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# *In vitro* Investigation of Antibacterial Activity of *Atlantia racemosa* Using Different Dye Assays

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**Abstract:** Hexane, chloroform, acetone, ethyl acetate and methanol leaf extracts of *Atlantia racemosa* were tested against two bacterial species such as *Staphylococcus aureus* and *Klebsiella pneumoniae*. Among all the extracts, methanolic extract of the leaves (LME) was found to exhibit significant antibacterial effect with Minimum Inhibitory Concentration (MIC) of 250µg/ml (zone diameter 17mm). This antibacterial activity of LME of *A. racemosa* was determined using well diffusion assay, broth dilution assay and dye assays such as, MTT, Neutral red and Kenacid blue. Also, the mode of action of LME was studied and found to be inhibition of intracellular bacterial proteins. Furthermore, chromatographic analysis of the crude extract revealed the presence of 7 distinct bioactive compounds. The results of our study suggest that the leaves of *A.racemosa* could be a potent source of natural drugs against these pathogenic bacteria and hence could lead to the development of effective drugs for the treatment of health effects caused by these test pathogens.

**Key words:** *Atlantia racemosa* · Antimicrobial activity · MTT

## INTRODUCTION

Herbalism is a traditional medicinal or folk medicine practice based on the use of plants and plant extracts. The scope of herbal medicine is sometimes extended to include fungal and bee products, as well as minerals, shells and certain animal parts [1]. In 2001, researchers identified 122 compounds used in mainstream medicine which were derived from "ethnomedical" plant sources; 80% of these compounds were used in the same or related manner as the traditional ethnomedical use [2].

Many of the herbs and spices used by humans to season food yield useful medicinal compounds [3, 4]. The screening of plant extracts and their products for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotic prototypes. Plant based antimicrobials represent a vast untapped source of medicines even after their enormous therapeutic potential and effectiveness in the treatment of infectious disease hence, further exploration of plant antimicrobials need to occur [5]. The selection of crude plant extracts for screening programs is potentially more successful in initial steps than the pure compounds [6]. Even though hundreds of plant species have been tested for antimicrobial properties; the vast majority of them have not yet been validated.

*Atalantia racemosa* var. *bourdillonii,* erect shrubs or medium-size trees 2-15 m tall found in evergreen forests in Tamil Nadu, Karnataka and Kerala at 60-600 m altitude. The new variety is closely allied to *A. racemosa* var. *henryi* and a key is given to distinguish them. The current study focuses on the validation of antimicrobial activity of *Atlantia racemosa*.

## MATERIALS AND METHODS

**Plant Material:** The fresh plants were collected from fields located in forest areas of Kerala. The same were

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verified and authenticated botanically by Mr. S. Aroumougame, University of Madras, Chennai. The leaves were then separated from the steam, carefully washed with tap water, rinsed with distilled water and air-dried for 1 hour. Then the leaves were shade dried in room temperature for one week. Then they were ground into powder and subjected to extraction with different solvents.

**Plant Extracts Preparation:** The finely ground leaves were extracted with different solvents such as, hexane, chloroform, acetone, ethyl acetate and methanol following the method of Eloff, 1998. Here, the extraction of the leaf powder was done with solvents in the ratio of 1:10 under shaking condition. The extracts were collected in different conical flasks and the same was repeated thrice to attain maximum extraction. Then the solvents were evaporated and condensed to concentrate the extracts obtained. The concentrated residues were weighed and re-dissolved in respective solvents to yield 10mg/ml solutions for further analysis.

**Antimicrobial Activity:** The crude extracts were subjected to antimicrobial screening against *Klebsiella pneumoniae (*MTCC 109) and *Staphylococcus aureus* (MTCC 96).

**Well Diffusion Assay:** Nutrient agar was prepared and poured in the Petri dish. 24 hours growing culture (*Klebsiella pneumoniae* and *Staphylococcus aureus*) were swabbed on it. The wells (10mm diameter) were made by using cork borer and the different concentrations of the crude extract were loaded in the wells. The plates were then incubated at 37°C for 24 hours. The inhibition diameter was then measured [7].

**Broth Dilution Assay:** Dilution assays are standard method used to compare the inhibition efficiency of the antimicrobial agents. 5ml of the Nutrient broth, 0.1ml of the 24hours growing culture (*Klebsiella pneumoniae* and *Staphylococcus aureus*) and the different concentration (100µg, 200µg….1000µg) of the drug dissolved in Dimethyl sulphoxide and added in the test tubes. The tubes were incubated at 37°C for 24 hours [8]. The optical densities were measured spectrometically at 600nm. The percentage of viable cells was calculated using the following formula.

$$\% \text{ viable cells} = \frac{\text{Contrl OD} - \text{TestOD}}{\mathit{ControlOD}} * 100$$

O.D – Optical Density

**Dye Assays:** The dye assays are most sensitive, rapid and accurate method to determine the cytotoxicity and cell viability. The dyes such as MTT, Neutral red and Kenacid blue were used in this assay. The activity was expressed in terms of percentage of cell viability as,

$$\% \text{ viable cells} = \frac{\text{Contrl OD} - \text{TestOD}}{\mathit{ControlOD}} * 100$$

O.D – Optical Density

**MTT - (3- (4, 5 – Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) Assay:** This is used to measure the activity of living cell by assessing the activity of the bacterial dehydrogenase enzymes. The bacterial dehydrogenase enzyme cleaves the tetrazolium ring. The MTT produces the purple formazan crystals which are insoluble in aqueous solution. And so the crystals are made to dissolve in Dimethyl sulphoxide. In the microtitre plate 5µl of the 17 hours growing culture (*K. pneumoniae, S. aureus*) and 95µl of the freshly prepared Muller Hinton Broth and the different concentration of the drug (100µg, 200µg……1000µg) were added to the wells and were incubated at 37°C for 24 hours. 10µl of MTT (5mg/mL) was added to each well and re-incubated for 4 hours. The contents were collected and centrifuged at 8000 rpm for 15 minutes and the pellets were dissolved with 100µl of Dimethyl sulphoxide. Then the contents were transferred to the appropriate well and read at 570 nm in the ELISA reader. The percentage of viable cells was calculated [9].

**Resazurin Assay:** Resazurin is used as the growth indicator. They detect the oxidative enzyme systems by acting as the electron acceptor. Bio reduction of the dye by the viable cells reduces the amount of its oxidized form (blue) and concomitantly increases the amount of its fluorescent intermediate (red), indicating the degree of cytotoxicity caused by the test agents. In the microtitre plate 5µl of the 17 hours growing culture (*K. pneumoniae, S. aureus*) and 95µl of the freshly prepared Muller Hinton Broth and the different concentration of the drug (100µg, 200µg……1000µg) were added and the plates were incubated at 37°C for 24 hours. 30µl of Resazurin (100µg/mL) was added to each well and incubated for 17 hours. Since the reduction of resazurin displays three different colours, the only way to determine the MIC or $IC_{50}$ by visible observation of change in colour to pink in live cell, the minimum concentration where there is no change of colour was recorded as MIC/ $IC_{50}$ of particular test pathogen [10].

**Kenacid Blue Assay:** The kenacid blue dye assay determines the total cellular protein by measuring the uptake of the dye kenacid blue. The amount of dye incorporated by the cells in the culture determines the degree of cytotoxicity caused by the crude extract. In the microtitre plate 5µl of the 17 hours growing culture (*K. pneumoniae, S. aureus*), 95µl of the freshly prepared Muller Hinton Broth and the different concentration of the drug (100µg, 200 µg…1000µg) and incubated at 37°C for 24 hours. 100µl of Kenacid blue (220µg/200mL) was added to it and again incubated for 4 hours. Then the contents were centrifuged (8000rpm/15 minutes), pellets were collected and treated with that 100µl of the ethanolic solvent mixture (96%ethanol: glacial acetic acid: water in the ratio of 2:1:17). The contents were then transferred to the appropriate wells and read in ELISA reader at 620 nm [11].

**Phytochemical Screening of *A. racemosa* Crude Extract:** Qualitative phytochemical tests for the identification of alkaloids, phenols, flavonoids, carbohydrates, glycosides, saponins, amino acids and proteins were carried out for all the extracts by the method described by [12, 13]. The freshly prepared extracts of *A.racemosa* were qualitatively tested for the presence of chemical constituents.

**Mode of Action of the Extracts**

**Protein Inhibition Assay:** Using protein inhibition assay the mode of action of the plant extract was studied. Here, the crude protein was extracted from the bacteria treated with the leaf extract (500µg/mL) of *A.racemosa* and the crude protein from untreated bacteria was used as control. The extracted proteins were run on SDS - PAGE and the bands were visually examined for determination of the mode of action.

**Thin Layer Chromatography:** The leaf extract was loaded on pre-coated silica plates which were then developed using the solvents methanol, ethyl acetate, chloroform in the ratio of 0.5:0.5:9. The spots were identified both in the UV light, far light and in the iodine chamber. Then $R_f$ value was calculated as the ratio of distance travelled by the solute to the distance travelled by the solvent [14].

## RESULTS AND DISCUSSION

The antibacterial property of *Atlantia racemosa* investigated by well diffusion assay infers that Leaf Methanolic Extract (LME) exhibits higher activity against the test pathogens when compared to other solvent extracts. The zone of inhibition of LME (250µg/mL) was found to be 17mm and 12mm (Table 1) respectively against *S. aureus, K. pneumoniae*. Further, the $IC_{50}$ of LME obtained from broth dilution assay was 600µg/ml and 700µg/ml, respectively against *K. pneumoniae* and *S. aureus* (Table 2). The results of broth dilution assay were further confirmed using values obtained from different dye assays which indicated that the $IC_{50}$ for LME against *K. pneumoniae* were in the range of 500µg/ml - 700µg/ml and those for *S. aureus* were in the range of 300µg/ml - 600µg/ml (Table 3 & 4). Among four different *invitro* assays (Including both classical inhibitory assay and dye assays), MTT was the best and very easy to perform with accurate reproducible results followed by Kenacid blue and Neutral red.

The results from protein inhibition assays suggest that the LME (500µg/ml) inhibited the test pathogens at protein level, which is evident from the bands seen on SDS - PAGE. The absence of clear bands in the protein obtained from bacteria treated with the LME indicates that the protein level inhibition of the extract (Figure 1).

The preliminary phytochemical screening of *A.racemosa* revealed the presence of phenolics and alkaloids in high amounts followed by saponins in trace. The carbohydrates, proteins and glycosides were completely absent (Table 5).

The chromatogram developed with methanol, ethyl acetate and chloroform in the ratio of 0.5:0.5:9 revealed the presence of seven major compounds at $R_f$ value of 0.97, 0.89, 0.82, 0.79, 0.56, 0.35 and 0.13 as visualized under iodine vapour and UV illumination (Figure 2).

Table 1: Well diffusion assay

| | Zone of the inhibition (mm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Staphylococcus aureus* | | | | | *Klebsiella pneumoniae* | | | | |
| Concentrations (µg) | Methanol | Ethyl acetate | Chloroform | Acetone | Hexane | Methanol | Ethyl acetate | Chloroform | Acetone | Hexane |
| 250 | 17 | 17 | 14 | 13 | 13 | 12 | 11 | 11 | 12 | - |
| 500 | 19 | 15 | 14 | 16 | 14 | 13 | 12 | 13 | 14 | - |
| 750 | 18 | 14 | 15 | 18 | 15 | 16 | 15 | 15 | 17 | 11 |
| 1000 | 21 | 20 | 17 | 17 | 16 | 18 | 15 | 15 | 18 | 13 |

Table 2: Broth Dilution Assay

| Concentration (µg/ml) | % for *K. pneumoniae* | % for *S. aureus* |
|---|---|---|
| 100 | 21 | 23 |
| 200 | 28 | 48 |
| 300 | 31 | 49 |
| 400 | 46 | 53 |
| 500 | 50 | 60 |
| 600 | 52 | 66 |
| 700 | 68 | 72 |
| 800 | 73 | 73 |
| 900 | 80 | 80 |
| 1000 | 95 | 92 |

Table 3: Determination of $IC_{50}$ concentration by MTT assay - (3- (4, 5 – Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) assay

| Concentration (µg/ml) | % for *K. pneumoniae* | % for *S. aureus* |
|---|---|---|
| 100 | 20 | 39 |
| 200 | 23 | 45 |
| 300 | 37 | 51 |
| 400 | 43 | 58 |
| 500 | 51 | 62 |
| 600 | 58 | 67 |
| 700 | 64 | 77 |
| 800 | 77 | 80 |
| 900 | 85 | 87 |
| 1000 | 98 | 97 |

Table 4: Determination of $IC_{50}$ concentration by Kenacid blue assay

| Concentration (µg/ml) | % for *K. pneumoniae* | % for *S. aureus* |
|---|---|---|
| 100 | 16 | 15 |
| 200 | 20 | 17 |
| 300 | 26 | 28 |
| 400 | 31 | 40 |
| 500 | 39 | 41 |
| 600 | 46 | 51 |
| 700 | 54 | 58 |
| 800 | 70 | 60 |
| 900 | 82 | 65 |
| 1000 | 90 | 73 |

Table 5: Phytochemical screening of *A. racemosa* methanol extract

| Detection of phytochemicals | Test/ Reagents | Result |
|---|---|---|
| Alkaloids | Mayer's | +++ |
| Carbohydrates | Fehling's | - |
| | Benidict's | - |
| Glycoside | Borntrager's | - |
| Saponins | Foam's | + |
| Protein and Aminoacids | Millon's | - |
| | Biuret | - |
| Phenolic compound | Ferric Chloride | +++ |
| | Lead Acetate | +++ |

+ + + : Present in high concentration;
\+ : Present in trace concentration;
\- : Constituents not detectable using the specified assay method

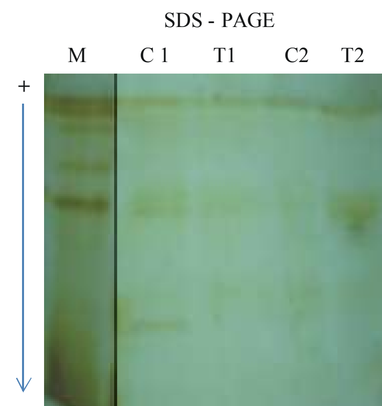


Fig. 1: Mode of action of LME of *A. racemosa* against bacterial pathogens

M – Molecular Marker; C1 – *K. pneumoniae* control; T1 – *K. pneumoniae* Treated; C2 – *S. aureus* Control and T2 - *S. aureus* Treated

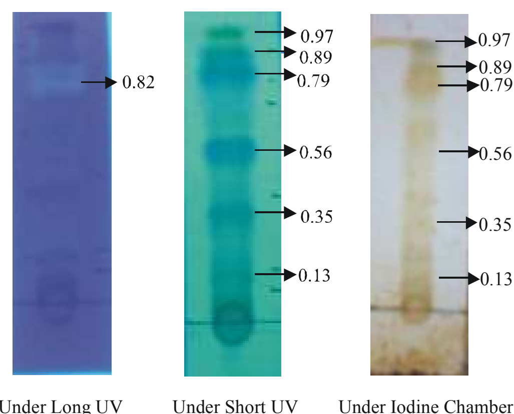


Fig. 2: Thin Layer Chromatography

## CONCLUSION

From the results obtained in this study, it is evident that the leaves of *A. racemosa* are effective against both *K. pneumonia* and *S. aureus.* In addition, the results of phytochemical screening supports the fact that the phenolics and alkaloids present in higher amounts could be responsible for the inhibitory action of the leaf extracts. Also, the chromatogram developed, suggests that seven major compounds are present in the leaf extract of *A.racemosa* which could contribute to its antibacterial activity. These results reveal that the leaves of *A.racemosa* could be a potential source of traditional medicine for infections caused by *K. pneumoniae* and *S. aureus*. Further investigation is necessary to elucidate the exact bioactive compound which is responsible for the destined antimicrobial action.

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