Chapter 3
Materials and Methods
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3.1. Plant Material

*Pajanelia longifolia* (Willd.) K. Schuman (Bignoniaceae) is widely distributed in the Southern Assam region of North East India in the three districts viz., Cachar, Karimganj and Hailakandhi. The leaves and bark of the plant is used in traditional medicine as a anti-microbial and hepatoprotective agent. Young leaves and stem bark of the plant were collected and air-dried. For record, a leaf twig with flowers was preserved on herbarium sheet and deposited to Assam University Herbarium, Department of Life Science, Assam University, Silchar.

3.2. Evaluation of Antimicrobial Activity

3.2.1. Anti-microbial Activity

*Micro-organisms used*

The test organisms (*Klebsiella* sp., *Streptococcus* sp., *Staphylococcus* sp., *Candida* sp., *Salmonella* sp., *Bacillus* sp., *E. coli* and *Proteus* sp. were obtained from Department of Microbiology, Silchar Medical College.

*Sterilization of glassware:*

Clean and dried glasswares were rinsed thoroughly with sterile double distilled water twice. Each of the glassware was rapped with paper and
autoclaved at 120°C at 15lb pressure for 15 min and dried in a hot air oven at 160°C.

**Preparation of media:**
Nutrient agar medium was used to maintain the culture. 1.49 g of the powdered medium was dissolved in sterile distilled water (500ml) in a conical flask. The content was mixed properly and allowed to dissolve by heating over a water bath. The flask were than tightly plugged and autoclaved at 120°C (15 lb pressure) for 15 min. The sterilized medium was then poured over Petri-plates and allowed to cool.

**Inoculum**
The micro-organisms were inoculated into Nutrient Agar Medium and incubated at 37°C prior to analysis.

**Preparation of filter paper discs**
For preparation of the filter paper discs, Whatman No. 1 filter paper was used. The discs containing the crude extracts and isolated pure compounds were placed over the nutrient agar plates and incubated at 37°C for 24h to 48h as per the method of Vincent and Vincent (1944). The zone of inhibition was recorded (no growth near the disc) and measured using a zone measurement scale (*HiMedia Labs, India*). The anti-bacterial activity of the plant was compared with standard antibiotics.
3.3. Evaluation of Hepatoprotective Activity

As per the accepted international protocol, the dried and milled bark extract (extracted with petroleum ether, ethyl acetate and 70% v/v ethanol) were taken for analysis of hepatoprotective activity. Swiss Albino mice were used as test animals. The animals were fed with standard pellet diet and supplied with sterile water *ad libitum*. Methyl cellulose (CMC) - 5% w/v in double distilled water was used as vehicle. The control groups received only the vehicle (1ml/kg body weight) by oral route. Standard group received Silymarin (100mg /kg body weight) once and then after 30 min received Carbon tetrachloride (CCL₄) and afterwards three times at 12h interval. Like standard group, the animals of the test group received plant extracts suspended in CMC vehicle in the dose of 200mg/kg body weight four times at 12h interval orally and received the toxicant once after 30min of the first dose of test drug (plant extracts) administration.

3.4. Collection of blood Samples

The blood samples were collected with the help of a syringe from the arterial vein of the mice or by making very small incision at the tip of the tail. The blood (~1ml) was collected and centrifuged at 3, 000 rpm for 15 min. The serum collected was stored at -80°C before use.
3.5. Estimation of serum bilirubin content

The bilirubin content was measured as per modified Jendrassik and Grof's method as suggested by Recommendations on a uniform bilirubin standard (1965). This spectrophotometric method is based on Diaz reaction.

3.6. Estimation of SGPT, SGOT and Alkaline Phosphatase

Serum Alkaline Phosphatase was measured as per the method of King (1965). The SGPT and SGOT levels were measured as per the method suggested by Reitman and Frankel (1957).

3.7. Qualitative Phytochemical Color Tests

A preliminary phytochemical screening (qualitative) was carried out for alkaloid, terpenoid, flavonoid, steroid, Saponin and tannin.

(a). Detection of Alkaloids:
To the solvent free extracts, few drops of dilute hydrochloric acid (HCl) were added followed by addition of Mayer’s Reagent, Dragandroff’s Reagent, Hager’s Reagent and Wagner’s Reagent in separate test tubes containing individual extracts. The observation exerted creamy off white, orange brown, yellow and reddish brown precipitate respectively.
(b). Detection of Flavonoids:
To the solvent free plant extract, 10% concentrated H₂SO₄ was added followed by addition of 1ml each of ammonia, sodium carbonate and sodium hydroxide in different test tubes. The observations exerted greenish yellow, pale yellow and yellow precipitate respectively.

(c). Detection of Saponin:
To the solvent free extracts, 20ml distilled water was added and shaken vigorously. A layer of foam formed detected the presence of Saponin. To the solvent free extracts, 1ml each of ammonia solution and lead acetate were added and shaken. Presence of black precipitate indicated the presence of Saponin.

(d). Detection of Tanin:
To the solvent free extracts, 1ml 5% ferric chloride solution was added. Bluish black or green black precipitate confirms the presence of Tanin.

(e). Detection of Steroid:
To the solvent free extracts, 1ml chloroform, 1ml acetic anhydride and 1ml concentrated H₂SO₄ were added. Development of reddish purple colour detected the presence of steroid.

3.8. Extraction and preparation of plant extracts
Leaf and bark of Pajanelia longifolia were collected from Cachar district (92°24’ E longitude and 24°22’N and 25°08’N latitude). Small sections
were air dried and milled. The milled leaf and bark materials were extracted using Soxhlet Apparatus (*JSGW, India*). About 5kg of the leaf and bark material were extracted in different phases. Firstly extraction was done with petroleum ether (b.p. 60-80°C) followed by extraction with ethyl acetate and 70% (v/v) ethanol to yield extracts with low polarity, medium polarity and high polarity. Other than these solvents absolute ethanol (100%) and methanol was also used. Each of the extraction was done with 10-15 siphoning cycles in the Soxhlet Apparatus. The material was taken and immediately evaporated using a Rotary Evaporator (*JSGW, India*) in vacuo, collected and stored for further analysis.

3.9. General Experimental Procedures

3.9.1. Thin Layer Chromatography (TLC)

The chromatographic chamber and plates were washed thoroughly with K₂Cr₂O₇ and H₂SO₄ mixture followed by washing with distilled water and dried in an oven. Silica gel slurry was prepared by mixing 20g of Silica Gel G with 40ml of distilled water, stirred with the help of a mortar and pestle. Coating of the silica gel slurry was done with the help of an applicator on clean dry glass plates. The plates were allowed to dry at room temperature and activated at 120°C for 30min. Uniform spots of the crude plant extracts were given over the silica gel coating
using clean capillary tubes. The glass plates were put gently inside the chamber and the chromatogram was developed by ascending technique till the solvent front moved 10-12 cm. The plates are then taken out and dry at room temperature and observed using iodine vapour.

3.9.2. Column chromatography (CC)

Isolation of the different components from leaf and bark of *Pajenelia longifolia* was carried out by column chromatography, where different combination of solvents like petroleum ether, *n*-Hexane, ethyl acetate, butanol, etc. were used as mobile phase and Silica gel (60-120 mesh) as stationary phase to yield different fractions. Distinct colour bands were visible during the separation process and the process continued with increasing polarity of the mobile phase.

3.9.3. Gas Chromatography

The gas chromatogram was developed using Clarus 500 GC-MS (*Perkin Elmer, USA*). The GC-MS was switched on for 2 hours prior to analysis. The plant extract was first filtered three times using Whatman No. 1 filter paper followed by filtration with Membrane filter. All the chemicals used were of spectroscopic grade (*Merck, Germany*). The chromatogram was developed by injecting 0.5μL of the sample to the GC. The mobile phase (gas) used was Helium. Each of the extract was studied in GC-MS for about 20 min and various peaks obtained in the
chromatogram were recorded. The whole operation was done using TurboMass 5.2. (Perkin Elmer, USA) software.

3.9.4. *Spectroscopic Analysis of Isolated Compounds*

The UV spectra were recorded on Thermo UV-Vis Spectrometer (*Merck, Germany*). The Infra Red (FT-IR) spectra were determined on Shimadzu IR Affinity Spectrometer in KBr pellets. The NMR (¹³C and ¹H) were recorded using CDCl₃ as internal standard on Bruker AM 400 Spectrometer. The mass spectra were recorded using Clarus 500 Mass Spectrometer (Perkin Elmer, USA).

3.10. **Analytical Reagents**

All the chemicals used were of analytical grade of Fisher Scientific, UK, Merck, Germany and Sigma, USA. Antibiotic discs used were procured from HiMedia Labs, India. All the glasswares used were of Borosilicate grade from Fisher (UK) and Merck (India & Germany).

3.11. **Drug Likeliness Score**

The molecular properties and drug likeliness of the isolated compounds was studied using online server www.molsoft.org.
3.12. Automated Pharmacophore Modeling

The pharmacophore modeling of the isolated compound was done in 
isico using LigandScout 2.0.

3.13. Docking Analysis

The docking was performed using AutoDock 4.0. Clinically approved 
drug targets were used. The key results in a docking log are the docked 
structure or conformations found at end of each run, the energies of the 
docked structures and their similarities to each other. The similarity of 
docked structure is measured by computing root mean square 
deviations (rmsd), between the coordinates of the atoms and creating 
clustering of the conformations based on these rmsd values. The 
docking results consist of the PDBQT of the Cartesian Coordinates of 
the atoms in the docked molecule, along with the state variable that 
describes this docked conformation and position of the docked energies.