Chapter 3
Materials and Methods

3.1. Plant Collection and Identification

Fresh leaves of *Gmelina asiatica* were collected from Scott Christian College Campus, Nagercoil, Kanyakumari District, South Tamilnadu, India and identified by using taxonomic keys (Gamble and Fischer, 1935; Brintha, 2012). The healthy and mature leaves were freshly collected and thoroughly washed with distilled water to remove all dust. The excess water in the sample is removed and spread over the filter paper and kept in shade at room temperature for about two weeks to dry. They were made into powder with the help of a mechanical grinder and sieved. The powdered leaves were used for extraction.

3.2. Preparation of Herbarium

The collected plant specimen was pressed using the pressboard and mounted over the standard size of herbarium sheet. The specimen was fixed into herbarium sheet and labelled. The voucher specimen was preserved in the Department of Botany, Scott Christian College, Nagercoil, Tamilnadu, India for future reference.

3.3. Taxonomic position of *Gmelina asiatica* Linn.

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subkingdom</td>
<td>Tracheobionta</td>
</tr>
<tr>
<td>Division</td>
<td>Spermatophyta</td>
</tr>
<tr>
<td>Sub division</td>
<td>Magnoliophyta</td>
</tr>
<tr>
<td>Class</td>
<td>Magnoliopsida</td>
</tr>
<tr>
<td>Subclass</td>
<td>Gamopetalae</td>
</tr>
<tr>
<td>Series</td>
<td>Bicarpellatae</td>
</tr>
<tr>
<td>Order</td>
<td>Lamiales</td>
</tr>
</tbody>
</table>
3.4. Habit and Botanical Description

*Gmelina asiatica* Linn. (Syn: *Gmelina parvifolia* Roxb.), is a deciduous large-sized bush or shrub, commonly growing to about 4 m to 8 m tall and much branched. The bark is thin, smooth and light yellowish or brownish white coloured. Wood is hard, grey coloured; branchlets horizontal, rigid, often compressed; twigs spinose, auxiliary spines sometimes leaf-bearing. Leaves are small with petioles 0.5-3 cm long, leaf-blades membranous, varying from oval to elliptic, obovate, sub rhomboid
or triangular in outline, ranging from 1-9.5 cm long (rarely to 13), 1.5-6 cm wide, entire or 3-5 lobed when young, basally mostly acute to cuneate, sometimes rounded, apically acute or obtuse, glabrous on both surfaces when mature, when young, dark and shiny above, pale green, glaucescent, and minutely white-glandular beneath, the glands round. Inflorescence is auxiliary and terminal, nodding or pendulous; bracts small, linear or lanceolate, 2-3 times as long as the calyx. Flowers are large, borne in short cymose in mostly terminal fulvous-tomentose racemiform panicles 2.5-5 cm long, corolla large, bright sulphur-yellow, 4-5 cm long, bilabiate, the tube narrow and curvate below, apically ampliate into a large ventricose throat, the limb 4 lobed, curved and bell shaped. Fruits are in drupes ovoid pyriform, yellow when ripe, with a watery, soapy exudates and contains 1-2 seeded (Plate 3.1).

3.5. General Habitat

_Gmelina asiatica_ L., commonly found in dry evergreen to dry deciduous forests, plains, wastelands, dry lands, agricultural lands and also on roadsides; occasional in scrub jungles, near villages and coastal areas.

3.6. Geographical Distribution

_Gmelina asiatica_ is a native of Southeastern Asia, distributed in temperate and coastal areas at an altitude of about 150-1200 ft. Globally, it is widely spread in Asian tropical countries such as Indian subcontinent: Bangladesh; Indo-china: Cambodia, Myanmar, Burma, Thailand, Vietnam; Indonesia, Malaysia; Asian Temperate regions including China, Srilanka and also in Oceanic Islands (Reunion and Mauritius, Andaman and Nicobar) (Plate 3.2).
In India, it is widely distributed in Bihar, Gujarat, Rajasthan, West Bengal, Utter Pradesh, Madhya Pradesh, Andhra Pradesh, Karnataka (Coorg, Mysore); Maharashtra (Pune, Raigad, Ratnagiri, Thane); Kerala (Kollam, Idukki, Kannur, Palakkad and Thiruvananthapuram) and Tamilnadu.

3.7. Pharmacognostic studies

Fresh healthy *G. asiatica* plants were collected from Scott Christian College campus, Nagercoil, Kanyakumari District, Tamilnadu, India. The required samples of different organs were cut and removed from the plant and fixed in FAA (Formalin-5 mL+ Acetic acid-5 mL + 70% Ethyl alcohol-90 mL). After 24 h of fixing, the specimens were dehydrated with graded series of tertiary tutyl alcohol as per Sass (1940). Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60°C) until TBA solution attained supersaturation. The specimens were cast into paraffin blocks.
3.7.1. Sectioning

The paraffin-embedded specimens were sectioned with the help of a rotary microtome. The thickness of the sections was 10-12 μm. Dewaxing of the sections was by customary procedure (Johansen, 1940). The sections were stained with Toluidine blue as per the method published by O’Brien et al. (1964). Since toluidine blue is a polychromatic stain, the staining results were remarkably good; and some cytochemical reactions were also noticed. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. Sections were also stained with safranin and Fast-green and IKI (for Starch) wherever necessary.

For studying the stomatal morphology, venation pattern and trichome distribution, paradermal sections (sections taken parallel to the surface of leaf) as well as clearing of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey’s maceration fluid (Sass, 1940) were prepared. Glycerine mounted temporary preparations were made for macerated/cleared materials. Powdered materials of different parts were cleared with NaOH and mounted in glycerine medium after staining. Different cell component were studied and measured.

3.7.2. Photomicrographs

Microscopic descriptions of tissues are supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon labphoto 2 microscopic Unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since
these structures have birefringent property, under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the scale-bars. Descriptive terms of the anatomical features are as given in the standard Anatomy books (Easu, 1964).

3.8. Preparation of extracts

The dried powdered sample (100 g) of *G. asiatica* was extracted with 1000 mL of solvents such as water, petroleum ether, chloroform, ethanol and acetone in a Soxhlet apparatus. The resultant filtrate was concentrated to powdered form through complete evaporation of the extraction solvent using Rotary evaporator. The solid residue of greenish brown colour obtained was designated as the extract, which was stored in a refrigerator until further analyses (Plate 3.3 and 3.4).

Plate: 3.3. Soxhlet apparatus
Hydrodistillation was conducted by using a standard procedure (Clevenger apparatus) with dried *G. asiatica* leaves which had previously been powdered in a mechanical grinder. The process was carried out continuously on a heating mantle at the temperature 60-80°C until no further oil was extracted. The essential oil was dried over anhydrous Na$_2$SO$_4$ and after filtration stored in a dark bottle at 4°C until analysis.

### 3.10. Preliminary phytochemical screening

Qualitative chemical tests were conducted to gain a general idea regarding the nature of constituents present in the extract. Aqueous, petroleum ether, chloroform, ethanol and acetone extracts were subjected to preliminary phytochemical investigations for detection of specific compounds as per the standard methods prescribed by Harborne (1998).
3.10.1. Tests for Carbohydrates

*Fehling’s Test:* 1 mL of Fehling’s A and 1 mL of Fehling’s B solution were added, mixed and boiled for 1 min. Now equal volume of test solution (aqueous, petroleum ether, chloroform, ethanol and acetone extracts) was added to the above mixture. The solution was heated in a boiling water bath for 5-10 min. A precipitate which was initially yellow turning to brick red colour indicated the presence of carbohydrates.

3.10.2. Tests for Glycosides

*Lugol’s Test:* Concentrated aqueous, petroleum ether, chloroform, ethanol and acetone extracts were treated with a few drops of 10% sodium hydroxide (NaOH) to make it alkaline. Then freshly prepared sodium nitroprusside was added to the solution. Formation of blue coloration indicated the presence of glycosides in the extracts.

3.10.3. Tests for Flavonoids

*Lead Acetate Test:* To a small quantity of extract lead acetate solution was added. Formation of yellow precipitate showed the presence of flavonoids.

3.10.4. Tests for Phenolic compounds

*FeCl₃ Solution Test:* Extracts were treated with 5% FeCl₃ solution. Formation of bluish black colour indicated the presence of phenols.

3.10.5. Tests for Saponins

*Foam Test:* Plant extracts were shaken vigorously with 2 mL of water. If the foam produced persisted for 10 min it indicated the presence of saponins.
3.10.6. Tests for Terpenoids

To the extract chloroform and conc. H$_2$SO$_4$ were added. Appearance of red colour indicated the presence of triterpenes.

3.10.7. Tests for Steroids

*Salkowski Test*: To 2 mL of extract, 2 mL of chloroform and 2 mL of conc. H$_2$SO$_4$ were added. The solution was shaken well. Formation of red chloroform layer and greenish yellow acid layer indicated the presence of steroids.

3.10.8. Tests for Alkaloids

The extract (1 mL) was treated with a few drops of diluted HCl and filtered. The filtrate was treated with 1 mL of Dragendorff’s reagent. Formation of reddish brown precipitate indicated the presence of alkaloids.

3.10.9. Tests for Quinones

1 mL of the extract was treated with alcoholic KOH solution. Presence of quinones was indicated by coloration ranging from red to blue.

3.10.10. Tests for Coumarins

Alcoholic extract (1mL) was treated with alcoholic NaOH solution; production of dark yellow colour indicated the presence of coumarins.

3.10.11. Tests for Proteins

*Xanthoproteic Test*: To a small quantity of extract 1 mL of conc. H$_2$SO$_4$ was added. This resulted in the formation of white precipitate which on boiling turned yellow. On addition of ammonium hydroxide (NH$_4$OH), the yellow precipitate turned orange.
3.10.12. Tests for Phytosterols

The extract was dissolved in 2 mL of acetic anhydride. To this 1 or 2 drops of conc. \( \text{H}_2\text{SO}_4 \) was added slowly along the sides of the tube and an array of colors was noticed, indicating the presence of phytosterols.

3.11. Quantitative phytochemical analysis

3.11.1. Estimation of Flavonoids (Evans, 1996)

Total flavonoid content was determined by the aluminium chloride method using gallic acid as the standard; 1mL of test sample and 4 mL of water were added to a volumetric flask (10 mL volume). After 5 min, 0.3 mL of 5% sodium nitrite and 0.3 mL of 10% aluminium chloride were added. After 6 min of incubation at room temperature, 2 mL of 1 M sodium hydroxide (NaOH) was added to the reaction mixture. Immediately the final volume was made up to 10 mL with distilled water. The absorbance of the reaction mixture was measured at 510 nm against a blank spectrophotometrically. Results were expressed as gallic acid equivalent (mg gallic acid/g dried extract).

3.11.2. Estimation of Tannins (Robert, 1971)

One millilitre of the extract was mixed with 5 mL of vanillin hydrochloride reagent a mixture of equal volumes of 8% HCl in methanol and 4% vanillin in methanol). The mixture was allowed to stand for 20 min and the absorbance measured at 500 nm. The standard graph was plotted for working standard catechin solution (0 to 250 µg/µL).
3.12. Fourier Transform Infrared Spectroscopic Analysis (FT-IR)

Oven-dried leaf samples (60°C) were ground into fine powder using a mortar and pestle. Two milligrams of the sample was mixed with 100 mg KBr (FT-IR grade) and then compressed to prepare a salt-disc (3 mm diameter). The disc was immediately kept in the sample holder and FT-IR spectra were recorded in the absorption range between 400 and 4000 cm\(^{-1}\). All investigations were carried out with a Shimadzu FT-IR spectrometer.

3.13. Gas Chromatography-Mass Spectrometry analysis (GC-MS)

Ethanolic extract of leaves of \(G. asiatica\) were subjected to GC-MS analysis. Extracts were dissolved in high-performance liquid chromatography (HPLC)-grade ethanol and subjected to JEOL GCMATE II GC-MS (Agilent Technologies 6890N Network GC system for gas chromatography). Helium was used as the carrier gas at a flow rate of 1mL/min. The temperature was programmed at 80°C for 5 min then increased to 300°C at the rate of 15°C /min. The temperatures of injector and EI detector (70 eV) were 280 and 300°C, respectively; 2 μL of plant extract was injected with a Hamilton syringe into the GC/MS manually.

3.13.1. Identification of Components

Interpretation of mass spectrum obtained from GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectra of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

Antihaemolytic activity of the sample extract was assessed by the method described by Naim et al. (1976). Blood samples were collected from rats under mild anaesthesia via cardiac puncture method into EDTA-coated tubes. The erythrocytes were separated by centrifugation and washed with 0.2 M phosphate buffer (pH 7.4). The erythrocytes were then diluted with phosphate-buffered saline to give 4% suspension; 250 µg of the sample extract in saline buffer was added to 2 mL of the erythrocyte suspension and the volume was made up to 5 mL with saline buffer. The mixture was incubated for 5 min at room temperature and then 0.5 mL of H$_2$O$_2$ solution in saline buffer was added to induce the oxidative degradation of the membrane lipids. The concentration of H$_2$O$_2$ in reaction mixture was adjusted to bring about 90% haemolysis of blood cells after 120 min. After incubation the reaction mixture was centrifuged at 1500 rpm for 10 min and the extent of haemolysis was determined by measuring the absorbance at 540 nm corresponding to haemoglobin liberation. The analysis was performed in triplicates and results were expressed in terms of percentage activity.

3.15. Antioxidant activity

The antioxidant activity of ethanolic leaf extracts of *G. asiatica* was determined by *in vitro* methods. 2,2-diphenyl-1-picrylhydrazyl free radical scavenging, hydroxyl radical scavenging, superoxide radical scavenging and 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) free scavenging assay methods were employed to assess the antioxidant potential of the extracts. All the assays were carried out in triplicate.
3.15.1. Determination of DPPH antiradical assay

The antioxidant activity of the sample was determined in terms of hydrogen-donating or radical-scavenging ability, using the stable radical DPPH, according to the method of Blois (1958). The sample extracts were taken at various concentrations (20-100 µg) and the volume was adjusted to 100 µL with methanol. Five millilitres of 0.1 mM methanolic solution of DPPH was added and allowed to stand for 20 min at 27°C. The absorbance of the sample was measured at 517 nm. Percentage radical-scavenging activity of the sample was calculated as follows:

\[
\% \text{ DPPH radical - scavenging activity} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100
\]

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC\textsubscript{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

3.15.2. Hydroxyl radical-scavenging Activity

The scavenging activity of the sample on hydroxyl radicals was measured according to the method of Klein \textit{et al.} (1991). Different concentrations of the extract (20-100 µg) were added 1 mL of iron-ethylenediamine tetraacetic acid (EDTA) solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 mL of EDTA solution (0.018%), and 1mL of dimethyl sulfoxide (DMSO) (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 80-90°C for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1 mL of ice-cold trichloroacetic acid (TCA) (17.5% w/v). Three millilitres of Nash reagent (75.0 g of ammonium acetate,
3 mL of glacial acetic acid, and 2 mL of acetyl acetone were mixed and the volume made up to 1 L with distilled water) was added and the mixture kept undisturbed at room temperature for 15 min. The intensity of the colour formed was measured spectroscopically at 412 nm against reagent blank. The % hydroxyl radical-scavenging activity (HRSA) was calculated as follows:

\[
\% \text{ HRSA} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100
\]

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC\(_{50}\)) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

3.15.3. Superoxide radical-Scavenging Activity

Superoxide radicals were generated by a modified method of Beauchamp and Fridovich (1971). The assay was based on the capacity of the sample to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system. Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, 12 mM EDTA, 0.1 mg NBT and various concentrations (20-100 µg) of sample extracts. Reaction was started by illuminating the reaction mixture with sample extract for 90 s. Immediately after illumination the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture kept under dark condition served as blank. The percentage inhibition of superoxide anion generation was calculated as:
\[
% \text{ Inhibition} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100
\]

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC\textsubscript{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

3.15.4. Free radical-Scavenging Activity on ABTS

The antioxidant activity of the samples was measured by ABTS radical cation depolarization assay according to the method of Re \textit{et al.}, (1999). ABTS\textsuperscript{**} was produced by the reaction of 7 mM ABTS aqueous solution with 2, 4 mM of potassium persulfate under dark condition for 12-16 h at room temperature. Prior to assay, this solution was diluted in ethanol (about 1:89 v/v) and equilibrated at 30°C to give an absorbance at 734 nm of 0.70 ± 0.02. The stock solution of the sample extracts were diluted and add 10-µL aliquots into the assay. It produces 20% and 80% inhibition of the blank absorbance. After the addition of 1 mL of diluted ABTS solution to 10 µL of sample (10-100 µg/ml), absorbance was measured at 734 nm exactly 30 min after the initial mixing. Samples were analyzed in triplicate. Percentage radical-scavenging activity of the sample was calculated as follows

\[
\% \text{ ABTS radical-scavenging activity} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100
\]

The analysis was performed in triplicate. The sample concentration provides 50% of inhibition (IC\textsubscript{50}) in the assay condition and was calculated from the graph of inhibition percentage against sample concentration.
3.16. Antidiabetic activity

3.16.1. In vitro inhibition of α-amylase (Miller, 1959)

The α-amylase (0.5 mg/mL) was premixed with the extract at various concentrations (100-500 μg/mL) and starch was added as a substrate as 0.5% solution to start the reaction. The reaction was carried out at 37°C for 5 min and terminated by adding 2 mL of 3,5-dinitrosalicylic acid (DNS). The reaction mixture was heated for 15 min at 100°C and diluted with 10 ml of distilled water in an ice bath. α-amylase activity was determined by measuring spectrum at 540 nm. The % α-amylase inhibitory activity was calculated by the following formula:

\[
\% \text{ Inhibition} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100
\]

The IC₅₀ value was defined as the concentration of the sample extract to inhibit 50% of α-amylase activity under assay condition.

3.16.2. In vitro inhibition of α-glucosidase (Miller, 1959)

The enzyme α-glucosidase inhibitory activity was determined by premixing α-glucosidase (0.07 Units) with 100-500 μg/mL of extract. Then 3 mM p-nitrophenyl glucopyranoside was added as a substrate. This reaction mixture was incubated at 37°C for 30 min and the reaction was terminated by addition of 2 mL of sodium carbonate. The glucosidase activity was determined by measuring the p-nitrophenyl release from p-nitrophenyl glucopyranoside at 400 nm. The % α-glucosidase inhibitory activity is calculated by the following formula:

\[
\% \text{ Inhibition} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100
\]
The IC\textsubscript{50} value was defined as the concentration of the sample extract to inhibit 50% of \( \alpha \)-glucosidase activity under assay condition.

### 3.17. Anticancer activity

#### 3.17.1. Cell line

Cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune. The cells were maintained in minimal essential medium (MEM) supplemented with 10\% foetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 \( \mu \)g/mL) in a humidified atmosphere of 50 \( \mu \)g/mL CO\textsubscript{2} at 37\(^\circ\)C.

#### 3.17.2. Reagents

Minimal Essential Media (MEM), foetal bovine serum (FBS), trypsin, Methylthiazolylidiphenyl-tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO) and MCF-7 were purchased from Hi-Media & Sigma Aldrich, Mumbai.

#### 3.17.3. \textit{In vitro} assay for cytotoxicity activity (MTT assay)

The anticancer activity of samples on MCF 7 was determined by the MTT assay (Mosmann, 1983). Cells (\( 1 \times 10^5 \)/well) were plated in 0.2 mL of medium/well in 96-well plates. Then the plates were incubated 5\% CO\textsubscript{2} incubator for 72 h. Then, various concentrations of the samples were added in 0.1\% DMSO for 24 h 5\% CO\textsubscript{2} incubator. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 20 \( \mu \)L/well (5 mg/mL) of 0.5\% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) in phosphate-buffered saline solution was added. After 4 h of incubation, 1 mL of DMSO was added. Viable cells were determined by the absorbance at 540 nm. Measurements were performed and the concentration required for a 50\% inhibition of viability (IC\textsubscript{50}) was determined.
graphically. The effect of the samples on the proliferation of MCF7 cells was expressed as the % cell viability, using the following formula:

\[
\% \text{Cell viability} = \frac{A_{540 \text{ of treated cells}}}{A_{540 \text{ of control cells}}} \times 100\%
\]

3.18. Larvicidal activity

3.18.1. Collection of eggs

The eggs of *Aedes aegypti* and the larvae of *culex quinquefasciatus* were collected from the Centre for Research in Medical Entomology (ICMR), Madurai, Tamilnadu, India.

3.18.2. Maintenance of eggs and larvae

The collected eggs were brought to the laboratory and transferred to 18 × 13 × 4 cm size enamel trays containing 500 mL water and kept for larval hatching. The freshly hatched larvae were fed with dog biscuits and yeasts in 3:1 ratio. They were added to the culture medium 24 h before adding the eggs. The feeding was continued till it reached the pupal stage. Homogenous population of larvae was produced (5 days old and 5 mm in length) from five to seven days later.

3.18.3. Larvicidal Bioassay

Three trials were carried out against vector mosquitoes for the following bioassays (Tonk *et al.*, 2006). Toxicity assays of the crude extract was conducted separately using the third instar larvae of *A. aegypti* and *C. quinquefasciatus*. Stock solution (1000ppm) was prepared by dissolving 100 mg of crude extract in 1 mL DMSO and volume raised to 100 mL with distilled water. From this, different dilutions of 10-100 ppm were prepared in 200 mL deionized water in 250 mL beaker
and third instar larvae 20 numbers were released in it and mortality was scored after 24 h. The beakers were kept in room temperature and the larvae were exposed to 200 mL water containing 0.1 mL of DMSO which served as control.

3.18.4. Larval susceptibility tests

The larval susceptibility tests were carried out according to the standard of WHO procedure (2005). The extract solutions of different concentrations were prepared and larvae of *Culex quinquefasciatus*, was placed in each test solution to observe the larvicidal property as per the following procedure. Groups of 20 larvae were placed in 200 mL of the extract solution. Control experiments without extract were run in parallel. The larvae in each solution were then left for 24 h. The numbers of dead larvae were counted after 24 h of exposure, and the percentage of mortality was recorded. The assay was extended to 24 h and the percentage of mortality was recorded. When control mortality ranged from 5-20 percent and it was calculated by Abbott’s (1925) formula.

\[
\text{Mortality(\%)} = \frac{\text{% mortality treated group} - \text{% mortality control group}}{\text{% mortality control group}} \times 100
\]

3.19. Determination of antibacterial activity

3.19.1. Bacterial strains

Seven human pathogenic bacterial strains were taken into consideration, four Gram-positive (*Actinomyces howelli* MTCC-3048, *Bacillus circulans* MTCC-9720, *Staphylococcus aureus* MTCC-3160 and *Streptococcus pyogenes* MTCC-1927) and three Gram-negative (*Escherichia coli* MTCC-9721, *Pseudomonas aeruginosa* MTCC-1688 and *Proteus vulgaris* MTCC-7299) bacteria were selected for
antibacterial activity assay. These species were collected from Microbial Type Culture Collection (MTCC), Chandigarh, India. The test organisms were sub cultured at 37°C for 24 hrs and maintained in their appropriate agar slants at 4°C throughout the study and used as stock cultures.

3.19.2. Preparation of standard inoculums, filter paper discs and plates

The microorganisms were inoculated into Muller Hinton broth (MHB) and incubated at 37°C for 24h. The resulting suspension was diluted with MHB to match with 1 McFarland turbidity standard. This level of turbidity is equivalent to approximately 3.0×10⁸ CFU/ mL, equivalent to 0.5 McFarland standards. The dried plant extracts were dissolved in 10% aqueous dimethylsulfoxide (DMSO) to a final concentration of 200 mg/mL and sterilized by filtration. Antimicrobial tests were then carried out by the disc diffusion method using the inoculum containing 10⁶ bacterial cells to spread on Muller-Hinton agar plates (1 mL inoculum/plate). The discs (diameter 6 mm) were impregnated with 50 µL of extract (10 mg/disc) at a concentration of 200 mg/mL.

3.19.3. Screening for antimicrobial activities

Antibacterial activities of G. asiatica leaf extracts were carried out by disc diffusion method using the Kirby-Bauer technique (Bauer et al., 1996). All the bacterial strains were maintained on nutrient agar (NA). Pure culture was inoculated into (Muller Hinton Agar) MHA plate and subcultured at 37°C for 24 h. Standardized inoculum was transferred and spread evenly on a MHA plate to yield a lawn culture. Sterile Whatman No.1 filter paper discs were impregnated with plant extracts (50 µg/disc) and inoculated in MHA plates. They were and allowed to diffuse for
30 min at 4°C and incubated at 37°C for 24 h. Kanamycin (10µg) served as a positive control. The plates were observed for the presence of inhibition of bacterial growth which was indicated by the clear zone around the disc. The size of the zone of inhibition (excluding disc) was measured in millimetres. The absence of zone inhibition was interpreted as the absence of activity. All the experiments were carried out in triplicates under strict aseptic conditions and the zone of inhibition around each disc was measured for sensitivity or resistance. The antibacterial activity was expressed as the mean zone of inhibition diameters (mm) ± standard deviation (S.D) produced by the plant extract.

3.19.4. Determination of Minimal Inhibitory Concentration (MIC)

The MIC of the crude extract was determined for each of the test strains in test tubes (Eloff, 2004); 0.5 mL of each of the test isolate was added to different concentrations of the leaf extracts (ranges from 5 to 50 µL/mL) containing 2 mL of nutrient broth. Similar tubes without leaf extract served as control. The cultures were then incubated at 37°C for 24 h. After incubation the tubes were examined for microbial growth by observing the turbidity. The tubes containing the least concentration of extract showing no visible sign of growth was considered as MIC.

3.19.5. Determination of Minimum Bactericidal Concentration (MBC)

To determine the MBC, for each of the test isolate 1 mL of the broth was collected from the tubes that showed no growth and inoculated in sterile nutrient agar. The plates were then incubated at 37°C for 24h. After incubation the concentration showed no visible growth was considered as MBC.
3.20. Statistical Analysis

Three replicates of each sample were used for statistical analysis and the values are reported as mean ± standard deviation (SD). Means, standard deviations and standard errors were calculated from replicates within the experiments and analyzed using Microsoft Excel XP. The percentage of mortality values for the third instar larvae *Aedes aegypti* and *Culex quinquefasciatus* treated with various concentrations (ranging from 10 to 100 ppm) of the leaf extract of *G. asiatica* was recorded and the percentage mortality was calculated and the data was analyzed using curve expert software for finding the LC$_{50}$, LC$_{90}$ and LC$_{95}$ values. The third degree polynomial fit was used as a suitable mathematic model in the curve expert software.