FT-IR coupled secondary metabolites profiling and biological activities of Neolamarckia cadamba leaves

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ABSTRACT: Neolamarckia cadamba is a pharmacologically significant tropical tree, exploited for various biological studies due to its rich secondary metabolites. In the present investigation, comparison of pharmacological and biological studies of five different extracts of Neolamarckia cadamba leaves have been carried out. This is the first report of phytochemical studies of five different extracts, coupled with FT-IR spectroscopy. Methanol extract (NCLE-D) was found to be rich in secondary metabolites. This extract also showed higher phenolic (279.02 GAE/g extract) and flavonoid (1067.48 QE/g extract) contents. Ethyl acetate extract (NCLE-C) and NCLE-D showed significant free radical scavenging activity with IC50 15.17 µg/mL and 16.12 µg/mL respectively compared to ascorbic acid (positive control) with IC50 5.22 µg/mL. NCLE-C exhibited antibacterial activity as well. These results suggest that Neolamarckia cadamba is capable of producing bioactive compounds especially from its leaves; hence, could be a valuable source of new drugs for treating diseases.

1. INTRODUCTION

Many plants are explored for potential sources with various pharmacological properties. Most of them are still unknown or less explored. One such tree mentioned in the Indian Ayurveda (He et al., 2020) is Neolamarckia cadamba (Pandey and Negi, 2018). Neolamarckia cadamba (Roxb.) Bosser is included in the “Rubiacae” family; called “kadamb” in Hindi and “wild cinchona” in English. It grows in the humid habitat with high temperature. This miraculous tree is a fast-growing species that grows, both, in fertile, loose and humid soil and in humid sandy soil (Wang et al., 2021). Its wood is commercially important for biomass utilization (Tian et al., 2018), pulp and paper production (Misbahuddin et al., 2019), wood board making, furniture, and other construction purposes (Hao et al., 2020). It is recommended in Ayurveda for uterine problems, leprosy, blood disorders, and dysentery (Zhao et al., 2014). In spite of its traditional use for treating diseases like diabetes (George et al., 2021; Munira et al., 2020), this tree is still understudied scientifically. Neolamarckia cadamba contain various biologically active phytochemicals (Pandey & Negi, 2016). Leaves contain a variety of phytochemicals, including alkaloids, flavonoids, steroids (Kumar et al., 2015), as well as glycosidic and nonglycosidic alkaloids such as cadambine and iso-cadambine (Zhao et al., 2021).

This research study focuses on the enhancement of knowledge of bio-potency of Neolamarckia cadamba leaves. With the view of comparative study, various solvents of different polarity were used to compare extraction efficiency of bioactive phytochemicals. Non polar solvents included petroleum ether and chloroform, mid polar included ethyl acetate whereas polar solvents used were methanol and water. This is the first report of Fourier Transform Infrared Spectroscopy (FT-IR) coupled phytochemical investigation of various extracts of its leaves. Present work also estimates the total phenolic, flavonoid as well as chlorophyll content. Antioxidant property of extracts was compared by performing in vitro DPPH assay (2, 2- Diphenyl-1-picrylhydrazyl). Resazurin Microtiter assay was used to measure the antibacterial activity (Kasare et al., 2019).

Phytochemical screening methods that are based mainly on physical observations often fail to provide final conclusions. Under such circumstances, FT-IR scan for functional groups can contribute widely and served as a solution where confusions regarding the presence of phytochemicals existed. This is the first ever report of phytochemical screening of five different extracts of Neolamarckia cadamba leaves coupled with FT-IR spectroscopy. The study contributed significantly to confirm the presence of various functional groups in secondary metabolites, such as hydroxyl (sharp) to very broad peaks...
corresponding to secondary metabolites such as sterols, long chain alcohols, phenolics and saponins), carbonyl (acids, methyl esters of fatty acids, steroids, amides and lactones), -CH groups (aromatic metabolites, steroids, long chain alkanes and alkenes), nitrogen containing compounds (alkaloids, amines and amides), etc. Our findings confirm the importance of FT-IR spectroscopy in identifying secondary metabolites found in plant extracts. So, this paper highlights the potential of Neolamarckia cadamba and identifies the most biopotent leaf extract.

2. MATERIALS AND METHODS

2.1. Chemicals and instruments

Extrapure analytical grade chemicals were purchased from SDF Mumbai. Other chemicals and reagents used include Gallic acid, Quercetin, anhydrous sodium carbonate (Na₂CO₃), Sulphuric acid (H₂SO₄), α-Naphthol, Magnesium turnings, Hydrochloric acid (HCl), Lead acetate, Aluminum chloride, Ferric chloride (FeCl₃), Dragendorff's reagent, Mayer's reagent, Folin-Ciocalteu reagent, Fehling's A and B, solvents such as Acetone, Ethyl acetate, Petroleum ether, Chloroform, Dimethyl Sulphoxide (DMSO), Methanol, and Sodium hydroxide (NaOH). DPPH used was from Sigma Aldrich. Solvent to sample ratio was 10:1(v/w) which has been used in rising polarity order included petroleum ether, followed by chloroform, then ethyl acetate, next methanol and finally water. Solvent to sample ratio was 10:1(v/w) which has been used as an ideal one for extractions. The efficiency of extraction was boosted via ultrasonication, i.e., by exposing the solution to ultrasonic waves for 30 min at regular intervals for eight hours (Frequency: 40 KHz and Power: 100 W). The extracts were decanted, followed by filtration using Whatman Grade 1 filter paper, and evaporated at r.t. until completely dry. This retained the possible thermolabile compounds present in the extract. Their percentage yields were calculated (Table 1), phytochemicals were screened, total phenolics, flavonoids and biological activities were determined.

2.5. Phytochemical Screening

Freshly prepared crude extracts of leaves of Neolamarckia cadamba were screened for various active phytoconstituents by employing following standard methods. (Harborne & B, 1998; Prashant et al., 2011; Tease et al., 1989)

2.5.1 Test for Carbohydrates

Molisch's test: Few drops of 1% alcoholic α-Naphthol and 3 mL conc. H₂SO₄ in 1 mL of test solution containing 50 mg of extract in 5 mL double distilled water confirms the presence of carbohydrates by the appearance of a reddish violet or purple ring at the intersection of two liquids.

Fehling's test: 1 mL of Fehling's (A+B) solution was added to test solution with 2 mg of extract in 1 mL double distilled water. The solution was shaken briskly and heated on waterbath for 10 minutes. Carbohydrates were confirmed by the appearance of brick red precipitate.

2.5.2 Test for Flavonoids

Shinoda's test: Magnesium turnings in 5 drops of conc. HCl were added dropwise to 1 mL of test solution. After few minutes, a pink, scarlet or crimson red colour confirms flavonoids.

Alkaline reagent test: 5 drops of 5% NaOH is added to 1 mL of test solution. Intensity of yellow colour increases becoming colourless on adding few drops of 2M HCl. This indicates flavonoids.

2.5.3 Test for Saponins

Foam test: In a test tube, extracts were dissolved in water and shaken vigorously. A stable honey comb like froth persistent for about 10 minutes indicates saponins.

2.5.4 Test for Phytosterols

Salkowski’s test: 2 mg of extract was mixed in chloroform (CHCl₃) and filtered. To this, conc. H₂SO₄ was added slowly along the sides of test tube. Red colouration, when allowed to stand, indicates phytosterols.

2.5.5 Test for terpenoids

Salkowski’s test: 2 mg of extract was shaken with 1 mL of CHCl₃ and few drops of conc. H₂SO₄ was added. Red brown colour formed at the interface shows terpenoids present in the extract.
2.5.6 Test for Tannins

Lead acetate test: Formation of white precipitate on mixing few drops of lead acetate solution to 2 mL of test solution indicates the presence of tannins in the extracts.

2.5.7 Test for Phenolic compounds

Ferric chloride (FeCl₃) test: Bluish black colour formed after adding few drops of 5% FeCl₃ to 2 mL of test solution indicates the presence of phenolic compounds.

2.6 FT-IR Spectral analysis

The extracts were also characterized by FT-IR technique. Spectra obtained were analysed and presence of functional groups such as carbonyl, hydroxyl, etc. were confirmed.

2.7 Quantitative Estimation

2.7.1 Determination of total phenolic content

Folin Ciocalteu's method was used to estimate total phenolic content. 1 mL of aliquots and gallic acid solutions (10, 20, 40, 60, 80, 100 μg/mL) was taken in each test tubes. 0.5 mL of Folin Ciocalteu's reagent was added to 5 mL double distilled water. Post 5 minutes, 1.5 mL of 20 % sodium carbonate was added. Solution was diluted to 10 mL with double distilled water, incubated for 2 hours at r.t., developing intense blue colour. Absorbance was measured at 750 nm. The procedure was performed in duplicates. Solvent was used as blank. Standard drug was gallic acid. Total phenolic content was expressed as mg of gallic acid equivalent weight (GAE)/g dry mass (Bhalodia et al., 2010).

2.7.2 Determination of total flavonoid content

Aluminium chloride colorimetric method (Zhishen et al., 1999) was used to measure total flavonoid content. An aliquot (1 mL) of extracts and quercetin solutions (20, 40, 60, 80 and 100 mg/L) was added to 4 mL double distilled water in a10 mL volumetric flask. To the flask was added 0.3 mL of 5% NaNO₂, 0.3 mL of 10% AlCl₃ was added 5 min later. At 6th min, 2 mL of 1 M NaOH was added. Solution was diluted to 10 mL with double distilled water, mixed well and absorbance was measured against blank at 510 nm. Standard drug was quercetin. Total flavonoid content was calculated from linear equation derived from calibration curve and stated as mg quercetin equivalents (QE)/g fresh mass (Matotoka et al., 2018).

2.7.3 Estimation of total chlorophyll content

The amount of chlorophyll was evaluated by spectrophotometry by Arnon's method (Kousar et al., 2007). 200 mg of fresh leaf tissue was homogenized in mortars using pestle in 30 mL 80% acetone. The homogenate was filtered using Whatman Grade 1 filter paper. It was centrifuged for 5 min at 5000 rpm. The supernatant dark red fluid from brief centrifugation was collected. Absorbance was read at 645 nm (A645) and 663 nm (A663) against the blank. The amount of chlorophyll pigment present in the sample was calculated according to Arnon's formula,

\[ \text{Chlorophyll a (mg/L)} = \left[ \left( \frac{12.7 \times A663}{} \right) - (2.6 \times A645) \right] \times 10^3 \text{ml acetone /mg leaf tissue} \]

\[ \text{Chlorophyll b (mg/L)} = \left[ \left( \frac{22.9 \times A645}{} \right) - (4.68 \times A663) \right] \times 10^3 \text{ml acetone /mg leaf tissue} \]

\[ \text{Total Chlorophyll (mg/L)} = \text{Chlorophyll a} + \text{Chlorophyll b} \]

2.8 Biological Evaluations

2.8.1 Antioxidant Activity

DPPH Free radical scavenging assay (Brand-Williams et al., 1995)

Briefly, 100 mL of 0.2 mM DPPH solution was prepared in methanol. 2 mL of extracts of various concentrations in methanol was added to 2 mL of above DPPH solution. The mixture was shaken briskly and allowed to stand for 30 min. Absorbance was measured at 517 nm. Samples were analysed in duplicates. Inhibition of DPPH free radical (%I) was calculated as (Kim et al., 2019),

\[ \% I = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \]

where control contains all reagents except test compound.

2.8.2 In vitro antibacterial activity

2.8.2.1 Preparation of microbial culture

All the five extracts of *Neolamarckia cadamba* leaves were assessed for antibacterial activity. Multidrug resistant (MDR) bacteria may live for lengthy periods of time and spread epidemics. Among the most dangerous and common MDR bacteria are Staphylococcus aureus, Escherichia coli and Bacillus species. Other important MDR pathogens include Salmonella species (WHO, 2012). Hence, these four species were considered for our antibacterial studies (Abreu et al., 2012). Cultures of all four microbial strains were revived, maintained on Nutrient agar slants at 4°C. The procedure was done on 16-hour-old cultures.

2.8.2.2 Determination of minimum inhibitory concentration (MIC)

Resazurin microtiter assay (Bhuyan et al., 2017), endorsed by the World Health Organization (Katawera et al., 2014), was used to estimate MIC of extracts. Resazurin is a redox indicator dye, previously reported for determining the minimum inhibitory concentration of essential oils (Mann & Markham, 1998) from extracts. Faster antibacterial assays such as agar disk-diffusion method, Etest, cross streak method, etc., use expensive equipments, media and skilled microbiologists. Resazurin assay, on the other hand, is a simple, reliable, rapid and inexpensive colorimetric technique to detect MDR isolates, particularly non-water-soluble plant extracts, and can be easily adapted in low-income countries like India (Palomino et al., 2002; Teh et al., 2017). Nutrient agar sub-cultured test organisms were used to prepare microbial suspensions in nutrient broth. They were incubated at 37°C for 16 hours. The turbidity of suspension was adjusted to 0.5 which represents McFarland standard number. Extracts were dissolved in DMSO and diluted with
water to get a concentration of 1000 μg/mL. Two-fold dilutions in series gave concentration range 3.90-1000 μg/mL. Microtitre plates with 96 well were used for this purpose. They were inoculated with 50 μL of microbial strains. Chloramphenicol (standard drug) was positive control and DMSO, negative. The microtitre plates were and incubated at 37°C for 24 hours post sealing with parafilm. Resazurin (50 mL, 0.2 mg/mL in sterilized double distilled water) was added to wells and plates were reincubated for 30 min. Bio-active microorganisms were detected by resazurin's colour change from purple to pink. Lowest concentration of extracts with no colour change was the MIC.

3. RESULTS AND DISCUSSION

![Figure 1](image-url)  
*Figure 1. The antibacterial activity of Neolamarckia cadamba leaf extracts*

<table>
<thead>
<tr>
<th>Tests</th>
<th>NCLE-A</th>
<th>NCLE-B</th>
<th>NCLE-C</th>
<th>NCLE-D</th>
<th>NCLE-E</th>
</tr>
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<tbody>
<tr>
<td>Alkaloids Dragendorff’s test</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Mayer’s test</td>
<td></td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Carbohydrates Molisch’s test</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Fehling’s test</td>
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<td></td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Flavonoids Shinodi’s test</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkaline reagent test</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Saponins</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>−</td>
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<td>Phenolics 5% FeCl₃ test</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Tannins Lead acetate test</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ Present, − Absent

| Table 2 Phytochemical screening of Neolamarckia cadamba leaf extracts |
|-----------------------|------------------|------------------|------------------|
| Tests                  | NCLE-A | NCLE-B | NCLE-C | NCLE-D | NCLE-E |
| Alkaloids Dragendorff’s test | − | − | − | + | + |
| Mayer’s test | − | + | + | + | − |
| Carbohydrates Molisch’s test | − | + | + | + | − |
| Fehling’s test | − | − | − | + | + |
| Flavonoids Shinodi’s test | − | − | − | + | + |
| Alkaline reagent test | − | + | + | + | − |
| Saponins | − | + | + | + | + |
| Phytosterols Salkowski’s test | − | + | + | + | − |
| Terpenoids Salkowski’s test | − | + | + | + | − |
| Phenolics 5% FeCl₃ test | − | + | + | + | − |
| Tannins Lead acetate test | − | + | + | + | + |

| Table 1 Percentage yield of Neolamarckia cadamba leaf extracts |
|-----------------------|------------------|
| Extracts | % yield | Colour | Consistency |
| NCLE-A | 5.86 | Yellowish brown | Sticky |
| NCLE-B | 2.91 | Yellow | Solid |
| NCLE-C | 0.56 | Dark yellow | Sticky |
| NCLE-D | 8.64 | Brown | Sticky |
| NCLE-E | 12.76 | Dark brown | Solid |

3.1. Percentage yield of Neolamarckia cadamba leaf extracts

Table 1 shows the percentage yield, colour and consistency of each extracts obtained after successive extraction. Aqueous extract (NCLE-E) was obtained in high yield (12.76%) in solid form, followed by sticky methanol (NCLE-D, 8.64%), petroleum ether (NCLE-A, 5.86%) and chloroform (NCLE-B, 2.91%) extracts. All extracts ranged from yellow to brown in colour.

| Table 3 FT-IR spectral peak values and functional groups obtained for Neolamarckia cadamba leaf extracts |
|-----------------------|------------------|
| Extracts | Peak values (cm⁻¹) | Functional groups |
| NCLE-A | 1214 1449 2925 | C-N asymmetric stretching, C=C stretching, C-H stretching |
| | | Long chain alkenes, ethers, alkanes |
| NCLE-B | 1253 1273 1452 1686 2916 3394 | C-N stretching C-N stretching, C=C aromatic ring stretching, C=O carbonyl group C-H stretching, -OH group |
| | | Phenols, sterols, triterpenoids, poly hydroxy compounds, aromatic compounds, alkaloids |
| NCLE-C | 1074 1375 1451 1708 2849 3383 (broad) | C-N stretching C-N stretching, C=C aromatic ring stretching, C=O carbonyl group C-H stretching, -OH group |
| | | Sterols, triterpenoids, tannins, phenols, poly hydroxy compounds, aromatic compounds, alkaloids |
| NCLE-D | 1067 1365 1440 1605 2918 3292 (very broad) | C-N stretching C-N stretching, C=C aromatic ring stretching, C=H stretching, -OH group |
| | | Phenols, sterols, triterpenoids, poly hydroxy compounds, alkaloids |
| NCLE-E | 1047 1600 3268 (very broad) | C-N stretching C=C stretching, -OH group |
| | | Saponins, sterols, poly hydroxy compounds, alkaloids |
3.2. Phytochemical screening

Tannins and phenolics were found in NCLE-B, NCLE-C, NCLE-D and NCLE-E during their preliminary phytochemical screening (Table 2). Sterols and terpenoids were observed in NCLE-B, NCLE-C and NCLE-D. Only NCLE-E showed positive test for saponins. Flavonoids were present in NCLE-D and NCLE-E. NCLE-C, NCLE-D and NCLE-E showed the presence of carbohydrates. Presence of alkaloids was prominent in none of the extracts though they showed colour change non-spontaneously with little intensity.

3.3. FT-IR Spectral data interpretation

FTIR is feasibly the most effective tool to distinguish chemical bond forms (functional groups) present in compounds. The wavelength of the absorbed light is typical of chemical bonds. These chemical bonds are elucidated by interpreting the generated spectrum (Ashokkumar and Rangaswamy, 2014). Our study produced the FT-IR spectrum profile for Neolamarckia cadamba (Figures 1S to 5S). Major peak values, corresponding functional groups data and secondary metabolites present in compounds. The chemical bonds are elucidated by interpreting the generated spectrum (Ashokkumar and Rangaswamy, 2014). Our study produced the FT-IR spectrum profile for Neolamarckia cadamba (Figures 1S to 5S). Major peak values, corresponding functional groups data and secondary metabolites present in compounds.

3.4. Total phenolic and flavonoid content

Table 4 gives total amount of phenolic and flavonoids content in Neolamarckia cadamba leaves. Total phenolic content (mg GAE/g extract) varied from 39.25 mg/g to 279.023 mg/g, which was obtained with the help of standard curve for Gallic acid (R² = 0.94477, Figure 6S (b)). Total phenolic content was the highest in NCLE-D, followed by NCLE-E and NCLE-C. NCLE-B had the lowest phenolic content. Using the standard curve generated for quercetin (R² = 0.96929, Figure 6S (a)), the highest total flavonoids content (mg QE/g extract) was found to be in NCLE-D with 1067.48 mg/g. NCLE-E was also rich in flavonoid content (683.60 mg/g).

3.5. Total Chlorophyll content

Amount of Chlorophyll in leaf tissue of Neolamarckia cadamba is tabulated in Table 5. Chlorophyll b was twice in quantity than chlorophyll a proving the abundance of light harvesting chlorophyll-protein complex (LHClI) than other complexes of Photosystem II (Kitajima et al., 2003); subsequently, a lower Chlorophyll a/b ratio of 0.496.

Table 6

In vitro antioxidant activity of Neolamarckia cadamba leaf extracts

Table 7

Antibacterial activity of Neolamarckia cadamba leaf extracts, in vitro

3.6. Biological evaluations

3.6.1 DPPH Free radical scavenging assay

Table 6 shows IC₅₀ values for all the extracts and reference drug ascorbic acid. The study found that ethyl acetate and methanol extracts proved to be excellent antioxidants with good free radical scavenging compared with butylated hydroxytoluene (BHT), a synthetic standard antioxidant.
3.6.2 In vitro antibacterial assay

MIC for potential extracts and standard drug Chloramphenicol is shown in Table 7. It was found that all extracts, except aqueous, possessed comparable antibacterial activity.

4. CONCLUSION

Percentage yield of extracts produced by successive extraction of Neolamarckia cadamba leaves was in the order: NCLE-E > NCLE-D > NCLE-A > NCLE-B > NCLE-C. Preliminary screening of these extracts confirmed the abundance of secondary plant metabolites such as terpenoids, tannins and phenolics. This matched the FTIR spectrum profiles generated for each extracts. Phenols and flavonoids were quantitatively higher in the methanol extract being rich in phenols and flavonoids of Neolamarckia cadamba leaves. High frequency regeneration of plants via callus-mediated organogenesis from cotyledon and hypocotyl cultures in a multipurpose tropical tree (Neolamarckia cadamba). Scientific Reports. 10(1), 4558. https://doi.org/10.1038/s41598-020-61612-z


CONFLICTS OF INTEREST

None was declared by authors.

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