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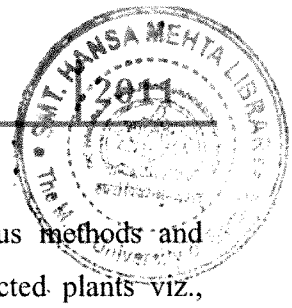
Chapter-3

Material & Methods



3. EXPERIMENTAL

The present section deals with the detailed description of various methods and techniques adopted for carrying out different studies on the selected plants viz., leaves, stem bark or root bark of *Feronia limonia*; leaves and stem bark of *Tecomella undulata.*

3.1 Pharmacognostical studies and proximate analysis

3.1.1 Collection and identification of plant materials

Feronia limonia leaves, stem bark and root bark were collected in the months of September–October, 2008 from the campus of The M.S. University, Vadodara (Gujarat), India. They were authenticated in the Botany Department and a voucher specimen (No. Pharmacy /FL / 08-09/01/MJ) has been deposited in the Pharmacy Department of The M. S. University of Baroda, Vadodara, Gujarat, India.

Tecomella undulata leaves and stem bark were collected from village Rajpipla, Vadodara, Gujarat, India. They were authenticated in the Botany Department and a voucher specimen (No. Pharmacy/TU/ 09-10/02/MJ) has been deposited in the Pharmacy Department, The M. S. University of Baroda, Vadodara, India.

3.1.1 a Preparation of powdered materials

The aerial and underground part of both selected plants were first properly cleaned and then dried under shade. The dried materials were then subjected to size reduction using a mechanical pulveriser to a coarse powder, which was used in further studies.

3.1.2 Macroscopical studies

The leaves, stem barks and root barks of *Feronia limonia* were examined macroscopically using reported methods (Wallis, 1985)

3.1.3 Microscopical studies

Microscopic evaluation of the different parts of *Feronia limonia* and *Tecomella undulata* were carried out. For the anatomical studies leaves were collected fresh from the plant and fixed in FAA (formalin: acetic acid: 70 % ethyl alcohol). Twenty four hours later, the samples were dehydrated with a graded series of tertiary-butyl alcohol (TBA). Infiltration of the samples was carried out by gradual addition of paraffin wax melting point 58 - 60 °C) until TBA solution attained supersaturation. The sample was then successively embedded into paraffin blocks (Brain and Turner, 1975).

The paraffin-embedded samples were sectioned with the aid of an MC 930 advanced precision rotary microtome. Serial sections were cut at 10 - 12 µm thickness. Dewaxing of the sections was performed by treating the specimen slides sequentially with xylol, xylol + alcohol, alcohol, water and finally, with staining fluid (Johansen, 1940). Wherever necessary, sections were also stained with safranin, fast-green and iodine in potassium iodide in order to evaluate starch, stomatal morphology, venation pattern, and trichome presence and distribution. The peels were made by scraping pieces of fresh or softened dried leaves (Glycerine: Water mixture) with the help of safety razor blade and stained it with the safranin and mounted in glycerine. Epidermal peels were also obtained by boiling leaf fragments cut from the mid portion of the lamina and boiled in 10% KOH (Sass, 1940) and stained in safranin after thoroughly washing in distilled water. Glycerin mounted temporary preparations were made for cleared materials. Adaxial and abaxial leaf surfaces from both the species were studied at×400 magnification and individual cells were identified and measured by micrometer. 20-25 peels were made from each species of several dozen of leaves. All the peels were examined and the representative areas were photographed using Leica research microscope using ×40 objective.

Further the micro powder analysis of the samples were done according to the standard procedures mentioned in text (Brain and Turner, 1975b; Kokate, 1986; Lala, 1981) and there diagnostic features were recorded.

3.2 Proximate Analysis

Physical and physicochemical standards are to be determined for the crude drugs, wherever possible. These were determined using standard procedures (Indian Pharmacopoeia, 1996; WHO, 1998). Fluorescence analysis was carried out according to the method (Chase and Pratt, 1994).

Following determinations were made:

3.2.1 Loss on drying

An excess of water in medicinal plant material will encourage microbial growth and deterioration following hydrolysis. Limits for water content should therefore be set for every given plant material. Loss on drying is determined as mentioned below:

Accurately weighed 5 gm of the dried plant material was placed in a weighing bottle which was previously dried and tared. The samples were dried at 100- 105°C

until two consecutive weighing does not differ by more than 5 mg. The loss in weight was calculated with reference to the air dried plant materials.

3.2.2 Total Ash

Controlled incineration of plant drugs results in an Ash residue. It usually represents the mixture of inorganic salts and silica naturally occurring in the plant drug and adhering to it, but it may also include inorganic matter added for the purpose of adulteration.

For determining the total ash about 2 gm of the powdered drug was weighed, in a previously weighed silica crucible, and sprayed as a fine layer to the bottom of the crucible. The crucible was incinerated at a temperature not exceeding 450 °C until it became free from carbon. Then it is cooled and weighed. The procedure was repeated till a constant weight was obtained. The % of the total ash was calculated with reference to the air dried drug.

3.2.2 a Acid insoluble ash

The ash obtained above (Total Ash) was boiled with 25 ml of hydrochloric acid (HCl) for 5 mins. The insoluble ash was collected on an ash less filter paper and washed with hot water. The insoluble ash was transferred into weight silica crucible, ignited, cooled and weight. The procedure was repeated to get a constant weight. The % of acid insoluble ash was calculated with reference to the air dried drug.

3.2.2b Water Soluble Ash

The total ash (obtained as above) was boiled for 5 min with 25 ml of hot water. The insoluble matter was collected on an ash less filter paper and washed with hot water. The insoluble ash was transferred into a weighed silica crucible and ignited at a temperature not exceeding 450°C. The procedure was repeated till a constant weight of the insoluble matter was detected from the weight of the total ash. The difference in weight was considered as water soluble ash. The % of water soluble ash was calculated with reference to air dried drug.

3.2.3 Extractive values

3.2.3 a Alcohol Soluble extractive value

Extraction of plant (drug) material with solvent yields a solution of different components and the composition of this solution will depend upon the constituents present in the drug and the solvent used.

Method: Powdered plant material (5 gm) was macerated with 100 ml of methanol (90% v/v) in a closed flask for 24 hrs. The contents of the flask were shaken for first 6 hrs and then set aside for 18 hrs. The contents were filtered and 25 ml of the filtrate was evaporated to dryness in a weighed flat bottom shallow dish, finally dried at 105 °C and weighed. The % of methanol soluble extractive was calculated with reference to the air dried plant material.

3.2.3 b Water soluble extractive value

Powdered plant material (5 gm) was macerated with 100 ml of chloroform water in a closed flask for 24 hrs. Contents of the flask were shaken for first 6 hrs and then set aside for 18 hrs. The contents were filtered and 25 ml of the filtrate was evaporated to dryness in a weighed flat bottom shallow dish, finally dried at 105 C and weighed. % of water soluble extractive was calculated with reference to the air dried plant material.

3.2.4 Estimation of in-organic elements

Elemental contents of different organs of *Feronia limonia* and *Tecomella undulata* were estimated on atomic absorption spectrophotometer at Vaibhav Analytical laboratory, Ahmadabad. For estimation of heavy metals about 5 gm of powdered drug material was ignited in muffle furnace to obtain total ash. 100 mg of the ash was dissolved in 10 ml of 1 N HCl and then the solution was filtered and diluted to 50 ml with distilled water and used for quantitative determination of heavy metals by the atomic absorption spectrophotometer (AAS , Systronic128), coupled with hydride generator and hollow cathode lamps for different elements .

***3.2.5* Determination of Total Phenolic content**

Total phenolic content was determined as per the reported method described by Singleton and Rossi (1965). This method was performed for total methanolic extracts and aqueous extracts of various parts of *Feronia limonia* and *Tecomella undulata.*

Preparation of reagents

- ✓ Folin ciocaltu reagent :
  1: 2 dilution of the readymade reagent with distilled water was prepared.
- ✓ 20 % sodium carbonate solution:

20 gm of anhydrous sodium carbonate was dissolved in 100 ml of distilled water.

- ✓ Preparation of standard solution :
  A stock solution of the gallic acid was prepared by dissolving accurately weighed 1 mg of standard gallic acid in 10 ml methanol. The stock solution (100 µg/ml) was diluted further to get the solutions containing different concentrations of gallic acid

- ✓ Preparation of test samples:
  Seperately, stock solutions of methanolic and aqueous extracts of both plant parts were prepared by dissolving 10 mg of extracts in 10 ml of methanol

Preparation of Calibration curve

From the stock solution of standard 0.5, 0.75, 1, 1.25, 1.5, 1.75, and 2 ml were taken in 25 ml volumetric flask, which gave 50, 75, 100, 125, 150, 175, 200 µg/ml concentrations respectively. To this, 10 ml of water and 1.5 ml of Folin Ciocalteu reagent were added. The above mixtures were kept for 5 min and then 4 ml of 20 % sodium carbonate solution was added and made the volume up to 25 ml with the distilled water on each flask. These mixtures were kept for 30 minutes and the absorbance of the blue color developed were measured at 765 nm, using Shimadzu 1800 spectrophotometer.

Analysis of samples solution

Percentage of total phenolics was calculated from calibration curve of gallic acid and total phenolic content was expressed as mg/g gallic acid equivalent using the following equation based on the calibration curve: $y = 0.1216x$, $R2 = 0.9365$ [where x was the absorbance and y was the gallic acid equivalent (mg/g)].

3.2.6 Determination of total flavanoid content

Flavonoids with various biological activities are considered as one of the key components in the plants. Therefore a quantitative determination of flavonoids was conducted according to the method described by Chung et al., 2005.

The total flavanoids content was determined by aluminum chloride colorimetric methods. The flavanoid content was determined in the methanol and water extracts of various plant parts of *Feronia limonia* and *Tecomella undulata.*

The sample solutions of 1.5 mg/ml and 5 mg/ml were prepared for estimation of total flavanoids content by Aluminium chloride colorimetric method.

Aluminum chloride colorimetric method:

The principle of aluminium chloride colorimetric method is that aluminium chloride forms stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonol. In addition, aluminium chloride forms acid labile complexes with ortho-dihydroxyl groups in the A or B - ring of flavanoids. Though the apigenin, kaempferol and quercetin can be used for the calibration curve, quercetin was used for this purpose in the experiment because of its maximum absorbance after reaction with aluminium chloride in the selected concentration range.

Preparation of reagents

- ✓ Preparation of standard solution:
  A stock solution, 1 mg/1 ml of quercetin was prepared in methanol.
- ✓ 10 % aluminium chloride:
  10 gm of aluminium chloride was dissolved in 100 ml of distilled water.
- ✓ 1 M Potassium acetate:
  9.814 gm of potassium acetate were dissolved in 100 ml distilled water.
- ✓ Preparation of standard solution:
  Quercetin was used to make the stock solution (100 µg/ml) for the calibration curve.
- ✓ Preparation of test samples:
  Seperately stock solutions of samples were prepared by dissolving 10 mg of extracts of various plant parts of *Feronia limonia* and *Tecomella undulata* in 10 ml of methanol.

Preparation of calibration curve

- For the stock solution of standard 0.1, 0.2, 0.3, 0.4, 0.6, 0.7, 0.8 and 1 ml were taken in 25 ml of volumetric flasks which gave 10, 20, 30, 40, 60, 70, 80 and 100 µg/mL concentrations respectively.
- The standard solution were separately mixed with 1.5 ml of methanol, 0.1 ml of 10 % aluminium chloride, 0.1 ml of 1 M potassium acetate and 208 ml of distilled water.
- After incubation at room temperature for 30 min. the absorbance of reaction mixture was measured at 415 nm with Shimadzu 1800 spectrophotometer.
- For the blank analysis 10 % aluminum chloride was substituted by the same amount of distilled water.

Analysis of the samples

Similarly, 1.5 ml of the sample solutions were reacted with Aluminum chloride for determination of total flavonoids content as described in above procedure. The percentage of total flavonoids was expressed as mg/g querecetin equivalent using the following equation based on the calibration curve: y = 0.0255x, R2 = 0.9812 [where x was the absorbance and y was the querecetin equivalent (mg/g)].

3.2.7 Estimation of total flavonols content

Total flavonols in the plant extracts were estimated using the method of Kumaran and Karunakaran. To 2.0 mL of samples (standard), 2.0 mL of 2% $AlCl_3$ ethanol and 3.0 mL (50 g/L) sodium acetate solutions were added. The absorption at 440 nm was read after 2.5 h at 20°C. Extract samples of various plant parts of *Feronia limonia* and *Tecomella undulata* were evaluated at a final concentration of 0.1 mg/ ml. Total flavonols content was calculated as quercetin (mg/g) using the following equation based on the calibration curve: y = 0.0255x, R2 = 0.9812 [where x was the absorbance and was the quercetin equivalent (mg/g)] (Jimoh et al., 2008).

3.3 Phytochemical studies

3.3.1 Successive solvent extraction of plant drugs

A) Petroleum ether extract

The coarsely powdered parts of F. limonia and T. Undulata (100 gm) were extracted with petroleum ether (60-80) via hot extraction process (Soxhlet apparatus) for 4 hrs after completion of extraction, the solvents was removed by distillation and concentrate in vacuums.

B) Toulene extract

The marc left after petroleum ether extraction was dried in air and extracted in toulene by hot extraction process (Soxhlet apparatus) for 4 hrs after completion of extraction, the solvents was removed by distillation and concentrate in vaccum.

C) Chloroform Extract

The marc left after toluene extraction was dried in air and extracted in chloroform by hot extraction process (Soxhlet apparatus) for 4 hrs after completion of extraction, the solvents was removed by distillation and concentrate in vacuum.

D) Ethyl acetate extract

The marc left after chloroform extraction was dried in air and extracted in Ethyl acetate by hot extraction process (Soxhlet apparatus) for 4 hrs after completion of extraction, the solvents was removed by distillation and concentrate in vaccum.

E) Methanol Extract

The marc left after ethyl acetate extraction was dried in air and extracted in methanol by hot extraction process (Soxhlet apparatus) for 4 hrs after completion of extraction, the solvents was removed by distillation and concentrate in vaccum.

F) Aqueous Extract

The marc left after methanol extraction was dried in air and extracted with chloroform water by maceration process. After completion of extraction, the solvents was removed by distillation and concentrate in vaccum colour, consistency and % yield of the extracts were noted. The extracts were preserved under vaccum for further phytochemical studies.

3.3.2 *Qualitative evaluation of successive extracts*

Following qualitative chemical tests were carried on vaccum dried successive extracts of different parts of *F. limonia* and *T. undulata* to identify the presence of various chemical constituents. (Kokate, 2005; Harborne, 1998)

Test for Alkaloids

Mayer's reagent

Dragendorff's reagent

Wagner's reagent

Hager's reagent

Test for Steroids/Terpenoids

Libermann Burchard test

Salkowaski test

Test for Glycosides (Anthraquinones)

Borntrager test

Modified Borntrager test

Test for Tannins and Phenolic compounds

Dilute ferric chloride solution

Lead solution acetate

Test with gelatin solution

Test for Flavanoids

Shinoda test (Mg/HCl test- Magnesium turnings test)

Test for Carbohydrates

Fehling's test

Benedict's test

Test for Protein and Amino acids

Biuret test

Ninhydrin test

Test for Saponins

Foam test

Test for Gums and Mucilage

Precipitation with alcohol

Molish's test

3.3.3 Thin layer chromatography studies on the successive extracts of leaves, stem bark and root bark of *Feronia limonia*

The various extracts obtained in the successive extraction process were subjected to thin layer chromatographic studies using silica gel 60 F_{254} pre coated plates (Wagner et al, 1983), to confirm the presence of various constituents.

A Camag TLC system equipped with Camag Linomat V an automatic TLC sample spotter, Camag glass twin trough chamber (20 X 10 cm), Camag scanner 3 and integrated win CATS 4 Software were used for the analysis. TLC was performed on a pre-coated TLC plate silica gel 60F254 (20 cm X 20 cm). Samples and standards were applied on the plate as 8 mm wide bands with an automatic TLC sampler (Linomat V) under a flow of N_2 gas, 10 mm from the bottom and 10 mm from the side and the space between two spots were 15 mm of the plate. The linear ascending development was carried out in a Camag twin trough chamber (20 cm X 10 cm) which was presaturated with 20 mL mobile phase (hexane/chloroform/methanol; 1-4.75- 0.25) for 20 min at room temperature (25 ± 2 ^{0}C and 40% relative humidity). The length of the chromatogram run was 8 cm. Subsequent to the development, TLC plates were dried under stream of hot air and then subjected to densitometric scanning using a Camag TLC scanner III (Camag, Switzerland) with win CATS software (version 1.4.1) in the absorbance- reflectance scan mode. Qualitative evaluation of the plate was performed in absorption-reflection mode at 254, 366 and 540 nm.

All the successive extracts of different part of *Feronia limonia* (root, stem and Leaf) were subjected to TLC and detection was done at UV 254, 366 and after derivatization with AS reagent at 540 nm.

3.3.4 High Performance Liquid chromatography studies on the methanolic extract of leaves stem bark and root bark *Feronia limonia*

The HPLC analysis of methanolic extract was carried out at The chromatographic system (Shimadzu, Kyoto, Japan) consisted of a Shimadzu LC-20 AT Prominence solvent delivery module, a manual Rheodyne injector (PerkinElmer, Mumbai, India) with a 20 mL fixed loop, and an SPD-20A Prominence (Shimadzu) UV-Vis detector. The separation was performed on a Hypersil C18 column (particle size 5 mm; 250 ′ 4.6 mm id; Thermoquest, Cheshire, UK) preceded by an ODS (Thermoquest) guard column (10 mm, 10 ′ 5 mm id) at an ambient temperature. Chromatographic data

were recorded and processed using a Spinchrom Chromatographic Station® CFR Version 2.4.0.193 (Spinchrom Pvt. Ltd, Chennai, India). Peak purity analysis was carried out using an SPD M20A photo-diode array (PDA) detector from Shimadzu.

The methanolic extract of different parts of *Feronia limonia* (root, stem and Leaf) were subjected to HPLC studies using (mobile phase, methanol-water; 1:1, flow rate; 2ml/min, detection; 254 and 366 nm) shows that various constituents are present in it which is confirmed by the various peaks present in the chromatogram at different retention time (in mins).

3.3.5 Extraction, fractionation and isolation of compound from leaves, stem bark and root bark of *Feronia limonia*

The methanolic extract of leaves of *Feronia limonia* was subjected to liquid-liquid partition, column chromatography and preparative TLC, method is described in section 4.3.1.a of chapter 4.

The methanolic extract of stem barks of *Feronia limonia* was subjected to liquid-liquid partition, column chromatography and preparative TLC, method is described in section 4.3.1.b of chapter 4.

The methanolic extract of root barks of *Feronia limonia* was subjected to liquid-liquid partition, column chromatography and preparative TLC, method is described in section 4.3.1.c of chapter 4.

3.3.6 Thin layer chromatography studies on the extracts, fractions and isolated constituents of *Feronia limonia*

The extracts, fractions and compound were then subjected to TLC studies in order to detect separation of constituents present in extract and their fractions.

Qualitative fingerprinting of extracts, fractions and isolated compound of leaf *Feronia limonia* was performed by thin layer chromatography (TLC). TLC analyses were carried out on A Camag TLC system equipped with Camag Linomat V an automatic TLC sample spotter, Camag glass twin trough chamber (20 × 10 cm), Camag scanner 3 and integrated win CATS 4 Software were used for the analysis. TLC was performed on a pre-coated TLC plate silica gel $60F_{254}$ plates (Kieselgel$60F_{254}$, Merck, Germany) (Jain et al, 2010), using the mobile phases of toluene -ethyl acetate (85:15). Detection of chemical constituent was done under UV at 365nm as reported by Wagner et al., 1983.

TLC studies of extracts, fractions and compound from stem barks of *Feronia limonia* was performed by using mobile phase, hexane- chloroform- methanol (1: 4.75: 0.25) for detection of chemical constituent present in extract and fractions.

TLC studies of extracts, fractions and compound from root barks of *Feronia limonia* was performed by using solvent system, chloroform –methanol (98: 2) for detection of chemical constituent present in extract and fractions.

3.3.7 Qualitative determination of secondary metabolite in bioactive extracts and fractions from *Feronia limonia* by TLC

The active extracts and their fractions were then subjected to TLC studies in order to detect separation of various types of constituents in different solvents using specific reagents (Wagner et al., 1983)

Flavanoids were developed using ethyl acetate: formic acid: acetic acid: water (100: 11: 11: 27) as mobile phase and detected by spraying with Natural product-polyethylene glycol reagent as visualisation agent and observing under UV 366 nm. Total tannin as well as total phenols were developed using (n-butanol- acetic acid-water, 40:1:5) as mobile phase and detected by spraying with Ferric chloride reagent as visualisation agent and observing under UV 540 nm.

3.3.8 Determination of content marker in methanolic extract of stem barks of *Feronia limonia* by HPTLC

Marmesin is one of the marker constituent isolated and identified from the stem barks. Therefore, to ensure identity and quality of this plant a simple, sensitive, specific and reproducible HPTLC method was developed for the quantification of marmesin. The method is described in detail in section 4.3.5.a of chapter 4.

3.3.9 Determination of content of marker in methanolic extract of leaves, stem bark and root barks of *Feronia limonia* by HPLC

Marmesin is one of the marker constituents identified from the leaf, stem and root barks. Therefore, to ensure identity and quality of this plant a simple, sensitive, specific and reproducible HPLC method was developed for the quantification of marmesin. The method is described in detail in section 4.3.5.b of chapter 4.

3.3.10 Characterisation of isolated compounds

The isolated compounds MR-1 and MR-2 were then subjected to determinations of various physical characteristic like melting point, solubility index and absorption maxima in UV light etc. to elucidate the structures. The FT-IR, ^{1}H NMR, 13C DEPT

NMR (Distortion less Enhancement through polarization Transfer) CHN analysis and Mass spectra of individual's compounds were performed. The various observations were recorded to elucidate the structure of the isolated compounds.

3.4 *Tecomella undulata*

3.4.1 Thin layer chromatography studies on the successive extracts of *Tecomella undulata*

The various extracts obtained in the successive extraction process were subjected to thin layer chromatographic studies using silica gel 60 F_{254} pre coated plates (Wagner et al., 1983), to confirm the presence of various constituents. The successive extract of stem and leaves were then subjected to TLC studies in order to detect separation of various types of constituents in different solvents using specific reagents (Wagner et al., 1983)

Total alkaloids were developed using (toluene- ethyl acetate- diethyl amine, 70:20:10) as mobile phase and detected by spraying with dragandorff reagent as visualisation agent and observing under UV 540 nm. Flavanoids were developed using ethyl acetate: formic acid: acetic acid: water (100: 11: 11: 27) as mobile phase and detected by spraying with Natural product-polyethylene glycol reagent as visualisation agent and observing under UV 366 nm. Triterpene were developed using (toluene- chloroform- ethanol, 40:40:10) as mobile phase and detected by spraying with AS reagent as visualisation agent and observing under UV 540 nm.

3.4.2 High Performance Liquid chromatography studies on the methanolic extract of *Tecomella undulata*

The methanolic extract of different parts of *Tecomella undulata* (stem and Leaves) were subjected to HPLC studies using (mobile phase, Acetonitrile-water; 75:25, flow rate; 2ml/min, detection; 254 and 366 nm) shows that various constituents are present in it which is confirmed by the various peaks present in the chromatogram at different retention time (in mins).

3.4.3 Extraction and fractionation of leaves *Tecomella undulata*

Unsaponified fraction

Coarsely powdered leaves of *T. Undulata* were extracted with petroleum ether in soxhlet apparatus until exhaustion; the extract was concentrated in vacuo by rotary

evaporator and dried in desiccator. The dried petroleum ether extract was saponified to obtain the unsaponifiable matter (Khandelwal, 2002).

Chloroform fraction

Coarsely powdered leaves of *T. Undulata* were extracted with methanol in soxhlet apparatus until exhaustion; the extract was concentrated in vacuo by rotary evaporator and dried in desiccator. The dried methanol extract subjected to liquid-liquid partition by using chloroform, fraction was collected and concentrated in vacuo by rotary evaporator and dried in desiccator.

3.4.4 Identification of content marker in methanolic extract of leaves *Tecomella undulata* by HPLC

Betulinic acid is one of the marker constituents identified from the leaves, therefore, to ensure identity and quality of this plant a simple, sensitive, specific and reproducible HPLC method was developed for the identification of Betulinic acid. The method is described in detail in section 5.3.2 of chapter 5.

3.4.5 Extraction, Fractionation and isolation of compound from stem bark of *Tecomella undulata*

The pet ether extract of stem bark of *Tecomella undulata* was subjected to liquid-liquid partition, column chromatography and preparative TLC, method is described in section 5.3.3 of chapter 5. The defatted methanolic extract of stem bark of *Tecomella undulata* was subjected to liquid-liquid partition, column chromatography and preparative TLC, method is described in section 5.3.3 of chapter 5.

3.4.6 HPLC studies on the extracts, fractions and isolated constituents from stem bark of *Tecomella undulata*

The extracts, fractions and compound were then subjected to HPLC studies in order to detect separation of constituents present in extract and their fractions.

The HPLC analysis was carried out at the chromatographic system (Shimadzu, Kyoto, Japan) consisted of a Shimadzu LC-20 AT Prominence solvent delivery module, a manual Rheodyne injector (PerkinElmer, Mumbai, India) with a 20 mL fixed loop, and an SPD-20A Prominence (Shimadzu) UV-Vis detector. The separation was performed on a Hypersil C18 column (particle size 5 mm; 250 ′ 4.6 mm id; Thermoquest, Cheshire, UK) preceded by an ODS (Thermoquest) guard column (10 mm, 10 ′ 5 mm id) at an ambient temperature. Chromatographic data were recorded and processed using a Spinchrom Chromatographic Station® CFR Version 2.4.0.193

(Spinchrom Pvt. Ltd, Chennai, India). Peak purity analysis was carried out using an SPD M20A photo-diode array (PDA) detector from Shimadzu.

HPLC studies of extract, fraction and compound from stem barks of *Tecomella undulata* was performed by using mobile phase, Acetonitrile - methanol (85: 15); flow rate, 1 ml/min for detection of chemical constituent present in extract and fraction was done at UV 205 nm.

3.4.7 Determination of content of marker in methanolic extract of stem barks of *Tecomella undulata* by HPLC

Lapachol is one of the marker constituent's isolation and identified from the stem barks. Therefore, to ensure identity and quality of this plant a simple, sensitive, specific and reproducible HPLC method was developed for the quantification of lapachol. The method is described in detail in section....of chapter...(Results and Discussion)

3.4.8 Characterisation of isolated compounds

The isolated compounds MR-1 and MR-2 were then subjected to determinations of various physical characteristic like melting point, solubility index and absorption maxima in UV light etc. to elucidate the structures. The FT-IR, Mass spectra, UV spectra and melting point of individual's compounds were performed. The various observations were recorded to elucidate the structure of the isolated compounds.

3.5 Biological Studies

***3.5.1 In vitro* hepatoprotective studies/ HepG2 cell lines**

3.5.1 a Cell culture

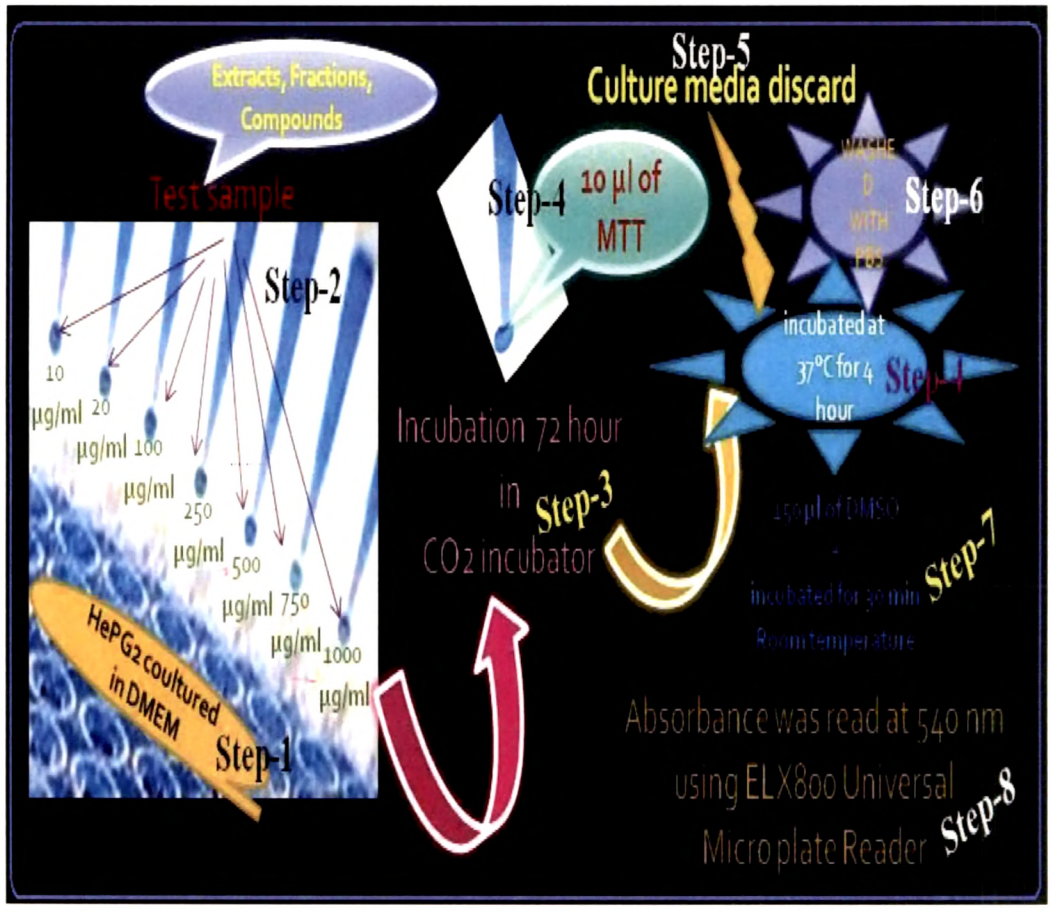
Human liver hepatoma cells (HepG2) (obtained from National Centre for Cell Sciences, Pune, India) were seeded (1×105cells/T 25Flask) and cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% for 24 h at 37°C with 5% CO2 (Thermo scientific, forma II water jacketed CO2 incubator). Cells were sub-cultured every third day by trypsinization with 0.25 % Trypsin-EDTA solution. All the reagents were sterile filtered through 0.22μ filter (Laxbro Bio-Medical aids Pvt. Ltd, Mumbai, India) prior to use for the experiment (Jain et al, 2011).

3.5.1 b In vitro Cytotoxicity assay

HepG2 cells (5.0 X 103cells /well) were maintained in 96 well culture plate (Tarson India Pvt Ltd) for 72 hr in presence of extracts, fractions or isolated compounds of

Feronia limonia at the concentrations of 10, 20, 100, 250, 500, 750 and 1000 µg/ml. At the end of incubation period, 10 µl of MTT (5 mg/ml in PBS) was added to wells and the plate was incubated at 37°C for 4 h. At the end of incubation, culture media was discarded and the wells were washed with PBS (Himedia Pvt Ltd, Mumbai, India). Later, 150 µl of DMSO was added to all the wells and, were incubated for 30 min at room temperature with constant shaking. Absorbance was read at 540 nm using ELX800 Universal Microplate Reader (Bio-Tek instruments, Inc, Winooski, VT) and subsequently % cell viability was calculated (Jadeja et al., 2010).

Figure 3.1 Schematic diagram of *in vitro* cytotoxicity assay



3.5.1 c CCL_4 induced hepatotoxicity assay in HepG2 cells

HepG2 cells (5.0 X 103cells /well) were maintained in culture media containing 1% CCL4 in presence or absence of extracts, fractions and isolated compounds from different parts of *Feronia limonia* and *Tecomella undulata* stem barks or sylimarin at the concentrations of 10, 20, 50, 100, 200µg/ml for 24 hr. Later, supernatants from

each well were removed and activity levels of aspartate transaminase (AST) and alanin transaminase (ALT) were determined while total protein was estimated in cell pellet using commercially available enzymatic kits using Merck microlab300 semi-autoanalyzer as per the instruction of manufacturer (Jain et al., 2011).

3.5.1 d Acridine orange/ ethidium bromide assay

At the end of the experimental period, cells were collected using a cell scraper. One microlitre of dye mixture (1 mg/ml Acridine orange (AO) and 1 mg/ml ethidium bromide (EB) in PBS) was mixed with 9 µl of cell suspension (0.5 X106 cells/ml) on a clean microscope slide. The suspension was immediately examined using Leica DMRB florescence microscope and photographed.

***3.5.2 In vivo* studies**

3.5.2 a In vivo acute toxicity study

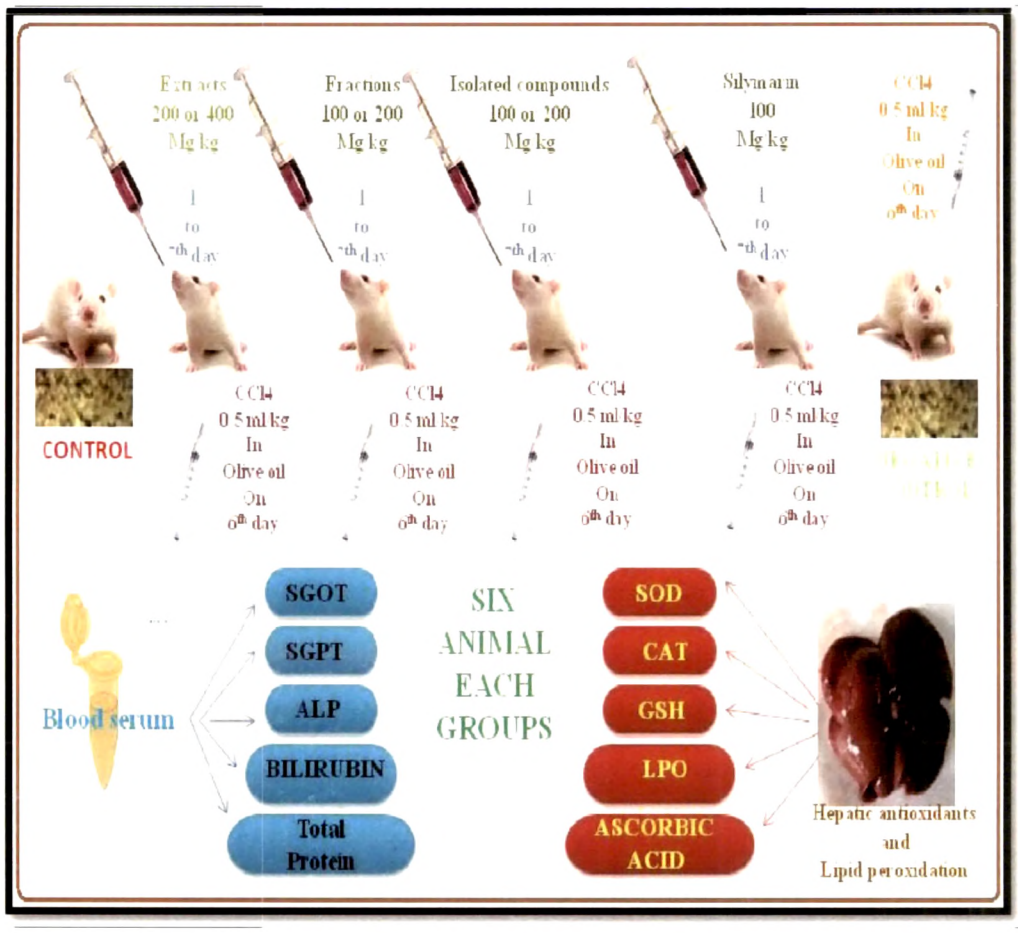
Acute oral toxicity study was conducted using the limit test procedure as per the OECD test guidelines on acute oral toxicity test 401 (OECD, 2001). Thirty two *Wistar* rats of either sex were divided into three groups (n=8) and were orally administered with a single dose of 1000mg, 2000mg, 3000mg or 5000mg body weight (b/w) of extracts, fractions 50, 300 and 2000 mg/kg, and isolated compounds 5, 50 and 300 mg/kg. After administration of extracts, fractions and isolated compounds, animals were observed for possible behavioural changes such as tremors, convulsions, sleep, altered feeding, salivation, altered somato motor activities and diarrhoea.

3.5.2 b In vivo hepatoprotective studies

Wistar rats were randomly divided into different groups of 6 each. Group – I (CON) served as normal control and was orally given 0.5% carboxy methyl cellulose (CMC) solution daily for 7 days. Group – II (CCl_4) were given 0.5% CMC solution daily for 7 days. Group – III and IV (CCl_4+ extracts 200 mg/kg and CCl_4+ extracts 400 mg/kg), Group – V and VI (CCl_4+ fractions 100 mg/kg and CCl_4+ fractions 200 mg/kg) and Group – VII and VIII (CCl_4+ isolated compounds 100 mg/kg and CCl_4+ isolated compounds 50 mg/kg) were orally given once daily for 7 days respectively. Group-IX (CCl_4+SYL) were orally given silymarin (100 mg/kg) as per our earlier report (Jadeja et al., 2011) once daily for 7 days. Groups II, III, IV, V, VI, VII, VIII, IX were injected with a single dose of CCl4 (0.5 ml/kg *i.p.*) on 7th day of the study (Jadeja et al., 2011).

At the end of the experimental period, blood sample were collected via retro-orbital sinus puncture under mild ether anaesthesia and plasma was separated for further biochemical analysis. Later, animals were sacrificed by cervical dislocation under mild ether anaesthesia and liver was excised and stored at -80°C for further estimations.

Figure 3.2 Schematic protocol of *in vivo* hepatotoxicity studies



3.5.2 c Plasma biochemical assays

Plasma SGOT, SGPT, ALP, total bilirubin and total protein were assayed using commercially available kits (Reckon diagnostics, Baroda, India).

***a) Quantitative estimation of glutamate pyruvate transminase activity in plasma* (IFCC method) -** The primary transaminase reactions was coupled with lactate dehydrogenase and reduced nicotinamide adenine dinucleotide (NADH) (Scan J, 1974).

PIPETTE IN TO TEST TUBES	TEST
Working reagent	1 ml
Sample	0.05 ml

***b) Quantitative estimation of glutamate oxaloacetate transminase activity in plasma* (IFCC method)** - The primary transaminase reactions was coupled with malate dehydrogenase (MDH) and reduced nicotinamide adenine dinucleotide (NADH) (IFCC, 1986).

PIPETTE IN TO TEST TUBES	TEST
Working reagent	1 ml
Sample	0.05 ml

c) Quantitative estimation of Total bilirubin in plasma- Bilirubin react with diazotized sulphanilic acid to form an azocompound the colour of which is measured at 546 nm (530-560 nm) and is proportional to the concentration of bilirubin. For total bilirubin the reaction is accelerated by caffeine reagent. The readings for total bilirubin are taken after 5 mins (Jendrassik and Grof, 1938).

	Sample Blank	Test
Sample	50 µl	50 µl
Working solution 1	-	100 µl
Solution 2	100 µl	-
Solution 3	1.0 ml	1.0 ml

3.5.2 d Hepatic antioxidant activity and lipid peroxidation

Liver of control and treated animal were excised, weighed and homogenized in chilled Tris buffer (10 mM, pH 7.4) at a concentration of 10% (w/v). The homogenates were then centrifuged at 10,000×g at 0 °C for 20 min in high speed cooling centrifuge. Supernatant was used for the assay of superoxide dismutase (SOD; CAT; GSH; total protein; LPO whereas, ascorbic acid content was determined in sediment by Roe and Küether (Roe and Kuether, 1943).

- Superoxide dismutase (SOD, EC 1.15.1.1) in the MF was assayed by the method of Kakkar *et al.* (1984) involving assay of NADH-PMS-NBT formazan complex. A mixture of phosphate buffer (0.052M), PMS (186 µM), NBT (30µM), NADH (780 nM) and 10% homogenate was incubated for 90sec at 37°C. Acetic acid and n butanol were added, shaken vigorously followed by centrifugation at 2000 rpm for 10 min and read at 560nm.
- Catalase (CAT, EC 1.11.1.6) activity in MF was measured spectrophotometrically at 240 nm by calculating the rate of degradation of hydrogen peroxide (Aebi, 1974). 10% homogenate was mixed with H2O2 (7.5 mM) and read at 240nm for 3 min at 30 sec interval.
- Reduced glutathione (GSH) content in MF was measured spectrophotometrically using Ellman's reagent (DTNB) as a colouring reagent, as per the method described by Beutler et al., 1963. 10% homogenate was mixed with precipating reagent and incubated for 5 min at room temperature. It was then centrifuged at 3000 rpm for 15 min, the supernatant was mixed with phosphate solution and DTNB was added.
- Hepatic mitochondrial lipid peroxidation (LPO) was determined by estimating malondialdehyde (MDA) content using thiobarbituric acid (TBA) reactive substances as per the method of Buge and Aust (1975). Commercially available 1, 1, 3, 3-tetraethoxypropane (Sigma-Aldrich Ltd) was used as a standard for calculation of MDA content.
- Glutathione peroxidase (GPx, EC 1.11.1.9) activity in MF was assayed by the method (Rotruck et al., 1973) based on the reaction between glutathione remaining after the action of GPx and DTNB to form a complex. A mixture of phosphate buffer (0.3M), sodium azide (10mM), reduced glutathione (4mM) and 10% homogenate was mixed well and H_2O_2 (0.2mM) and distilled water were added. It was then incubated for 10 min at 37°C and 10% TCA was added followed by centrifugation at 3000 rpm for 10 min. The supernatant was mixed with phosphate buffer and DTNB was added and read at 412 nm.

3.5.2 e Histopathological evaluations of liver

Liver samples were fixed in 4% buffered paraformaldehyde, dehydrated in graded alcohol series and embedded in paraffin wax. Five µm thick sections were cut and

stained with hematoxyline and eosin and examined for gross structural changes. Some tissue sections were stained with Weigert's haematoxylin for 8 minutes washed and counterstained with picro-sirius red for one hour. Observations were done under Leica microscope and photographs were taken with Canon power shot S72 digital Camera (200 X)

3.6 Statistical analysis

Data was analysed for statistical significance using one way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test and results were expressed as mean ± S.E.M using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California USA.