test | Negative | Positive | Positive | Positive |

**Test For Tanins**

| S.NO | TESTS | Hexane Extract | Acetone Extract | Methanol extract | Water extract |
|---|---|---|---|---|---|
| **1** | $Fecl_2$ Test | Negative | Positive | Positive | Positive |
| **2** | Lead acetate Test | Negative | Positive | Positive | Positive |
| **3** | Pot. dichromate Test | Negative | Positive | Positive | Positive |

**Test For Saponins**

| TESTS | Hexane Extract | Acetone Extract | Methanol extract | Water extract |
|---|---|---|---|---|
| Vigorous Shaking Test | Negative | Positive | Negative | Positive |

**Test for Anthocyanins**

| TESTS | Hexane Extract | Acetone Extract | Methanol extract | Water extract |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| Ammonia-HCl Test | Negative | Negative | Positive | Negative |

**Test for Leuco-Anthocyanins**

| TESTS | Hexane Extract | Acetone Extract | Methanol extract | Water extract |
|---|---|---|---|---|
| Iso Amyl Alcohol Test | Negative | Negative | Negative | Positive |

**Test For Coumarins**

| TESTS | Hexane Extract | Acetone Extract | Methanol extract | Water extract |
|---|---|---|---|---|
| NaOH Test | Negative | Negative | Positive | Positive |

**Test For Reducing Sugars**

| TESTS | Hexane Extract | Acetone Extract | Methanol extract | Water extract |
|---|---|---|---|---|
| Keller-Kiliani Test | Negative | Positive | Positive | Positive |

**Phenol Quantitative Determination**

The total phenolic content of 4 extracts of *Acacia leucophloea* was estimated, among the 4 extracts the methanol extract showed high amount of phenolic compounds followed by water extract and acetone showed moderate range and hexane extract showed low range of phenolic compounds. Methanol extract showed a result of 120.7µg/ml phenolic compound at a concentration of 500µg/ml. Aqueous, acetone and hexane extracts recorded simultaneously 105.1µg/ml, 100.2µg/ml and 58.6µg/ml phenolic compound at a concentration of 500µg/ml. (Figure.2)

**Anti -bacterial activity**

The anti-bacterial activity of methanol, acetone, hexane and aqueous extracts of *Acacia leucophloea* against nine bacterial strains was studied. The studies recorded high activity against the tested organisms at 500µg/ml. Among them methanol extract showed high antibacterial activity followed by Acetone and aqueous extracts. Hexane showed low antimicrobial activity when compared with the other three extracts. (Table 2)

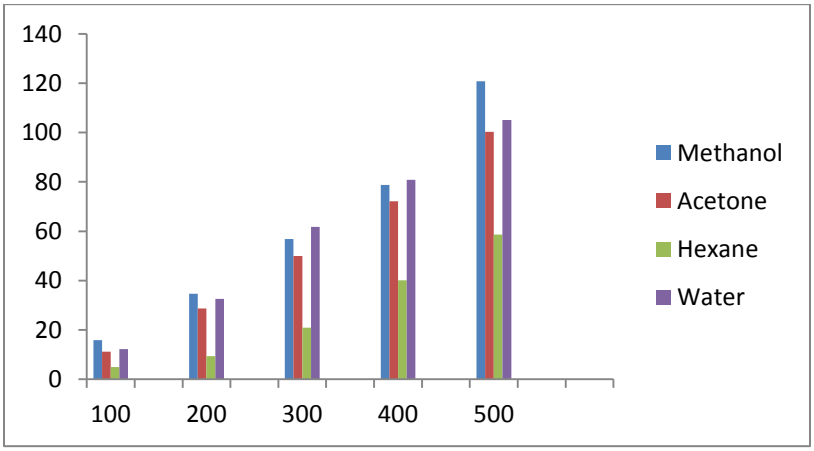


**Figure 2: Graphical representation for Total phenolic content**

**Table-2: Anti-bacterial activity (zone of inhibition in mm)**

| Microorganism | **Methanol** | **Acetone** | **Hexane** | **aqueous** |
|---|---|---|---|---|
| *Staphylococcus aureus* | 18 | 16 | 14 | 17 |
| *Streptococcus mutans* | 14 | 14 | 12 | 14 |
| *Lactobacillus casei* | 16 | 16 | 13 | 16 |
| *Lactobacillus acidophilus* | 16 | 14 | 14 | 14 |
| *Enterococcus faecalis* | 14 | 14 | 13 | 13 |
| *Bacillus megaterium* | 16 | 14 | 13 | 16 |
| *Xanthomonas campestris* | 17 | 15 | 13 | 17 |
| *Escherichia coli* | 16 | 14 | 13 | 14 |
| *Pseudomonas aeruginosa* | 14 | 13 | 12 | 13 |

**Anti- fungal activity**

Anti- fungal activity was studied using all the four extracts against four fungal strains like *Candida albicans, Aspergillus niger, Rhizopus oryzae and Candida rogasa.* Among them methanol extract is having good antifungal activity for *Candida albicans* with 19 mm of zone of inhibition. Acetone and aqueous extracts are having moderate zone of inhibition. Acetone is good with 14 mm zone of inhibition against *Candida albicans* and aqueous extract showed 16mm zone of inhibition against *Candida albicans* and *Aspergillus niger*. Poor activity was recorded by hexane extract against both bacterial and fungal cultures. (Table 3)

**Table 3: Antifungal activity (zone of inhibition in mm):**

| Microorganism | **Methanol** | **Acetone** | **Hexane** | **Water** |
|---|---|---|---|---|
| *Candida albicans* | 19 | 14 | 10 | 16 |
| *Aspergillus niger* | 12 | 14 | 12 | 12 |
| *Rhizopus oryzae* | 12 | 11 | 11 | 16 |
| *Candida rugosa* | 13 | 12 | 12 | 13 |

**Estimation of Free-radical scavenging activity by DPPH assay**

DPPH radical scavenging activity of *Acacia leucophloea* plant extract was compared with standard ascorbic acid in this study. The estimation of radical scavenging activity was analyzed by DPPH activity. The 4 extracts of *Acacia leucophloea* were diluted with appropriate solution aliquots. Aqueous extract showed highest antioxidant activity followed by methanol then by acetone and least by Hexane extracts (Table 4). The $IC_{50}$ values is least for aqueous extract followed by methanol, acetone and hexane extracts. However the $IC_{50}$ of aqueous extract is not as good as standard ascorbic acid, but the compound responsible for antioxidant activity in aqueous extract may show much better activity when purified.

**Table 4: Antioxidant activity of *Acacia leucophloea***

| Conc. (µg/ml) | Hexane extract (% of inhibition) | $IC_{50}$ | Acetone extract (% of inhibition) | $IC_{50}$ | Methanol extract (% of inhibition) | $IC_{50}$ | Water extract (% of inhibition) | $IC_{50}$ | Standard % of inhibition (Ascorbic acid) |
|---|---|---|---|---|---|---|---|---|---|
| 100 | 18.7 | 644 | 29.6 | 417 | 37 | 318.4 | 45 | 197.3 | 50.67 |
| 200 | 23.6 | | 32.4 | | 45.6 | | 49.4 | | 53.25 |
| 300 | 25.8 | | 37.2 | | 48.2 | | 55.9 | | 79.08 |
| 400 | 37.6 | | 45.6 | | 54.7 | | 62.4 | | 83.51 |
| 500 | 42 | | 53.8 | | 60.2 | | 68.7 | | 91.26 |

## CONCLUSION

The results of our study suggest that *Acacia leucophloea* plant extract are rich in bioactive compounds and have good biological activities. However, further detailed investigations are needed to ascertain the mechanisms and constituents behind its biological actions.

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