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PRELIMINARY PHYTOCHEMICAL INVESTIGATION AND BIOLOGICAL EVALUATION OF THE LEAVES OF *NEOLAMARCKIA CADAMBA*

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ABSTRACT

Natural compounds can be a lead compounds, allowing the design and rational planning of new drugs, biomimetic synthesis development and the discovery of new therapeutic properties not yet attributed to known compounds (S.M.K. Rates, 2011). The present study has made an attempt to evaluate the microscopic characters of *Neolamarckia cadamba* by determining leaf constants, trichomes and stomata, Phytochemical screening by using Qualitative chemical tests & column chromatography. The study includes biological evaluation of antibacterial and antifungal activity. Phytochemical screening of the crude methanolic extract of the leaves of *Neolamarckia cadamba* showed the presence of Alkaloids, Tannins, Saponins, Steroids and Glycosides. In Biological Evaluation, the antibacterial and antifungal activities of extracts (50, 75, 100 µg/ml) of *Neolamarckia cadamba* were tested against Gram-positive—*Staphylococcus aureus,* Gram-negative—*Escherichia coli.* Zone of inhibition of extracts were compared with that of standards like Amikacin for antibacterial activity and fluconazole for antifungal activity Post hoc analysis showed the remarkable inhibition of the bacterial growth was shown against the anti-bacterial organisms and a minute inhibition for anti-fungal organism.

KEYWORDS: *Neolamarckia cadamba,* Anti-bacterial activity, Anti-fungal activity, Methanolic Extract, Column Chromatography.

INTRODUCTON

In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants. This interest in drugs of plant origin is due to several reasons namely, conventional medicine can be inefficient, abusive and/or incorrect use of synthetic drugs results in side effects and other problems (Raju S et al. 2011). However, the potential use of higher plants as a source of new drugs is still poorly explored, of the estimated 250,000-500,000 plant species, only a small percentage has been investigated phytochemically and even a smaller percentage has been properly studied in terms of their pharmacological properties; in most cases, only pharmacological screening or preliminary studies are carried out. It is estimated that 5000 species have been studied for medical use (Sirigiri Chandra Kala, 2016).

Plant profile: *Neolamarckia cadamba* is a tree which belongs to Rubiaceae –madder family. It can grow in dry areas with as little as 200 mm rain/year. It is very light demanding and intolerant to frost. It can grow on a variety of soils and tolerates periodic flooding.

Fig 1: Leaves of *Neolamarckia Cadamba.*

Taxanomical Classification

- **Kingdom:** Plantae – plants
- **Subkingdom:** Tracheobionta - vascular plants
- **Superdivision:** Spermatophyta – seed plants
- **Division:** Magnoliophyta – flowering plants
- **Subclass:** Asteridae

- **Order:** Rubiales
- **Family:** Rubiaceae – madder family
- **Genus:** Neolamarckia F. Bosser
- **Species:** Neolamarckia cadamba (Roxb.) F. Boss

Vernacular names

- **English:** Kadam, Cadam
- **Hindi:** Kadamb
- **Kannada:** Kaduavalatige
- **Malayalam:** Vellakadumbu, Kodavara, Kadambu
- **Tamil:** Vellaikkatampu
- **Telugu:** Rudrakskamba
- **Others:** Indulam, Arattam, Vellai kadambu, Kadam

Botanical Description: Tree up to 45 m tall, without branches for more than 25 m. Diameter up to 100 (-160) cm but normally less, sometimes with buttresses. The crown is umbrella-shaped and the branches are characteristically arranged in tears.

Description of the Leaf: Leaves simple, 13-32 cm long.

Industrial uses: Kadam is a lightweight hardwood with poor durability. It is mainly used for pulp, producing low- and medium-quality paper. The wood can be used for light construction work but only indoors as it is perishable in contact with the ground (Pandey A et al., 2016). It is fast growing and suitable for reforestation in watersheds and eroded areas and for windbreaks in agro forestry systems. It is also excellent as a shade tree for dipterocarp line planting. Leaves and bark are used in medicine.

MATERIALS AND METHODS

Plant Collection and Identification: The specimens of the leaves was collected from the *Neolamarckia cadamba* tree located at Mindivanipalem surrounding area, Anandapuram, Visakhapatnam, Andhra Pradesh.

Quantitative Methods (leaf constants)

(i) *Stomatal Number and Stomatal Index*

The upper epidermis of the leaf between midrib and lamina was peeled and transparent area was cleared with Lacto Phenol and mounted on glass slide. Microscope is calibrated using

stage micrometer (D.L Royer, 2001). The stomata and epidermal cells were traced on black sheet using prism type camera lucida under high power (45x). The number of epidermal cells and stomata were counted and calculated per sqmm. The experiment was repeated for five times and Stomatal number was directly calculated**.**

Stomatal index was calculated using formula

$$\text{Stomatal index} = \frac{Number of stomata}{Number of stomata + Epidermal cells} \times 100$$

Average values were determined and results were expressed per sq.mm.

(ii) *Vein-Islet Number*

The leaf portion between midrib and margin was macerated in concentrated Lacto phenol solution for 24 h and decolorized with bleaching solution (5% calcium chloro-hypochlorite). The cleared lamina portion was mounted on glass slide and vein islet was traced on black sheet between 0.5 mm square using low power (5x). The process was repeated and values were determined per sq.mm of leaf area between midrib and margin (Dinesh Kumar et al., 2012).

Qualitative Methods

Phytochemical screening of crude leaf extract of *Neolamarckia Cadamba*

Drying of leaves: The plant leaves were dried under shade for a period of 12 days and then it was subjected to size-reduction by using domestic mechanical grinder and finally the powder is processed for extraction by using Soxhlet apparatus.

Preparation of Crude Extract of the Leaves: 500 grams of dried leaf powder was taken into a Soxhlet tube and to it suffient volume of methanol was added. The extraction process was processed. It was runned for about 3-4 cycles. The solvent obtained by filtration was concentrated by evaporating the methanol to get thick semi solid. This concentrated crude extract was transferred into a china dish and was dried in desiccators over a week (Azwanida NN, 2015).

Qualitative Chemical Tests for Phyto-Constituents

1. Test for steroidal Triterpenes

- Salkowski Test: Few drops of concentrated sulphuric acid was added to the chloroform extract, shaken and on standing, lower layer turns red in colour.

2. Tests for Saponins

- Foam Test: Small amount of extract is shaken with little quantity of water; the foam produced persists for 10 minutes. It confirms the presence of saponins.

3. Tests for Alkaloids

- Dragendroff's Test (potassium bismuth iodide): The acid layer with few drops of Dragendroff's reagent gives reddish brown precipitate.
- Mayer test (Potassium-mercuric-iodide solution): Alkaloids give cream colour precipitate with this reagent.
- Wagner test (iodine-potassium-iodide solution): Alkaloids give Brown colour precipitate with this reagent.
- Hager reagent test (Saturated solution of picric acid): Alkaloids give yellow colour precipitate with this reagent.

4. Test for carbohydrates)

- Molish's Test: The extract is treated with molish's reagent and concentrated sulphuric acid along the sides of the test tube, a reddish violet ring shows the presence of carbohydrate.
- Benedict Test: The extract on heating with Benedicts reagent, brown precipitate indicates the presence of sugar.

5. Test for flavonoids

Shinoda test: The alcoholic solution with few fragments of magnesium ribbon and concentrated hydrochloric acid produced management color after few minutes.

6. Test for Tannins

- Ferric Chloride Test: A 5% solution of ferric chloride in 90% alcohol was prepared. Few drops of this solution was added to a little of the above filtrate. If dark green or deep blue color is obtained, tannins are present.
- Lead Acetate Test: A 10% w/v solution of basic lead acetate in distilled water was added to the test filtrate. If precipitate is obtained, tannins are present.
- Potassium Dichromate Test: If on an addition of a solution of potassium dichromate in test filtrate, dark color is developed, tannins are present.

7. Test for Glycosides

- Keller-Kiliani Test: A solution of glacial acetic acid (4.0 ml) with 1 drop of 2.0% $FeCl_3$ mixture was mixed with the 10 ml aqueous plant extract and 1 ml H_2SO_4 concentrated. A brown ring formed between the layers which showed the entity of cardiac steroidal glycosides.
- Legal Test: The test is employed for digitoxose containing glycosides. The extract of drug is dissolved in pyridine, sodium nitroprusside solution is added to it and made alkaline, pink or red color is produced.
- Baljet Test: To the extract, sodium picrate solution is added. It shows yellow to orange colour (Monika Gupta et al., 2013), (Rahman_Gul et al., 2017).

Separation of Chemical Constituents by Column Chromatography

The adsorbent(silica gel)column grade is made into slurry with n-hexane in 1:6 ratio and placed in a cylindrical tube the 2g of crude extract is mixed with silica gel and placed in the column that is plugged at the bottom by a piece of cotton. The mixture to be separated was dissolved in a suitable solvent such as n-hexane and introduced at the top of the column and is allowed to pass through the column. As the mixture moves down through the column, the components are adsorbed at different regions depending on their ability for adsorption. The different components are collected separately by adding solvent like n-hexane, chloroform and methanol from the top of the column. The different fractions are collected separately distilled and evaporated the solvent from the different fractions to give the pure components (M. Danot et al., 1984).

The methanolic residue (2 g) was subjected to column chromatography over silica gel.
Weight of the residue: 2g
Weight of the silica gel (ACME 100-200 #): 30g
Volume of the Eluant: 300 ml

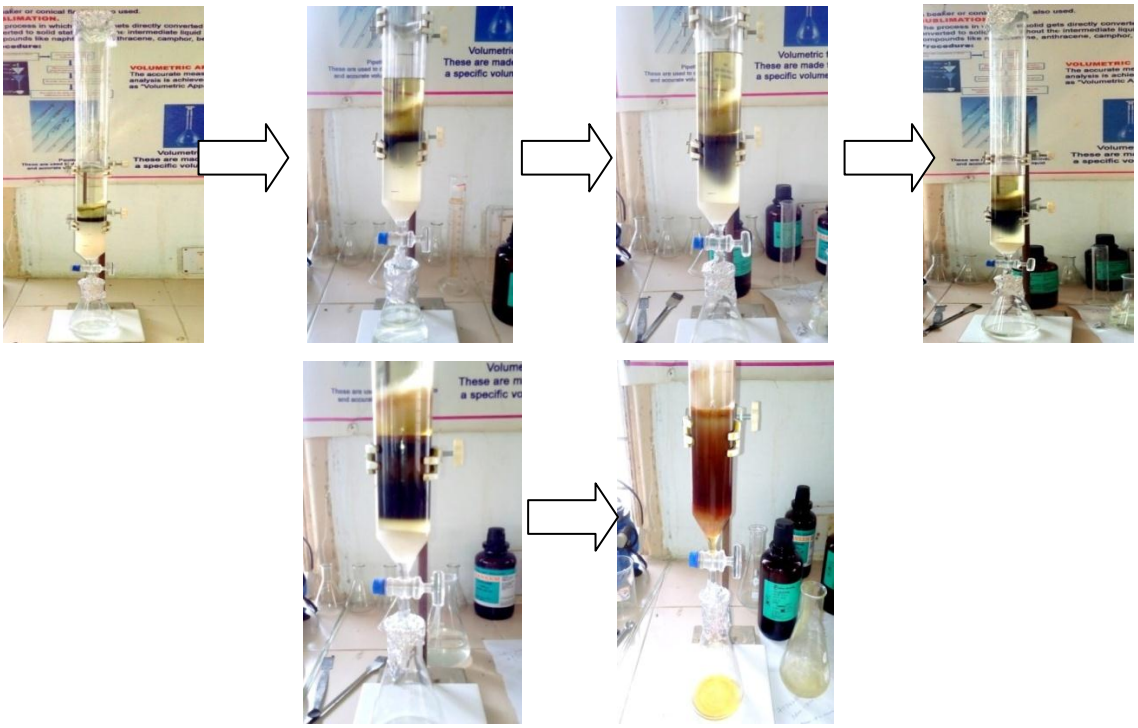


Fig 2: Separation by Column Chromatography.

Biological Screening

Anti-Bacterial Activity

The methanol and aqueous extracts of leaves were screened for the antibacterial activity against two Gram-positive bacteria viz., *Staphylococcus aureus* and two Gram-negative bacteria viz., *Escherichia coli* by using the cup plate method. Amicacin was used as reference standard for comparing the results.

Culture medium: Nutrient broth was used for the preparation of inoculum of the bacteria and nutrient agar was used for the screening method.

Composition of Nutrient agar medium

Peptone	5.0 gm
Sodium chloride	5.0 gm
Beef extract	1.5 gm
Yeast extract	1.5 gm
Agar	15.0 gm
Distilled water (q.s)	1000 ml
P^H	7.4± 0.2

The test organisms were sub cultured using nutrient agar medium. The tubes containing sterilized medium were inoculated with the respective bacterial strain. After incubation at 37ºC±1 ºC for 18 hrs, they were stored in a refrigerator. The nutrient agar medium was sterilized by autoclaving at 121ºC (15 lb/sq.inch) for 15 min. The petriplates, tubes and flasks plugged with cotton were sterilized in hot-air oven at 160ºC, for an hour. Into each sterilized petriplate (10 cm diameter), was poured about 40 ml of molten nutrient agar medium which was already inoculated with the respective strain of bacteria (1 ml of inoculum to 40 ml of nutrient agar medium) aseptically. The plates were left at room temperature aseptically to allow the solidification. Methanol extract of Neolamarckia *Cadamba* (5 mg) was dissolved in 5 ml methanol, to give a concentration of 1000 µg/ml. Amikacin solution was also prepared to give a concentration of 1000 µg/ml in sterilized distilled water. The pH of all the test solutions and control was maintained in between 2 to 3 by using conc. HCl. The Extract was tested at dose levels of 25µg (0.02 ml), 50µg (0.04 ml), 75µg (0.06 ml), and 100 µg (0.1 ml) and methanol used as a control. The solutions of extract of different concentrations mentioned above, control and reference standard (0.05 and 0.1 ml) were added separately in the cups and the plates were kept undisturbed for at least 2 hrs in a refrigerator to allow diffusion of the solution properly into nutrient agar medium. Petri dishes were subsequently incubated at 37 ± 1^0C for 24 hrs. After incubation, the diameter of zone of inhibition surrounding each of the cups was measured with the help of an antibiotic zone reader. All the experiments were carried out in duplicate (Hugo J.de Boer et al., 2005).

Anti-Fungal Activity

The extracts screened earlier for antibacterial activity were also tested for their antifungal activity. The fungi employed for the screening was Aspergillus *niger*. Fluconazole was employed as standard to compare the results. The test organisms were sub-cultured using Potato-Dextrose-Agar (PDA) medium. The tubes containing sterilized medium were inoculated with test fungi and kept at room temperature for obtaining growth. After that, they were stored at 4ºC in a refrigerator (Hugo J.de Boer et al., 2005).

Composition of Potato-Dextrose-Agar medium

Peeled potato	50.0 gm
Dextrose	5.0 gm
Agar-agar	4.0 gm
Distilled water upto	200 ml

The test organisms were sub-cultured using PDA medium. The tubes containing sterilized medium were inoculated with respective fungal strain and kept aside at room temperature for growing the organism. After confirming the growth, they were stored in a refrigerator. The inoculum was prepared by aseptically transferring 10 ml of sterile water into freshly sub-cultured slants of the test fungi and making a suspension by scraping the growth with an inoculation medium.

The PDA medium was sterilized by autoclaving at 121ºC (15 lb/sq. inch) for 15 min. The petri plates, tubes and flasks plugged with cotton, were sterilized in hot-air oven at 160ºC, for an hour. Into each sterilized petri plate (20 cm diameter), poured about 125 ml of molten PDA medium which was already inoculated with the respective strain of fungi (5 ml of inoculum to 250 ml of nutrient agar medium) aseptically. The plates were left at room temperature aseptically to allow the solidification. After solidification, the cups of each of 7 mm diameter were made by scooping out medium with a sterilized cork borer from a petridish and labeled accordingly.

Each test compound (5 mg) was dissolved in methanol (5 ml)to give a concentration of 1000 µg/ml. Fluconazole solution was also prepared at a concentration of 1000 µg/ml in sterilized distilled water. The pH of all the test solutions and control was maintained at 2 to 3 by using concentrated HCl. The extract was tested at dose levels of 25 µg (0.02 ml), 50 µg (0.04 ml), 75 µg (0.06 ml), and 100 µg (0.08 ml) and methanol used as a control. The solutions of each test compound, control and reference standards (0.05 and 0.1 ml) were added separately in the cups and the plates were kept undisturbed for at least 2 hrs in a refrigerator to allow diffusion of the solution properly into the PDA medium. Petri dishes were subsequently kept at room temperature for 48 hrs. After that, the diameter of zone of inhibition in mm surrounding each of the cups was measured with the help of an antibiotic zone reader. All the experiments were carried out in triplicate.

RESULTS

I) Microscopic evaluation

- The TS of leaf shows some distinguishing features having a vascular bundles and phloem fibers. The significant feature is that, the epidermal layer in thick and spreads all over the leaf and on petiole.

- The guard cells are bean-shaped in surface view, while the epidermal cells are irregular in shape.
- The guard cells contain chloroplasts, so they can manufacture food by photosynthesis (The epidermal cells do not contain chloroplasts).
- Number of stomata per sq.mm is 1.23
- Vein islet number is 3.33
- Stomatal index of a Neolamarckia cadamba leaf is 25.6%

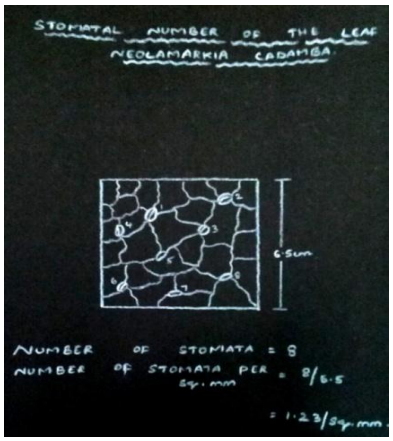


Fig 3: Stomatal Number

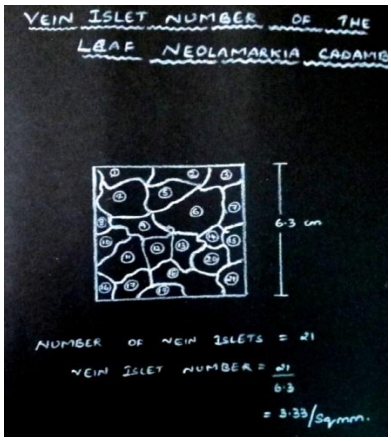


Fig 4: Vein-Islet Number

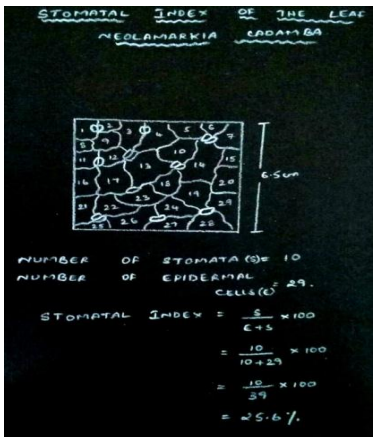


Fig 5: Stomatal Index

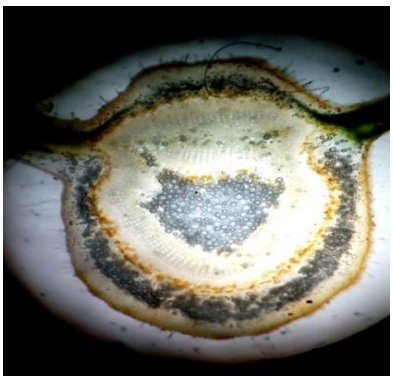


Fig 6: T.S of Leaf

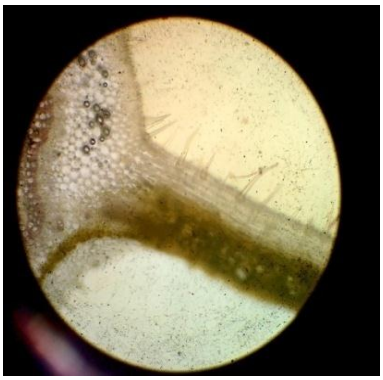


Fig 7: Trichomes

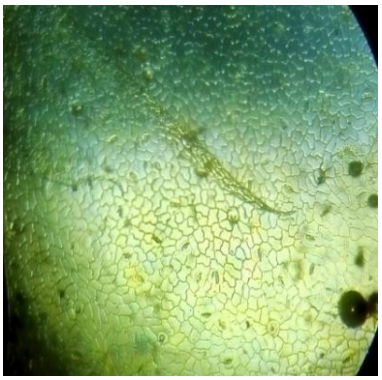


Fig 8: Stomata

II) Phytochemical screening of crude leaf extract of *Neolamarckia Cadamba:*

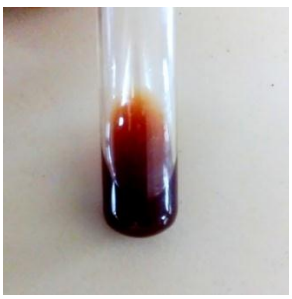


Fig 9: Test for Steroidal Triterpines.

Fig 10: Test for Glycosides.

Fig 11: Test for Tannins.

Fig 12: Test for Alkaloids.

Fig 13: Test for Saponins.

Table 1: Results for Phytochemical Screening

S.No	Secondary Metabolites	Results in *Neolamarckia Cadamba*
1	Alkaloids	+
2	Tannins	+
3	Flavanoides	-
4	Saponinns	+
5	Steroids	+
6	Glycosides	+
7	Coumarin	-
8	Carbohydrates	-
9	Starch	-
10	Inulin	-
11	Amino acid	-

(+) Indicates Presence, (-) Indicates Absence

III) Separation of Chemical Constituents

Fig 14: Samples collected through Column Chromatography.

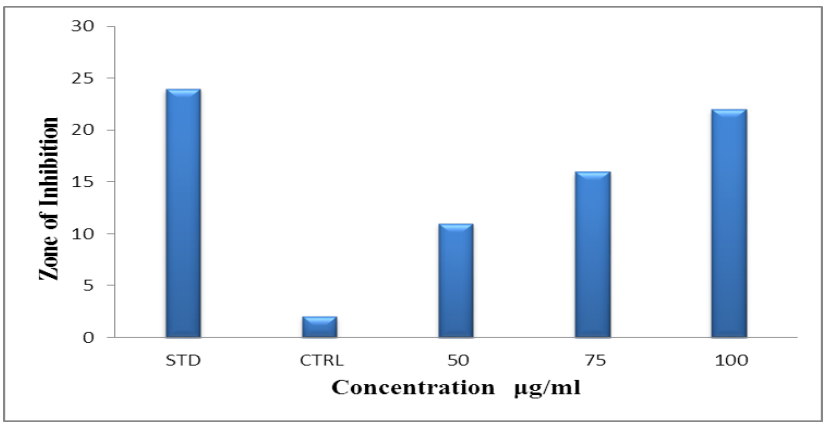
Table 2: Results for separation of Chemical Constituents.

S. NO	FRACTION	ELUANT	COMPOUND	INFERENCE
1	1-06	Pure hexane	Colourless solution	_
2	07-12	Hexane: Chloroform 50%	Green colour solution	Chorophyll
3	13-18	Pure Chloroform	Colourless solution	_
4	19-24	Chloroform : Methanol 50%	Red Mixture	Steroidal triterpines, glycosides and tannins
5	25-25	Pure Methanol	Yellow solution	Saponins and alkaloids

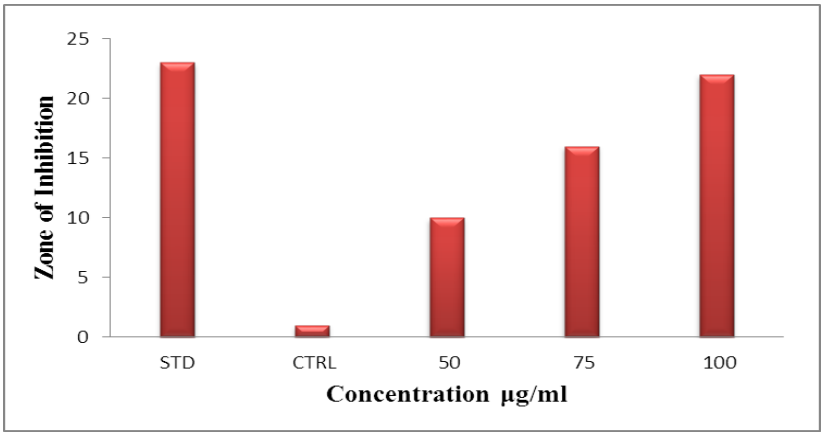
IV) Biological Screening

Table 3: Results for anti-bacterial activity

Conc.in µg/ml	Inhibition Zone of Gram-positive *Staphylococcus aureus* (mm)	Inhibition Zone of Gram-negative *Escherichia coli* (mm)
Standared (Amikacin)	24	20
Control (methanol)	2	8
50	11	5.6
75	16	10
100	22	17



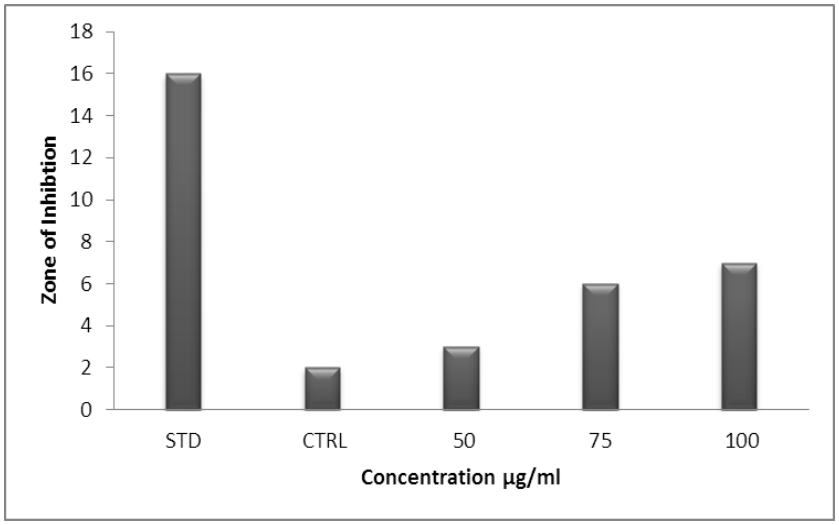
Graph 1: Inhibition Zone of Gram-positive bacteria (*Staphylococcus aureus*).



Graph 2: Inhibition Zone of Gram-negative bacteria (*Escherichia coli*).

Table 4: **Results for anti-fungal activity.**

Concentration µg/ml	Inhibition Zone of *Aspergillus niger* (mm)
Standard(flucanozole)	16
Control(methanol)	2
50	3
75	6
100	7



Graph 3: Inhibition Zone of Anti-fungal bacteria (*Aspergillus niger*).

DISCUSSION

From the Quantitative microscopy, the leaf constants like stomatal number, stomatal index, vein-islet number were calculated which was not known till date and also we have found the shape of the guard cells and epidermal cells. Whereas in Phytochemical screening, the methanolic extract of *Neolamarckia cadamba* showed positive results for Steroidal triterpines, Glycosides, Saponins, Tannins, Alkaloids and these were proved by performing chemical tests. Separation of chemical constituents were done by performing Column Chromatography by using different solvents like n-hexane, chloroform and methanol in different ratios and maximum separation was observed in chloroform and methanol ratios. We also noted that the leaves of Neolamarckia cadamba showed remarkable anti-bacterial activity and a minute anti-fungal activity.

CONCLUSION

The transverse section of the leaf of *Neolamarckia Cadamba* showed distinguishing features like vascular bundles and Phloem fibers and with a significant feature of having the epidermal layer in thick which spreads all over the leaf and on petiole. We have also

concluded that there is an absence of chloroplasts in epidermal cells. The crude extract of Neolamarckia cadamba showed anti-bacterial activity and minute anti-fungal activity. The extract has Secondary metabolites which were separated by column chromatography that results for performing further studies and also in the development of new Pharmaceutical activities.

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