Materials and Methods
3 MATERIALS AND METHODS

3.1 Macroscopic study

3.1.1 Collection of plant material

_Gmelina asiatica_ Linn. was collected from Palayamkottai, Tirunelveli district, Tamil Nadu during August 2010. The plant was identified by Dr. Sasikala, Deputy Director, The Central Research Institute for Siddha and Ayurveda, Arumbakkam, Chennai. The plant was deposited in General Herbarium collection Voc. No. BD-V-047, Government Siddha Medical College, Arumbakkam, Chennai (Appendix I).

Mature, fresh and healthy plant materials were used for macroscopic evaluation. The nature of the aerial (leaf, stem, flower, fruit and seed) and sub aerial parts (root) of _G. asiatica_ were analyzed by careful observation according to standard procedure (WHO, 2002).

3.2 Microscopic study

Microscopic analysis of leaf, stem, petiole and root was carried out using semi-permanent preparations (Johansen, 1940). The anatomical features were described as given in the published literature (Esau, 1964 a, b).

The plant parts of _Gmelina asiatica_ (stem, root, internode and leaf) were selected from the mature twigs for taking sections. The required parts were sliced into bits of desirable sizes. The bits were washed in distilled water. Then the bits were placed in formalin - acetic - alcohol for 12 hours (alcohol used – 70%). Then the materials were rinsed quickly in distilled water. Then they were transferred to commercial hydrofluoric acid (48 %) for 120 hours. The plant material was removed from hydrofluoric acid. Then it was washed in running tap water for one hour.

The plant material was transferred to 50% tertiary butyl alcohol solution and then transferred and retained in Safranin stain for 96 to 120 hours before taking section. The material was dehydrated using tertiary butyl alcohol solution, from 20% to 100% at time intervals of two hours. Then they were transferred to 10%, 25%, 50%, 70%, 85%, 95% and 100% of toluene series made up in tertiary butyl alcohol at two hour time intervals. Then they were transferred to solution of equal parts of xylene and toluene.
Finally the bits were transferred to 100% xylene. The specimen bottle with the material was placed in the oven for one and a half hours, allowing xylene to warm up. The materials were infiltrated in a good grade of rubber paraffin. This was repeated several times with intervals of three hours. The materials were removed after complete infiltration and allowed to set in room temperature. The materials were sectioned at 10 microns and the paraffin was removed from slide using xylene. The sectioned material was transferred to solution of equal parts of xylene and absolute alcohol and retained for 45 seconds with constant agitation. The sectioned material was transferred to absolute ethyl alcohol and the agitation was continued for 45 seconds. The counter stain was done with Fast Green and agitated for about 30 to 45 seconds. Then the slides were transferred to xylene and alcohol and kept for one minute (one part xylene, one part clove oil and one part of 100% ethyl alcohol), later they were cleared in synthetic methyl salicylate for about two times at two minutes interval each, and mounted using DPX mountant.

3.3 Pharmacognostic study

Plant parts such as leaf, stem and root were used for the following pharmacognostic study.

3.3.1 Organoleptic evaluation

Organoleptic characters such as colour, size, odour, taste and texture were observed based on the description by Nadkarni, (1982).

3.3.2 Physico-chemical evaluation

Disease-free plant parts (root, stem and leaf) were shade dried, powdered and stored in airtight containers. Physico-chemical evaluation (foreign matter, loss on drying, total ash, acid insoluble ash tests) were carried out by employing standard methods as per WHO guidelines (Anonymous, 1998; Indian Pharmacopoeia, 1996). The extractive values (alcohol and water soluble) of the powdered drug were determined according to the procedure described by Mukherjee, (2002).

The various physicochemical parameters like ash values and extractive values were performed as per the standard procedures. In physical evaluation, moisture content, ash values viz., total ash, acid insoluble ash, and extractive values viz., alcohol soluble extractive value, water soluble extractive values were determined. The ash value represents
the inorganic salts present in the drug. Extracts obtained by exhausting crude drugs are indicative of approximate measures of certain chemical compounds they contain, the diversity in chemical nature and properties of contents of drug. The determinations were performed in triplicate and results are expressed as mean ± standard error. The percentage w/w values were calculated with reference to the air-dried drug.

**Total ash**

About 4 g of the plant powder was accurately weighed in a previously ignited and tarred silica dish. The sample was ignited in a muffle furnace at 600ºC to convert completely into ash. The dish was repeated to get constant weight. The percentage of total ash was calculated.

\[
\text{Percentage of Total Ash} = \frac{\text{Weight of Ash}}{\text{Weight of Sample}} \times 100
\]

**Acid insoluble ash**

To the dish containing total ash, 15 ml of 1:5 Hydrochloric acid was added thrice and gently heated for 5 minutes and filtered through Whatman No. 1 filter paper. The filter paper along with the insoluble matter was transferred to silica dish and ignited in muffle furnace at 600ºC to get constant weight. The dish was cooled in desiccator and weighed. The percentage of acid insoluble ash was calculated.

\[
\text{Percentage of Acid Insoluble Ash} = \frac{\text{Weight of Ash}}{\text{Weight of Sample}} \times 100
\]

**Water soluble extractive**

About 4 g of the plant powder was accurately weighed in glass stoppered 250 ml flask. 100 ml of distilled water was added and shaken occasionally for 6 hours and then allowed to stand for 18 hours. The solution was filtered using Whatman No.1 filter paper. About 25 ml of the filtrate was pipette out into a pre weighed 100 ml beaker. The filtrate was evaporated on a water bath and the beaker was dried in a hot air oven at 105ºC for 6 hours. It was cooled in a desiccator and weighed. The percentage of the water soluble extractive was calculated.
Alcohol soluble extractive

About 4 g of the plant powder was accurately weighed in glass stoppered 250 ml flask. 100 ml of distilled alcohol (95%) was added and shaken occasionally for 6 hours and then allowed to stand for 18 hours. The solution was filtered using Whatman No.1 filter paper. About 25 ml of the filtrate was pipetted out into a pre weighed 100 ml beaker. The filtrate was evaporated on a water bath and the beaker was dried in a hot air oven at 105ºC for 6 hours. It was cooled in desiccator and weighed. The percentage of the alcohol soluble extractive was calculated.

Lignin (Krishnamurthy, 1988)

Phloroglucinol (1, 3, 5 Benzene triol) is a white or yellowish crystalline powder (melting point of 218°C) soluble in ethanol and water. The reagent was prepared by dissolving 1g of Phloroglucinol in 100ml of ethanol (10 g/Lt). It is not only used for staining sections, but also for vegetable powders and deposits (Phloroglucinol method-Johansen, 1940; Siegel, 1953). Fresh plant materials like stem and root were sectioned uniformly using sharp razor and mounted on a clean slide. Saturated aqueous solution of Phloroglucinol in 20% of HCl was poured on the mounted sections. The sections were allowed to be in Phloroglucinol solution for five minutes. Then the excess solution was drained and observed under the microscope.
Starch (Krishnamurthy, 1988)

2 g of potassium iodide was dissolved in 100 ml of distilled water. 0.2 g of iodine was mixed with above potassium iodide solution. This solution was used for staining the starch grains deposited inside the tissues (Potassium Iodide method). The fresh sections of stem and root were placed on a clean glass slide separately. A few drops of iodine-potassium iodide solution were poured on the sections. The sections were allowed to stay in for five minutes. Then the sections were mounted on the microscope one by one to view the colour change.

Polyphenols (Krishnamurthy, 1988)

Toluidine blue (0.05 g) was dissolved in 200 ml of benzoate buffer. Benzoate buffer was prepared by dissolving 0.25 g of benzoic acid and 0.29 g of sodium benzoate in 200 ml of water. The solution was allowed to saturate for 10 to 15 minutes (Toluidine blue method). The sections were stained with 0.05% of toluidine blue for 5 to 10 minutes. After rinsing, the slides were observed under the microscope.

Tannins (Krishnamurthy, 1988)

20% solution of ferric chloride or ferric sulphate (20 g/100ml) was mixed with 10% of formalin solution (10/100 ml). The sections were mounted in a clean glass slide and the prepared reagents were sprayed. Then the excess of solution were drained and observed under the microscope.

Protein (Krishnamurthy, 1988)

One ml of sodium hydroxide solution (40%), 2 drops of copper sulphate solution (1%), were poured on the sections till a blue color is produced.

Cutin and suberin

Sudan IV solution and ethanol 70% were mixed thoroughly and allowed to stand for 10 minutes. The cell walls and periderm wall layers were stained with this solution. A drop of this stain was dropped on sections and observed for colour change.

Pharmacognostic evaluation is usually carried out by observation of the external features of the fresh or dried plant parts and by analyzing and interpreting their
distinguishing characters. In the present study, the vegetative parts like root, stem and leaf were analyzed separately.

**Lignified wall layers**

The woody plants normally have lignified wall layers in their periderm, cork cells, vascular elements and stone cells. This can be observed by using specific stains and their colour change.

**Vascular bundles**

The vascular elements comprise water conducting tissues of xylem and food conducting tissues of phloem. The arrangement of these tissues varies species to species. Hence using vascular elements for identification and analyzing is one of the evaluating techniques.

**Determination of leaf constants - palisade ratio**

The different parameters like stomatal number, stomatal index, vein islet number and palisade ratio were determined as per standard procedure (Wallis, 1997).

Small pieces of leaf from the apex were selected, the middle and basal portion of lamina, were taken from young or old leaves, the lower epidermis was peeled off and then cut into pieces. It was boiled gently in a test tube taking 4 - 5 such leaf pieces in about 5 ml of strong solution of chloral hydrate till the green colour goes off. The pieces were kept separately on a glass slide with its upper epidermal layer visible at top. It was focused under high power and the palisade cells just below the epidermis were observed under the microscope.

**Stomatal index and stomatal number**

The stomatal number and stomatal index are very specific criteria for identification and characterization of leafy crude drugs.

**Vein islet number**

This was another criterion for identification and evaluation of herbal drugs. It could be defined as the small area of green tissues surrounded by the vein islets. It is determined by counting the number of vein-islets in an area of 4 sq.mm of the central part of the leaf.
between the midrib and the margin. This value remains constant within the plant species and is of diagnostic value in differentiating the species.

Small pieces of leaf were selected from the middle and basal portion of lamina. These pieces were boiled gently in a test tube taking 4 - 5 such leaf pieces in about 5 ml of strong solution of chloral hydrate till the green colour goes off. The pieces were kept separately on a glass slide with its upper epidermal layer visible at top. It was focused under high power and vein endings were observed under the microscope.

3.3.4 Powder microscopy

Shade dried stem, leaf and root were powdered with the help of an electric grinder till a fine powder was obtained. This fine powder of the stem, leaf and root were subjected to powder microscopy, as per standard procedures mentioned (Wallis, 1997).

3.3.5 Qualitative evaluation of phytochemicals

In order to identify the efficacy of the vegetative parts (stem, leaf and root) of the *Gmelina asiatica* plant, the extracts were prepared separately using polar and non-polar solvents.

**Preparation of plant material**

The mature vegetative parts of *Gmelina asiatica* were collected and shade dried completely, then used for extraction. The stem, leaf and root of the plant were separately cut and air-dried at room temperature for seven days and pulverized in mortar and pestle. The powdered samples were sieved into fine powder and stored in an air-tight zip bags. The extracts were prepared using soxhlet apparatus using non polar solvents. The aqueous solvents extracts were prepared using lyophilizer. The residues were lyophilized to get dry solid mass.

**Solvents used for extraction**

The orders of solvents used were chloroform, ethyl acetate, ethanol, methanol and water.

**Extract preparation**

The powdered plant parts were extracted using chloroform, ethyl acetate, ethanol, methanol and water by sequential extraction (1:4, w/v). The crude extract of the plant parts
were prepared in soxhlet apparatus. The plant parts (stem, root and leaves) were dried, powdered and weighed. Powdered plant parts (50 g) were soaked in 200 ml of organic solvent using soxhlet apparatus. Briefly the extraction was carried out at room temperature until exhaustion. The extracts were filtered and air dried at 40°C. The weight of each dry residue was recorded. The crude extract was reconstituted with dimethyl sulphoxide (DMSO) whenever used. All extracts were prepared and stored at 4°C until used.

**Aqueous extract**

Lyophilization is a dehydration process typically used to preserve a perishable material or make the material more convenient for transport. Freeze-drying works by freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to sublime directly from the solid phase to the gas phase. In a lab, this is often done by dry ice and methanol.

The drying phase aims to remove unfrozen water molecules, since the ice was removed in the primary drying phase. After the freeze-drying process is complete, the vacuum is usually broken with an inert gas, such as nitrogen, before the material is sealed. At the end of the operation, the final residual water content in the product is extremely low, around 1% to 4%. Lyophilized (freeze dried) products retain their original properties. By adding water or other solvents they can be reconstituted easily and very quickly to a usable solution. The extracted residues were collected in the following order: chloroform extract, ethyl acetate extract, ethanol extract, methanol extract and aqueous extract. The extractive values were tabulated, correlated and interpreted.

**Screening of phytochemicals**

The concentrated extracts were subjected to qualitative tests for the identification of alkaloids (Mayer’s test and Dragendorff’s test), carbohydrates (Molish’s, Fehling’s and Benedict’s test), glycosides (Borntrager’s test), proteins (Millions test), saponins (Foaming test), phenols (Ferric chloride, Gelatin and Lead acetate test) and flavonoids (Ammonia test) as per the standard procedures described by Harborne (1984), Sofowara (1993) and Trease and Evans (1997). The intensity of the coloration determines the abundance of the compound present.
**Steroid (Liebermann-Burchard test for steroids)**

To a 0.2 g of sample extract, 2 ml of acetic acid was added; the solution was cooled well in ice followed by the addition of conc. H₂SO₄ carefully. Colour development from violet to blue or bluish-green indicated the presence of a steroid i.e. aglycone portion of cardiac glycoside (Sofowara, 1993).

**Triterpene (Test for terpenoids)**

Sample extract (0.2 g) was dissolved in ethanol. To this solution 1 ml of acetic anhydride was added followed by the addition of conc. H₂SO₄. A change in colour from pink to violet showed the presence of terpenoids (Sofowara, 1993).

**Flavonoids (Shinoda's test for flavonoids)**

About 0.5 g of sample extract was dissolved in ethanol, warmed and then filtered. Three pieces of magnesium chips was then added to the filtrate followed by few drops of concentrated HCl. A pink, orange, or red to purple colouration indicates the presence of flavonoids (Trease and Evans, 2002).

**Sugar (Molisch's test for Carbohydrates)**

Few drops of Molisch's reagent was added to about 0.2 g of sample extract and dissolved in 1 ml of distilled water, this was then followed by addition of 1 ml of conc. H₂SO₄ by the side of the test tube. The mixture was then allowed to stand for two minutes and then diluted with 5 ml of distilled water. Formation of a red or dull violet colour at the interface of the two layers was a positive test for sugar (Sofowara, 1993).

**Monosaccharides (Barfoed's test)**

About 0.5 g sample extract was dissolved in distilled water and filtered. 1 ml of the filtrate was then mixed with 1 ml of Barfoed's reagent in a test tube and then heated on a water bath for a period of 2 minutes. Reddish precipitate of cuprous oxide was considered as a positive test for monosaccharides (Sofowara, 1993).

**Reducing sugars (Fehling's test for free Reducing sugar)**

About 0.5 g sample extract was dissolved in distilled water and filtered. The filtrate was heated with 5 ml of equal volumes of Fehling's solution A and B. Formation of a red
precipitate of cuprous oxide was an indication of the presence of reducing sugars (Sofowara, 1993).

Reducing sugars (Fehling's test for Combined Reducing Sugars)

About 0.5 g sample extract was hydrolysed by boiling with 5 ml of dilute hydrochloric acid and the resulting solution neutralised with sodium hydroxide solution. To this, few drops of Fehling's solution was added and then heated on a water bath for 2 minutes. Appearance of a reddish-brown precipitate of cuprous oxide indicates the presence of combined reducing sugars (Sofowora, 1993).

Alkaloids (Test for alkaloids)

Sample extract (0.2 g) was stirred with 5 ml of 1% aqueous HCl on water bath and then filtered. Of the filtrate, 1 ml was taken individually into 2 test tubes. To the first portion, few drops of Dragendorff's reagent were added; occurrence of orange-red precipitate was taken as positive. To the second 1 ml, Mayer's reagent was added and appearance of buff-coloured precipitate will be an indication for the presence of alkaloids (Sofowora, 1993).

Tannins (Test for Tannins)

About 0.5 g sample extract was stirred with about 10 ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 ml of the filtrate, occurrence of a blue-black, green or blue-green precipitate indicates the presence of tannins (Trease and Evans, 2002).

Quinines (Borntrager's Test)

About 0.2 g sample extract was shaken with 10 ml of benzene and then filtered. 5 ml of the 10% ammonia solution was then added to the filtrate and shaken. Appearance of a pink, red or violet colour in the ammonical (lower) phase was taken as the presence of free anthraquinones (Sofowora, 1993).

Phenol

About 5 ml of aqueous plant extract was mixed with 2 ml of FeCl₃ solution and formation of deep bluish green colour indicates the presence of phenol.
Acid

About 0.2 - 0.5 g of sample was weighed in 250 ml of conical flask. 50 ml of Ethyl alcohol was added and heated in hot water bath. This solution was titrated against 0.1N KOH using phenolphthalein indicator. The end point was inferred from colourless to pink colour appearance.

\[
\text{Acid value} = \frac{A \times N \times 56.1}{W}
\]

Where \( A = \) ml of 0.1N KOH consumed for sample, \( N = \) Normality of KOH & \( W = \) Weight in g of the sample.

Saponins (Test for saponins)

One gram of sample was boiled with 5 ml of distilled water and filtered. To the filtrate, about 3 ml of distilled water was further added and shaken vigorously for about 5 minutes. Frothing which persisted on warming was taken as an evidence for the presence of saponins (Sofowora, 1993).

Coumarins (Test for coumarins)

Aqueous plant extract (0.5 g) was taken in a small test tube and covered with filter paper moistened with 1 N NaOH. The test tube was placed for few minutes in boiling water. Then filter paper was removed and examined in UV light for yellow fluorescence to indicate the presence of coumarins (Cao et al., 1998).

2.3.6 Quantitative evaluation of phytochemicals

The sample extracts which have shown positive for presence of phytochemicals were further quantitatively analysed. Hence the methanol and aqueous extracts of the leaf, stem and root were subjected to quantitative determination of phenols, carbohydrates, flavonoids, tannins, amino acids, proteins, photosynthetic pigments, and non-photosynthetic pigments.

Estimation of total phenol content (McDonald et al., 2001)

One g of the powder was extracted three times each with 20 ml of acetone: methanol: water (7:7:6 v/v/v) at room temperature. It was kept in shaker for 1 - 2 hours, filtered through Whatman No. 1 filter paper and centrifuged. To 1 ml of filtrate, 0.2 ml of
Folin Ciocalteau reagent (0.5 N) was added and incubated in room temperature for 15 minutes. 5 ml of 2% sodium carbonate solution was added and it was incubated at room temperature for 30 minutes. The absorbance was measured at 760 nm. Pyrocatechol (10 mg/100 ml) was used as a standard (Slinkard and Singleton, 1977) and the total phenolic content was expressed in terms of standard equivalent (mg/g) of the sample on dry weight basis.

**Estimation of total carbohydrates by Anthrone Method (Trevelyan and Harrison, 1952)**

One g of powder was homogenized using 10 ml of distilled water and centrifuged at 5,000 rpm for ten minutes. 0.5 ml of the supernatant was transferred into a fresh tube and the volume was made up to 1 ml with distilled water. 4 ml of 0.2% Anthrone reagent was added and heated in water bath for 10 minutes and allowed to cool. The formation of green colour was observed and the absorbance was measured at 630 nm. Glucose was used as the standard and the amount of total carbohydrates was expressed in terms of the standard equivalent (mg/g) of the sample on dry weight basis.

**Estimation of flavonoids (Boham and Kocipal-Abyazan, 1974)**

Five gram of the powder was taken and extracted with 50 ml of 80% (v/v) aqueous methanol and covered and kept in shaker for 1 - 2 hrs. Then filtered through Whatman No.1 filter paper and transferred to petriplates, was evaporated to dryness. After the extract dried, it was weighed and the presence of flavonoids was expressed as mg/g on dry weight basis.

**Estimation of tannins (Oyaizu, 1986)**

Total tannins were estimated using Folin-Denis method. The absorbance was measured at 760 nm using Shimadazu UV-1601 Spectrophotometer. Analyses were carried out in triplicate and quantification was calculated from a calibration curve obtained with tannic acid. Total tannins were expressed as g/100g of tannic acid equivalent.

**Test for total free amino acids by Ninhydrin method (Chinard, 1952)**

Sample powder was homogenized in 80% (v/v) ethanol and centrifuged at 5000 rpm for 10 minutes. 0.5 ml of the supernatant was taken and it was made up to 1 ml with distilled water and then 2 ml of 4% Ninhydrin was added and heated in boiling water bath
for 20 minutes. It was then cooled and observed for the change of colour to purple-violet and the absorbance was read at 570 nm in UV spectrophotometer (Systronics 117, India). Glycine was used as the standard and the total amino acid content was expressed in terms of the standard equivalent (mg/g) of the sample on dry weight basis.

**Estimation of total soluble proteins by Lowry et al., method (1951)**

The alkaline copper reagent was prepared by mixing 50 ml of solution A and 1 ml of solution B. Solution A (Phosphate buffer (pH 7.0)) is a mixture of 1M mono sodium phosphate monohydrate (138 g/L) and 1M di sodium phosphate (142 g/L). Solution B is a mixture of 2% Na₂CO₃ in 0.1N NaOH and 0.5% CuSO₄ in 1% sodium potassium tartrate. This mixture serves as alkaline copper reagent. Solution C is Folin Ciocalteau reagent (0.5N).

Sample powder was homogenized using 1 ml of 1M phosphate buffer (pH 7.0) and centrifuged at 5000 rpm for 10 minutes. 0.5 ml of the filtrate was made up to 1 ml using distilled water. To this 4.5 ml of alkaline copper reagent was added and incubated at room temperature for 10 minutes. To this 0.5 ml of Folin Ciocalteau reagent (0.5 N) was added and incubated for 30 minutes in dark, at room temperature. Appearance of bluish-black colour was observed and absorbance at 620 nm was read. Bovine serum albumin was used as the standard and the amount of total soluble proteins was expressed in terms of the standard equivalent (mg/g) of the sample on dry weight basis.

**Estimation of photosynthetic pigments by Lichtenthaler and Wellburn’s method (1983)**

Fresh sample of leaf (50 mg) was weighed and homogenized with pre-chilled 80% acetone solution. Then, it was centrifuged at 6000 rpm for 3 minutes. Resulting pellet was resuspended in acetone till the pellet became colourless successively, after each wash.

Chlorophyll \(a = (12.21 \times A463) - (2.81 \times A647)\)  
Chlorophyll \(b = (20.13 \times A647) - (5.03 \times A663)\)  
Total Chlorophyll \(= \) Chlorophyll \(a +\) Chlorophyll \(b\)  
Carotenoids \(= (1000 \times A470) - (3.27 \times \) Chlorophyll \(a) - (104 \times \) Chlorophyll \(b) / 229\)

Where \(A\) 470 = Absorbance at 470 nm; \(A\) 647 = Absorbance at 647 nm; \(A\) 663 = Absorbance at 663 nm.
Test for non-photosynthetic pigments by Mancinelli et al., (1975)

Fresh leaf sample (50 mg) was weighed and homogenized and left in 80% acidified methanol (80:20:1) overnight and kept in dark to extract the flavonoids in the given sample. This extract was centrifuged and then the supernatant was removed and the absorbance read at 530 nm, 670 nm and 315 nm.

\[
\text{Carotinoids} = (A530) - (0.38 \times A670)
\]

Where \( A530 \) = Absorbance at 530 nm; \( A670 \) = Absorbance at 670 nm.

2.4 Analytical evaluation

The two prominent uses of TLC in the standardization of plant materials include fingerprint profiling for the assessment of chemical constituents of a drug and quantitative analysis of markers in plant drugs. Hence the study was performed in methanol and aqueous extracts of \textit{Gmelina asiatica} aerial parts separately. Based on qualitative and quantitative evaluation of phytochemicals methanol and aqueous extracts of leaf, stem and root were taken for analytical evaluation.

2.4.1 Thin Layer Chromatography (TLC)

The methanol and aqueous extracts were tested through TLC and compounds were identified. About four grams of the sample was soaked in 40 ml of methanol, kept overnight, boiled for ten minutes and filtered. The filtrate was concentrated to 10 ml and made up to the mark in a 10 ml standard flask. About 10 and 15 µl of this solution was applied on Merck aluminium pre-coated plate 60 F\textsubscript{254} recoated with silica gel of 0.2 mm thickness and the plate was developed in toluene: ethyl acetate: formic acid; 5:1.5:0.5. After drying the plate was visualized under UV at 254 and 366 nm and photographs were taken. The plate was dipped in Vanillin-Sulphuric acid reagent and kept in oven at 105ºC till the colour of the spots appeared. The photographs were taken. The TLC of aqueous extracts was also performed in the same procedure.

2.4.2 High Performance Thin Layer Chromatography (HPTLC)

High performance TLC (HPTLC) plates use thin layers of adsorbent (100 µm instead of 200 - 250 µm) and smaller particles (5 - 6 µm versus 10 - 12 µm) of more homogeneous size (4 - 8 µm versus 5 - 20 µm). Moreover, they give better resolution (5 -
to 10-fold more) over shorter runs (3 - 6 cm versus 8 - 15 cm), reduce separation time (3 - 20 min versus 20 - 200 min), accommodate more samples per plate (more than double), use smaller sample volumes (0.1 - 0.5 μl versus 1 - 5 μl) with improved detection limits (100 - 500 pg), and significantly improve the precision, accuracy and sensitivity.

The study of TLC was further authenticated through the HPTLC analysis. The fingerprinting has been done using the following chromatographic conditions. Chromatography was performed on a 10 x 10 cm pre-activated HPTLC silica gel 60 F254 plates. Samples were applied to the plate as 6 mm wide band with an automatic TLC applicator (Linomat 5 with N2 flow - CAMAG, Switzerland, and 8mm from the bottom). Densitometric scanning was performed on CAMAG scanner III. The plates were pre-washed by methanol and activated at 60°C for 5 minutes and 20 minutes scanning speed was employed. 10 ml of mobile phase was used per chromatography. Linear ascending development was carried out in 20 cm x 10 cm twin glass chamber saturated with the mobile phase.

Approximately 1 g of sample was weighed and mixed with 5.0 ml of methanol. The samples were ultrasonicated for 30 minutes and filtered using a 0.45 μm membrane. The volume of the filtered supernatant was made up to 10 ml with methanol containing phosphoric anhydride (P2O5) and allowed to saturate for 2 hours. After saturation for 15 min. with mobile phase vapour, the plates were developed for 85 mm at ambient temperature of 16.5°C in a CAMAG twin-trough chamber with the lower layer of chloroform–ethyl acetate–methanol–water (20:40:22:10, v/v; for glycosides) and toluene–butyl acetate–formic acid (60:30:5, v/v; for aglycones) as mobile phase. Visualization of the chromatogram was performed by spraying with 10% H2SO4 in ethanol and heating at 105°C until the bands were visible. The plate was observed immediately in UV (366 nm) cabinet and the HPTLC image was documented. Then the corresponding digital scanning profile was generated with the self-developed software.

**Preparation of samples and testing methodology**

Sample : Root, stem and leaf sample prepared in methanol and aqueous extract

Sample concentration : 50 mg/ml.
Mobile phase: For aqueous extract is n-butanol: glacial acetic acid: water (7:2:1)
For methanol extract is toluene: chloroform: ethanol (28.5: 57: 14.5).

Stationary phase: TLC aluminum sheet pre-coated with silica gel 60 F₂₅₄, (10 x 10 cm) was used as stationary phase, obtained from MERCK

Instrument type: CAMAG TLC SCANNER III
“SCANNER 3_141205”S/N141205 (1.14.28)

Chamber for mobile phase: CAMAG twin trough chamber (10 x 10 cm)

Chamber saturation: Chamber saturation was done for 18 hours

Scanning wavelength: 254 nm and 366 nm

Applied volume: track 1 (5µl), track 2 (10µl)

Development mode: Ascending mode

The TLC plate was activated by heating at 1200°C for about 30 min prior to use. Alcohol extract solution (2 µl) and aqueous extract solution (2 µl), each were applied in duplicate, as tracks 1-6, with a band length of 7 mm each on a pre coated silica gel 60’ F 254 TLC plate, with Linomat V applicator using a Hamilton syringe. No prewashing of the plate was done. Chamber saturation time was 18 hrs. The TLC plate was kept for development to a migration distance of 77 mm.

Post derivatization has been done with vanillin-phosphoric acid. The derivatized plate was dried in hot air oven at 108 °C for 10 min. and scanned at 254 nm and 366 nm, band length 7 mm and source of radiation was Deuterium and Tungsten lamps for methanol and aqueous extracts respectively.
The RF and peak area of the spots were interpreted by using software. The derivatized plate was photo documented under 254 nm and 366 nm light using CAMAG Reprostar 3, equipped with 12 bit CCD camera. The results were interpreted.

2.4.3 Gas Chromatography and Mass Spectroscopy

Gas chromatography has a very wide field of applications. The principle of gas chromatography is adsorption and partition. Within the family of chromatography based methods, gas chromatography (GC) is one of the most widely used techniques. It was first described by James and Martin (1952), and has become one of the most important tools for the separation of volatile compounds.

Mass spectrometry (MS) is the most sensitive and selective method for molecular analysis and can yield information on the molecular weight as well as the structure of the molecule. Combining chromatography with mass spectrometry (GC-MS) provides the advantage of both chromatography as a separation method and mass spectrometry as an identification method. In mass spectrometry, there is a range of methods to ionize compounds and then separate the ions.

Gas chromatographic study includes important optimization processes such as: introduction of sample extract into the GC column, separation of components on an analytical column and detection of target analysis using Mass Spectrometric (MS) detector

Principle

Separation is due to differential distribution coefficients. In this chromatography, moving phase (or mobile phase) is a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen. The stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column. The instrument used to perform gas chromatography is called a gas chromatograph (or "aerograph", "gas separator").

The gaseous compounds are being analyzed to interact with the walls of the column, which is coated with different stationary phases. This causes each compound to elute at a different time, known as the retention time of the compound. Secondly, the
column through which the gas phase passes is located in an oven where the temperature of the gas can be controlled, whereas column chromatography (typically) has no such temperature control. Thirdly, the concentration of a compound in the gas phase is solely a function of the vapour pressure of the gas.

Sample preparation

The plant samples collected were dried and powdered, and after preparation of extract were analyzed through GC-MS.

Preparation of extract and testing methods

About 25 g of the powdered samples of stem, root and leaves were soaked separately in 95% methanol for 12 hrs. The extracts were then filtered through Whatman filter No.41 along with 2 g sodium sulphate to remove the sediments and traces of water in the filtrate.

Before filtering, the filter paper was made wet with 95% ethanol along with sodium sulphate. The filtrate was then concentrated by bubbling nitrogen gas into the solution. The extract contained both polar and non-polar phyto-components in the plant material. 2 μl of this solution was employed for GC-MS analysis (Merlin et al., 2009).

The plant powder was extracted with methanol and analyzed using GC-MS (GC Clarius 500 Perkin Elmer) analyzer. The data were obtained on an Elite-1(100% Dimethyl poly siloxane) column (30 m, 0.25 mm, 1 μm). Helium (99.999%) was used as the carrier gas with a flow rate of 1ml/min in the split mode (10:1). An aliquot of 2 μl of ethanol solution of the sample was injected into the column with the injector temperature at 250 ºC. GC oven temperature started at 110 ºC and holding for 2 min and it was raised to 200 ºC at the rate of 10 ºC/min, without holding.

Holding was allowed at 280 ºC for 9 min with program rate of 5 ºC/min. The injector and detector temperatures were set at 250 ºC and 280 ºC respectively. Ion source temperature was maintained at 200 ºC. The mass spectrum of compounds in samples was obtained by electron ionization at 70 eV and the detector was operated in scan mode from 45 - 450 amu (atomic mass units). A scan interval of 0.5 seconds and fragments from 45 to 450 Da was maintained. The total running time was 36 minutes.
Identification of components

Identification was based on the molecular structure, molecular mass and calculated fragments. Interpretation on mass spectrum was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The name, molecular weight and structure of the components of the test materials were ascertained. The relative percentage amount of each component was calculated by comparing its average peak area to the total area. The spectrum of the unknown component was compared with the spectrum of the component stored in the NIST library version (2005), software, Turbomass 5.2. This was done in order to determine whether this plant species contains any individual compound or group of compounds, which may substantiate its current commercial and traditional use as an herbal medicine. Further it helped to determine the most appropriate methods of extracting these compounds. These results were consequently discussed in the light of their putative biological or therapeutic relevance and interpreted.

Aqueous extracts of plant parts could not be analyzed through GC-MS, hence methanol extracts were analyzed by following the protocol of James and Martin (1952). The phytochemicals were identified based on the comparison of GC-MS chromatogram with the NIST Library database.

2.5 Biological activities of the extract

Methanol and aqueous extracts of leaf, stem and root of G. asiatica were tested for their antimicrobial, antifungal and antioxidant efficacy.

2.5.1 Antimicrobial evaluation (Bauer et al., 1966)

The present work encompasses the screening of aqueous and methanol extracts of plant species collected, for their antibacterial property against a wide array of microorganisms. Further, the most active plant extracts showing best antibacterial activity were screened for antifungal activity. The most promising plant extract was selected for further for biological evaluation.

2.5.2 Anti-bacterial activity

Plant parts selected for antibacterial study

The primary extraction of plant parts like root, stem and leaf were selected. The methanol and aqueous extracts of all the parts were screened for antibacterial activity.
Bacterial strains and growth conditions

The test microorganisms were *Bacillus cereus* (ATCC11778), *Citrobacter freundii* (ATCC10787), *Escherichia coli* (ATCC25922), *Klebsiella pneumoniae* (NCIM2719), *Proteus vulgaris* (NCTC8313), *Pseudomonas aeruginosa* (ATCC27853), *Salmonella typhi* (Ty21a), *Salmonella typhimurium* (ATCC23564), *Staphylococcus aureus* (ATCC25923) and *Vibrio cholerae* (MO10). Bacterial cultures were grown on Nutrient Broth (Hi-Media) at 37 °C for 24 hrs. All the microbial cultures were maintained at 4 °C on Nutrient Agar slants. In which basis selected the microbes

Preparation of inoculum

Stock cultures were maintained at 4 °C on slopes of nutrient agar. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of Mueller Hinton Broth (MHB) for bacteria. The cultures were diluted with fresh MHB to achieve optical densities corresponding to 2x10^6 colonies (CFU/ml) for bacteria.

Agar diffusion method

The screening of aqueous extracts of different plant species for antibacterial activity was determined by agar disc diffusion method (Bauer et al., 1966). *In vitro* antimicrobial activity was screened by using Mueller Hinton Agar (MHA) obtained from HiMedia (Mumbai). The MHA plates were prepared by pouring 15 ml of molten media into sterile petriplates. The plates were allowed to solidify for 5 minutes and 0.1% inoculum suspension was swabbed uniformly and the inoculum was allowed to dry for 5 minutes.

The different concentrations of plant extract (1000, 500 and 200 µg/well), standard drug (Ciprofloxacin 30 µg/well) and dimethyl sulfoxide (solvent control) were loaded on 6 mm agar wells. Then the loaded plates were kept at 4 °C for 30 minutes to allow the pre-diffusion of the antibiotic, drug and solvent control. The plates were then incubated at 37 °C for 24 hours. The results were read by measuring the zone of inhibition around the well.

2.5.3 Anti-fungal activity

Fungal strains and growth conditions

The test microorganisms include *Cryptococcus aureus* (ATCC32044), *Candida albicans* (ATCC2091), *Aspergillus niger* (ATCC6275), *Fusarium oxysporum* (70T01) and
Drechslera sp. (ICMP6807). The fungal strains were grown on Sabouraud broth and maintained on MGYP (Malt-extract-Glucose-Yeast extract-Peptone) slants at 4 °C. In which basis selected the microbes

**Preparation of inoculum**

The test fungal strains were inoculated into Sabouraud dextrose broth and incubated at 28 °C on a rotary shaker. The inoculum size was maintained as per the 0.5 McFarland standards (1 x 108 cfu/ml). The activated inoculum was used for antifungal assay.

**Antifungal susceptibility test**

*In vitro* antifungal activity was screened by using Sabouraud dextrose agar (SDA). The SDA plates were prepared by pouring 15 ml of molten media into sterile petriplates. The plates were allowed to solidify for 5 minutes and 0.1% inoculum suspension was swabbed uniformly and the inoculum was allowed to dry for 5 minutes. The different concentrations of plant extract (1000, 500 and 200 µg/well), standard drug (Ketoconazole 10 µg/well) and dimethyl sulfoxide (solvent control) were loaded on 6 mm agar well. Then the loaded plates were kept at 4 °C for 30 minutes to allow the pre-diffusion of the antibiotic, drug and solvent control. The plates were then incubated at 28 °C for 24 - 48 hours. The results were read by measuring the zone of inhibition around the well.

**2.5.4 Determination of Minimum Inhibitory Concentration (MIC)**

Minimum Inhibitory Concentration (MIC) was determined using the standard reference method (Nakamura *et al*., 1999) for bacteria and fungi. The initial concentration of the extract was 100 µg/ml. freshly prepared nutrient broth was used as diluents. Crude extract was diluted by two fold serial dilution method. 50 µl of the standard culture inoculums was added to each test tube except the negative control tube. All tubes were incubated at 37 °C for 24 hours. The tube content was sub cultured in fresh nutrient agar separately and minimum bactericidal concentration was determined as the concentration which showed no growth.

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation, and minimum bactericidal concentrations (MBCs) as the lowest concentration
of antimicrobial that will prevent the growth of an organism after subculture on to antibiotic-free media. MICs are used by diagnostic laboratories mainly to confirm resistance, but most often as a research tool to determine the in vitro activity of new antimicrobials, and data from such studies have been used to determine MIC breakpoints.

The extracts giving an inhibition zone of 12 mm in diameter were chosen to assay the minimum inhibitory concentration (MIC) with the agar dilution method. A stock solution of each extract was serially diluted twofold in pure DMSO and 0.2 ml of each dilution were incorporated in 20 ml of the appropriate melted agar medium and poured into a petriplate. The final concentrations of extracts in the medium ranged from 0.2 to 0.006 % (w/v) and the resulting DMSO concentration was 1 % (w/v). Overnight culture dilutions (1:100) were streaked in radial patterns on the surface of the plates. DMSO was used as controls. The MIC was defined as the lowest concentration of the extract inhibiting the visible growth of each micro-organism.

All the data regarding antimicrobial activity are the average of triplicate analyses were recorded. The antibiotic Ciprofloxacin (10 μg/ml) was used as reference standards as recommended by the National Committee for clinical laboratory standards. Antibacterial of extracts were assessed in terms of zone of inhibition. The results of the antibacterial activities were presented as per their measurement of zone of inhibition in mm. As compared with standard drugs (Ciprofloxacin) the results of bacteria was calculated for their antibacterial activity.

2.5.5 Antioxidant activity

The antioxidant activity of the plant extracts was tested using the following radical scavenging methods: nitric oxide scavenging activity; superoxide anion scavenging activity and hydroxyl scavenging activity. The reducing power and iron chelating activity of the drug was also evaluated to identify the antioxidant capability of the sample. The antioxidant activity was tested for the extracts in the concentration range of 0.4 to 2 mg.

Nitric oxide scavenging activity

The method of Garratt, (1964) was adopted to determine the nitric oxide radical scavenging activity of methanol and aqueous extract of the plant sample. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generate nitric oxide which interacts with oxygen to produce nitrite ions determined by the use of Greiss
reagent. 2ml of 10 mM sodium nitroprusside dissolved in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of plant extract at various concentrations (0.4 - 2 mg/ml). The mixture was incubated at 25 °C. After 150 min, 0.5 ml of incubated solution was withdrawn and mixed with 0.5 ml of Griess reagent [1.0 ml sulfanilic acid reagent (0.33% in 20% glacial acetic acid at room temperature for 5 min) with 1 ml of naphthyl ethylene diamine dichloride (0.1% w/v)]. The mixture was incubated at room temperature for 30 min. The absorbance was measured at 540 nm. The amount of nitric oxide radical was calculated using the following equation:

\[
\text{Percentage Inhibition of NO} = \frac{[A_0 - A_1]}{A_0} \times 100
\]

Where, \(A_0\) is the absorbance before reaction and \(A_1\) is the absorbance after reaction has taken place.

**Scavenging activity of superoxide anion**

The scavenging activity of superoxide anion was determined by the method of Yen and Chen (1995) for plant extracts of 0.4 to 2 mg concentrations. The reaction mixture consists of 1 ml of plant extract (1 mg/ml), 1 ml of Phenazine Metho Sulphate (PMS) (60 μM) prepared in phosphate buffer (0.1 M pH 7.4) and 1 ml of Nicotinamide Adenine Dihydrogen (NADH) (phosphate buffer) was incubated at 25 °C for 5 min, the absorbance was read at 560 nm against blank samples.

**Hydroxyl radical scavenging activity** (Deoxy ribose method, Elizabeth and Rao, 1990)

The hydroxyl radical scavenging activity was studied in concentration ranging from 0.4 to 2 mg of extracts. To the reaction mixture containing 0.2 ml of Deoxyribose (3 mM), 0.2 ml of ferric chloride (0.1 mM), 0.2 ml of Ethylene Diamine Tetra Acetic acid disodium salt (EDTA) (0.1 mM), 0.2 ml of ascorbic acid (0.1 mM) and 0.2 ml hydrogen peroxide (2 mM) in phosphate buffer (pH 7.4, 20 mM), was added 0.2 ml of various concentrations of the extract or standard in dimethyl sulphoxide (DMSO) to give a total volume of 1.2 ml.

The solutions were then incubated for 30 min at 37 °C. After incubation, ice cold 0.2 ml of trichloro acetic acid (15% w/v) and 0.2 ml of thio barbituric acid (1% w/v) in 0.25N hydrochloric acid were added to the incubated solution. The reaction mixture was
kept in a boiling water bath for 30 min, cooled and the absorbance was measured at 532 nm.

**Reducing power activity**

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity (Oktay et al., 2003). Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Yen and Chen, 1995).

The reducing power can be determined by the method of Athukorala et al. (2006). 1.0 ml extract is mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferric cyanide (30 mM) and incubated at 50°C for 20 min.

Thereafter, 2.5 ml of trichloroacetic acid (600 mM) is added to the reaction mixture, centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) is mixed with 2.5 ml of distilled water and 0.5 ml of FeCl₃ (6 mM) and absorbance is measured at 700 nm. Ascorbic acid was used as positive control.

**Iron chelating activity**

Ferrozine can quantitatively chelate with Fe²⁺ and form a complex with a red colour. This reaction is limited in the presence of other chelating agents and results in a decrease of the red color of the ferrozine - Fe²⁺ complexes. Measurement of the colour reduction estimates the chelating activity to compete with ferrozine for the ferrous ions (Soler-Rivas et al., 2000).

The chelation of ferrous ions is estimated using the method of Dinis et al., (1994). 0.1 ml of the extract is added to a solution of 0.5 ml ferrous chloride (0.2 mM). The reaction is initiated by the addition of 0.2 ml of ferrozine (5 mM) and incubated at room temperature for 10 min and then the absorbance is measured at 562 nm. Citric acid (Dinis et al., 1994) was used as the positive control.
2.6 Pharmacological evaluation

2.6.1 *In vivo* acute toxicity

Acute oral toxicity study was performed on Wistar Albino mice as per OECD-423 reference (Organization for Economic Cooperation and Development) guidelines (Turner, 1965). Plant powder (leaf, stem and root) of *G. asiatica* were administered in the form of suspension in water with 1% sodium carboxy methyl cellulose as suspending agent. The animals were left on fast overnight, after which the plant powder (aerial parts and sub aerial parts) was administered orally with a dose concentration of 50, 500, 1000 and 2000 mg/kg body weight.

The body weight and feed consumption of the animals were observed for 28 days. The kidney function was calculated using urea, uric acid and creatinine level. The liver function test was identified by SGPT (IU/L) (Substance from Glutamate Pyruvate Transaminase), SGOT (IU/L) (Substance Glutamic Oxaloacetic Transaminase) and AP (IU/L) (Alkaline Phosphatase).

**Experimental animals for in vivo study**

Wistar albino rats of either sex (140 - 200 g) were obtained from the inbred colony of Department of Pharmacology, A.J College of Pharmacy, Chennai. The animals were kept in polypropylene cages at 25 ± 2°C with relative humidity 45 - 55% under 12 hr light and dark cycles. They were fed with standard laboratory animal feed (Poultry Research Station, Tamil Nadu Veterinary and Animal Sciences University, Chennai, India) and tap water *ad libitum*

The toxicological and pharmacological experimental protocols were approved by the IAEC (Institutional Animal Ethics Committee) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) (AJ/IAEC/11/42) (Appendix II).

**Preparation of the drug**

The powdered drug was administered in the form of suspension in water with 1% sodium carboxy methyl cellulose as suspending agent. Acute toxicity study was performed as per OECD-423 guidelines (acute toxic class method). Wistar rats (n = 6) of both sex selected by random sampling technique was used for the study.
Test method

The animals were kept fasting for overnight providing only water, after which the plant powder was administered orally at the dose level of 5 mg/ kg of body weight by intragastric tube and observed for 14 days. If mortality was observed in 2-3 animals, then the dose administered was assigned as toxic dose. If mortality was observed in 1 animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for further higher doses such as 50, 500, 1000 and 2000 mg/ kg body weight.

Body weight and feed consumption of animals were observed every day. The animals were sacrificed on 29th day using excess dose of thiopental sodium and serum was separated. Liver function test like, estimation of alkaline phosphatase, SGPT and SGOT were carried out. Kidney function test like: Estimation of urea, creatinine and uric acid were carried out. Histopathalogy of liver and kidneys was also carried out.

Acute toxicity study of Gmelina asiatica plant powder sample did not produce any toxic symptoms or mortality at the dose level of 2000 mg/kg in rats and hence the plant sample was considered to be safe for pharmacological study. According to acute toxic class method (OECD – 423 guidelines) the LD$_{50}$ dose of 2000 mg/kg and above is categorized as ‘X’ (unclassified).

Effect of G. asiatica on albino mice

The effect of G. asiatica aerial and sub-aerial parts were tested on the major vital organs of albino mice through histopathological studies. Organs such liver, kidney and spleen were recovered from both control and plant sample treated animals and microscopical examination was carried out.

2.6.2 In vivo antiulcer activity

The accumulation of acidic gastric juice in the stomach causes ulceration and in this method several parameters were estimated. The antiulcer activity was carried out by three different models:

- Pylorus ligation induced ulcer model (Shay et al., 1945)
- Cold stress induced ulcer model (Qiu et al., 1996)
Aspirin induced gastric ulcers (Shay et al., 1945)

The antiulcer activity was performed by using methanol extract, aqueous extract and plant powders (leaf, stem and root) of *G. asiatica*. The ulcer protective effect was tested at doses of 100 mg/kg and 400 mg/kg against the standard Ranitidine of 10 mg/kg.

**Effect of *G. asiatica* vegetative parts on albino mice through pylorus ligation induced ulcer model (Shay et al., 1945)**

Albino mice weighing between 160 - 200 g were divided into 8 groups of 6 mice in each group. They were fasted in individual cages with measures taken to avoid coprophagy for 24 hours prior to the experiment with free access to water. One group was served as normal control given with vehicle only. Second group was treated with standard drug Ranitidine (10 mg/kg). Third to eighth groups were treated with low (100 mg) and high (400 mg) doses of methanol extract, aqueous extract and powdered sample of root, stem and leaf of *Gmelina asiatica*. The various groups were treated with vehicle/drug/ extracts 30 min prior to pylorus ligation.

Group I: Normal animals treated with vehicle only;
Group II: Standard Ranitidine (10 mg/kg i.p);
Group III: Low dose of methanolic root, stem & leaf extract / (100 mg/kg);
Group IV: High dose of methanolic root, stem & leaf extract / (400 mg/kg);
Group V: Low dose of aqueous root, stem & leaf extract / (100 mg/kg);
Group VI: High dose of aqueous root, stem & leaf extract / (400 mg/kg);
Group VII: Low dose of powdered root, stem & leaf extract / (100mg/kg);
Group VIII: High dose of powdered root, stem & leaf extract / (400 mg/kg)

**Experimental procedure - Ulcer index**

Under light ether anesthesia, the abdomen was opened and the pylorus ligation was performed and then sutured. Four hours after pylorus ligation all the animals were sacrificed with excess of anesthetic ether and the stomach of each rat was dissected out. Gastric juice collected into centrifuge tubes was centrifuged at 1000 rpm for 10 min and volume was noted. The pH of the gastric juice was recorded by pH meter. The gastric content was subjected for analysis of free and total acidity. The stomachs were washed under running tap water and then focused under microscope to note the ulcers in the
glandular portion. The numbers of ulcers per stomach were scored microscopically with the help of (10x) hand lens and the scoring is done as per standard procedure. Mean ulcer score for each animal is expressed as Ulcer Index. The percentage ulcer protection was calculated using the formula:

\[
\text{Percentage Ulcer protection} = \frac{Ut}{Uc} \times 100
\]

Where, \(Ut\) = Ulcer index of treated group and \(Uc\) = Ulcer index of the control group.

**Effect of *G. asiatica* vegetative parts on albino mice through cold stress induced ulcer model (Qiu et al., 1996)**

Cold stress induced ulcer was performed by oral administration of the samples [methanol, aqueous and powdered samples of root, stem and leaf (low dose 100 mg and high dose 400 mg)] to albino mice weighing between (150 – 200 g). After 30 min of oral administration, mice were placed in cold water vertically for 1 hour in individual restraint cages maintained at 22 ºC. Then, they were removed from water, dried and injected with 30 mg/kg Evans blue via the tail vein. Formol-saline (2% v/v) was then injected into the totally ligated stomach for overnight storage. The various groups were treated with vehicle/drug/ extracts 30 min prior to cold stress. One group was served as control and another was treated with standard drug Ranitidine 10 mg/kg. The next day, the stomach was opened along the greater curvature, washed in warm water and examined microscopically for ulcers.

- **Group I**: Normal animals treated with vehicle only;
- **Group II**: Standard Ranitidine (10 mg/kg i.p);
- **Group III**: Low dose of methanolic root, stem & leaf extract / (100 mg/kg);
- **Group IV**: High dose of methanolic root, stem & leaf extract / (400 mg/kg);
- **Group V**: Low dose of aqueous root, stem & leaf extract / (100 mg/kg);
- **Group VI**: High dose of aqueous root, stem & leaf extract / (400 mg/kg);
- **Group VII**: Low dose of powdered root, stem & leaf extract / (100mg/kg);
- **Group VIII**: High dose of powdered root, stem & leaf extract / (400 mg/kg)
Experimental procedure

The opened stomach was examined microscopically for ulcers with the help of hand lens (10x). Mean ulcer score for each animal is expressed as ulcer score, ulcer number and ulcer index. Gastric juice was collected and measured for pH, free and total acidity.

Effect of *G. asiatica* vegetative parts on albino mice through aspirin induced gastric ulcers (Shay et al., 1945)

Albino rats of either sex weighing between (150 - 200 g) each group containing 6 animals were divided into 8 groups. Each group was treated with low (100 mg) and high (400 mg) doses of methanol extract, aqueous extract and powdered sample of root, stem and leaf of *Gmelina asiatica*. After 30 minutes, aspirin was administered at a dose of 250 mg/kg and after 6 h mice were sacrificed by using anesthetic ether and their stomachs were dissected out for determination of gastric lesions, washed in warm water. One group was served as control and another was treated with standard drug Ranitidine 10 mg/kg.

Group I: Normal animals treated with vehicle only;
Group II: Standard Ranitidine (10 mg/kg i.p);
Group III: Low dose of methanolic root, stem & leaf extract / (100 mg/kg);
Group IV: High dose of methanolic root, stem & leaf extract / (400 mg/kg);
Group V: Low dose of aqueous root, stem & leaf extract / (100 mg/kg);
Group VI: High dose of aqueous root, stem & leaf extract / (400 mg/kg);
Group VII: Low dose of powdered root, stem & leaf extract / (100mg/kg);
Group VIII: High dose of powdered root, stem & leaf extract / (400 mg/kg)

Experimental procedure

The dissected and washed stomach was examined for ulcers microscopically with the help of hand lens (10 x). Mean ulcer score for each animal in each group is expressed as ulcer index. Gastric juice was collected and measured for pH, free and total acidity.

2.7 Statistical analysis

The acronym ANOVA refers to analysis of variance and is a statistical procedure used to test the degree to which two or more groups vary or differ in an experiment. In
most experiments, a great deal of variance (or difference) usually indicates that there was a significant finding from the research. The ANOVA test is used to determine the impact independent variables have on the dependent variable in a regression analysis.

**Analysis of variance (ANOVA)** is a collection of statistical models used to analyze the differences among group means and their associated procedures (such as "variation" among and between groups), developed by statistician and evolutionary biologist Ronald Fisher. In the ANOVA setting, the observed variance in a particular variable is partitioned into components attributable to different sources of variation. In its simplest form, ANOVA provides a statistical test of whether or not the means of several groups are equal, and therefore generalizes the $t$-test to more than two groups.

The results were subjected to statistical analysis by using one way ANOVA (Analysis of variance) followed by Dunnett’s -$t$- test to verify the significant difference if any among the groups. $P<0.05^*$, 0.01** and 0.001*** were considered significant.

The inhibitions of ulcer percent of each sample in low and high doses were checked with respect to the inhibition of ulcer percent of Ranitidine (30 mg). In this view the sample tested, if it showed less than 50% of inhibition with respect to Ranitidine is considered as non-significant. The values fall between 50 to 60% significant range with less effective, 60 to 70% significant with medium effective range and 70 to 100% significant with very effective range.