CHAPTER III

Phytochemical and Pharmacognostic Approach
3. PHYTOCHEMICAL AND PHARMACOGNOSTIC APPROACH

3.1 Introduction

The use of plant derived components as medicine ranges from the crude preparations, refined extracts and isolation of pure compounds. Plants are used as botanical drugs, herbal medicines, prescription medicines and food supplements. There is great increase in use of plants as a source of novel pharmacophores familiarize their chemical versatility and diversity, which cannot match with the synthetic chemical library (Fowler, 2006). Ethnobotany and ethnopharmacology have contributed widely in the discovery of various important herbal drugs due to popularity and special importance received by the green pharmaceuticals.

A complex approach which includes phytochemical, biological, molecular and botanical techniques is adopted from medicinal plant for drug discovery in the recent days. It is necessary to know the chemical constituents of a plant in order to understand the biological activity irrespective of its function as nutritive, medicinal or toxicity (Croteau et al., 2000). Chemical and biological assays play a vital role in ethnobotanical studies to lead the chemists towards isolation of different class of compounds (Gurib-Fakim, 2006). In the discovery and development of new pharmaceutical products, natural products derived from plants perform a significant function (McChesney et al., 2007).

The functions such as defense mechanism of plant against pathogen attack, herbivore, abiotic stress and inter-plant competition is due to the presence of phytochemicals which are responsible for bio-activity of natural products (Briskin, 2000; Ruba et al., 2013). The complementary mechanisms of action in the body such as stimulation of the immune system, modulation of enzyme actions, antioxidant effects,
modulation of hormone metabolism, interference with DNA replication, anti-bacterial & antiviral effect and physical actions are shown by phytochemicals where they bind to the cell walls of host thereby preventing the adhesion of pathogens to the host cell wall (Ngoci, et al., 2011). As a result, researchers in the present day are concentrating much on evaluation and characterization of plants and their constituents depending on their traditional claims for treatment against various diseases. Extraction of the new bioactive phytoconstituents is thus a challenging task for the researchers.

For drug development the utilization of bioactive phytochemical compounds is in the form of therapeutic agents, starting material for the synthesis of drugs or it may serve as model for pharmacologically active compounds. In the context of modern science, exploitation of traditional herbal medicines is needed for optimum and proper utilization of traditional plant derived drugs. In India, though there is lot of studies are being done on medicinal plants in a large number, yet the amount of phytochemical entities or herbal preparations with modern standards of safety and efficacy as useful marketable drugs are limited (Rao, 2007; Yadav and Dixit, 2008).

Identification of potential plants for combating a particular disease is the first step in the development of a phytomedicine followed by the preparation of a therapeutically valuable extract, crude homogenate or active fraction with respect to therapeutic use. The plants are usually selected based on ethnomedical value and is further evaluated for its efficacy in experimental animal models. The ethno medicinally used plant part is tested first and then the crude preparation or solvent extract is evaluated in two or three consecutive doses. The extract which has shown highest biological activity is selected and tested by at least three different extracts such as n-hexane, alcohol and water ranging
from non-polar to polar. The preferred method is to carry out each extraction separately from the original plant material or dried powder. Sequential solvent extraction method is the most common and potent method chosen by some researchers. To preserve the biological activity of compounds which are sensitive to heat, extraction is done at room temperature. The yield of the extract is established for each extract and one or two doses of each of the extracts is tested for pharmacological activity where dose selection depends on the activity in the original homogenate and also yield of extract. One among the three extracts is selected for further studies based on its bioactivity. The extracts prepared from different parts of the plant are tested in order to determine the most suitable plant part (Mendonca, 2006; Salim et al., 2008).

### 3.1.1 Choice of solvents

Successful isolation of bioactive compounds from plants is mainly dependent on type of solvent used in the extraction process. Low toxicity, ease of evaporation at low heat, preservative action, promotion of rapid physiologic absorption of the extract, inability to cause the extract to dissociate are the main characteristic features of a good solvent used in plant extractions. The choice of solvent is mainly dependent on ease of subsequent handling of the extracts, rate of extraction, quantity of phytochemicals to be extracted, diversity of different compounds extracted, diversity of inhibitory compounds extracted, potential health hazard of the extractants and toxicity of the solvent in the bioassay process (Eloff, 1998). The solvent should not interfere with the bioassay and should be non-toxic, as the end product has some traces of solvent. The choice of solvent also depends on the compounds targeted for extraction (Ncube et al., 2008; Das et al., 2010).
Ash is the inorganic residue remaining after either ignition or complete oxidation of organic matter in a foodstuff. To ensure reliable results, the characteristic of various ashing procedures and types of equipment also essential. There are two important types of ashing such as dry ashing and wet ashing; dry ashing is primarily used for proximate composition and specific mineral analyses and wet ashing is used for the general analysis of certain minerals. A microwave system is available both for dry and wet ashing in order to speed up the process. The dry samples such as cereals, whole grain, dried vegetables etc do not need any prior preparation but the fresh samples have to be dried before ashing. The ash content of foods is either expressed on a wet weight or dry weight basis.

The total mineral content present in food materials is represented by the ash value which is a part of proximate analysis useful for nutritional evaluation. In the process of preparing a sample for specific elemental analysis, ashing is done first and plays an important role because most of the samples are high in particular minerals. The ash content and elemental composition is constant. In animal samples but varies in plant sources. The ash content of dairy products vary from 0.5-5%, fats & oils is 0-4%, fresh fruits about 0.2-0.6% ash, fresh foods contain about 5% ash, dried fruits have 2.4-3.5%, wheat germ about 4.3% ash and pure form of starch has 0.3% (Maurice, 2010).

The present chapter deals with the broad spectrum screening of phytochemical and pharmacognostic parameters of *Feronia limonia* Linn which reveals the potential bioactivity of phytochemicals present in the extracts.
3.2 Materials and Methods

3.2.1 Plant material

The present work was carried out in the Department of PG studies and Research in Biotechnology, Gulbarga University, Kalaburagi with the object of screening the potential bioactivity of the plant. The plant material was collected in and around the Bellary district. The plant specimen was identified as *Feronia limonia* Linn belonging to the family Rutaceae and authenticated by a voucher specimen number HGUG-247 allotted by the Department of Botany, Gulbarga University, Kalaburagi. The plant materials such as fruits and leaves were chosen based on the therapeutic properties and traditional uses. After authentication the plant samples were brought to the laboratory, thoroughly washed and shade dried at 25± 2°C for about 6 days. The dried plant samples were ground into a fine powder in a mixer grinder and sieved to yield particle size of 50–120mm. The powder was stored at room temperature in air tight polythene bags before extraction.

The fruits and leaves were selected for further screening based on therapeutic properties and traditional uses of the plant.

3.2.2 Serial exhaustive soxhlet extraction

It is the common and widely used method which involves successive extraction of dried plant material by using organic solvents of increasing polarity from a non polar solvent such as hexane to a more polar solvent such as methanol and water to obtain a wide range of compounds of similar polarity. Great care must be taken for thermolabile compounds because extended heating leads to degradation of some compounds (Das et al., 2010).
25g of dried plant powder was weighed accurately, packed in Whatman filter paper no.1 and was extracted in a soxhlet apparatus using 175ml of Solvents such as Petroleum ether ($60^0\text{C}–80^0\text{C}$), Chloroform ($65^0\text{C}$), Methanol ($80^0\text{C}$) and Aqueous ($100^0\text{C}$) successively. The extracts were dried and were stored at $4^0\text{C}$ in a refrigerator.

### 3.2.2 Qualitative phytochemical screening

Phytochemical progress is aided by the development of rapid and accurate methods of screening plants for particular chemicals. Medicinal plants have physiologically active principles which are exploited for the treatment of various ailments. There is a reasonable likelihood that medicinal plants with a long history of human use will ultimately yield novel drug prototypes.

The different qualitative phytochemical analysis was performed using the procedures of Kokate (1994); Kokate and Purohit (2004) and Trease & Evans (1989) for establishing the profile of four different extracts of *Feronia limonia* Linn such as petroleum ether, chloroform, methanol and aqueous extracts. The following tests were performed on the different extracts to detect various phytoconstituents present in them.

**Detection of alkaloids**

About 25 mg of solvent free extract was mixed with few ml of dilute hydrochloric acid and filtered. The filtrate was tested sensibly with various alkaloid reagents as follows:

**Mayer’s test**

To a few ml of above filtrate, a drop or two of Mayer’s reagent was added along the side of the test tube. A white or creamy precipitate indicates the test as positive for the presence of alkaloids.
**Mayer’s reagent:** 3.5 g of Mercuric chloride was dissolved in 60 ml of water and potassium iodide (5.0 g) was dissolved in 10 ml of water separately. Both the solutions were mixed together and volume was made up to 100 ml with water.

**B. Wagner’s test**

To a few ml of filtrate, few drops of Wagner’s reagent were added along the side of the test tube. A reddish–brown precipitate confirms the test as positive for the presence of alkaloids.

**Wagner’s reagent:** 1.27 g of Iodine and 2 g of potassium iodide were dissolved in 5 ml of water and made up to 100 ml with water.

**C. Hager’s test**

To a few ml of filtrate, 1–2 ml of Hager’s reagent (saturated aqueous solution of picric acid) was added. A prominent yellow precipitate indicates the presence of alkaloids.

**D. Dragendorff’s test**

To a few ml of filtrate, 1–2 ml of Dragendorff’s reagent was added. A prominent yellow precipitate indicates the test as positive.

**Dragendorff’s reagent**

**Stock solution:** 5.2 g of Bismuth carbonate and 4 g of sodium iodide were boiled for few minutes in 50 ml glacial acetic acid. The precipitated sodium acetate crystals were filtered off using a sintered glass funnel after 12hrs of standing. 40 ml of clear reddish brown filtrate was mixed with 160 ml ethyl acetate & 1 ml water and stored in brown colored bottle.
**Working Solution:** 10 ml of Stock solution was mixed with 20 ml of acetic acid and volume was made up to 100 ml with water.

**Detection of carbohydrates**

25 mg of extracts were dissolved in 5 ml of water and filtered. The filtrate was subjected to the following tests.

**A. Molisch’s test**

2 ml of above filtrate was taken in a test tube and two drops of alcoholic solution of α-naphthol was added. The mixture was shaken well and slowly 1 ml of concentrated sulphuric acid was added by the sides of the test tube and allowed to stand for a while. The appearance of violet ring reveals the presence of carbohydrates.

**B. Fehling’s test**

One ml of filtrate was taken in a test tube and 1 ml each of Fehling solutions A and B were added and boiled on water bath for few minutes. A red precipitate denotes the presence of sugar.

**Fehling’s solution:** Fehling’s solution A: 34.66 g of Copper sulphate was dissolved in distilled water and volume was made up to 500 ml with distilled water.

Fehling’s solution B: 173 g of Potassium sodium tartarate and 50 g of sodium hydroxide was dissolved in water and volume was made up to 500 ml with distilled water.

**C. Barfoed’s test**

1 ml of filtrate was taken in a test tube and 1 ml of Barfoed’s reagent was added and heated on a boiling water bath for about 2 min. Red precipitate indicates the presence of sugar.
**Barfoed’s reagent:** 6.5 g of Copper acetate was dissolved in 8 ml of glacial acetic acid.

**D. Benedict’s test**

To 0.5 ml of filtrate, 0.5 ml of Benedict’s reagent was added. The mixture was heated on a boiling water bath for 2 min. A characteristic colored precipitate shows the presence of sugar.

**Benedict’s reagent:** 173 g of Sodium citrate and 100g of sodium carbonate was dissolved in 800 ml distilled water and boiled to make the solution clear. Copper sulphate (17.3 g) dissolved in 100 ml distilled water separately added to this solution.

**Detection of glycosides**

For detection of glycosides, 50 mg of extracts was hydrolysed with concentrated hydrochloric acid for 2 hrs on water both, filtered and the hydrolysate was subjected to the following tests.

**A. Borntrager’s test**

2 ml of filtrate was taken in a test tube and 3 ml of chloroform was added and shaken well. The chloroform layer was separated; to this 10% ammonia solution was added. Development of pink colour in the ammonical layer denotes the presence of glycosides.

**B. Legal’s test**

25 mg of the extracts was treated with sodium nitroprusside solution in pyridine and the solution was made alkaline by using 10 % sodium hydroxide. Presence of glycoside was indicated by pink colour.
Detection of Saponins

A. Froth test

Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of froth specifies the presence of saponins.

B. Foam test

0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

Detection of proteins and amino acids

50mg of the extract was dissolved in 10 ml of distilled water and filtered through whatman No.1 filter paper. The filtrate was subjected to the following tests for detection of proteins and amino acids.

A. Millon’s test

2 ml of filtrate was taken in a test tube and few drops of Millon’s reagent was added. A white precipitate appeared indicating the presence of proteins.

Preparation of millon’s reagent

1g of Mercury was dissolved in 9 ml of nitric acid. When the reaction was completed, equal volume of distilled water was added.

B. Biuret test

An aliquot of 2 ml of filtrate was treated with one drop of 2 % copper sulphate solution. To this, 1 ml of ethanol (95%) was added, followed by addition of potassium hydroxide pellets, appearance of pink colour in the ethanolic layer designates the presence of proteins.
C. Ninhydrin test

Two drops of ninhydrin solution (10 mg of ninhydrin in 200 ml of acetone) were added to two ml of aqueous filtrate. A characteristic purple colour reveals the presence of amino acid.

D. Xanthoproteic Test

The extracts were treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.

Detection of phytosterols

A. Salkowski’s test

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour points out the presence of triterpenes.

B. Libermann Burchard’s test

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid was added. Formation of brown ring at the junction between two components implies the presence of phytosterols.

Detection of fixed oils and fats

A. Spot test: A small quantity of extract was pressed between two filter papers. Oil stain on the paper indicates the presence of fixed oil.

B. Saponification test: Few drops of 0.5 N alcoholic potassium hydroxide solution was added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated on water bath for 2hrs. Formation of soap or partial neutralization
of alkali signifies the presence of fixed oils and fats.

**Detection of tannins**

**A. Ferric chloride test**

The extract (50 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution was added. A dark green colour denotes the presence of phenolic compounds.

**B. Gelatin test**

The extract (50 mg) was dissolved in 5 ml of distilled water and 2 ml of 1% solution of gelatin and 10 % sodium chloride was added to it. White precipitate indicates the presence of phenolic compounds.

**Detection of phenolics**

**A. Ellagic test**

Plant ethanolic extract was treated with few drops of acetic acid and 5% sodium nitrite solutions. Presence of phenolics was indicated by the development of muddy brown precipitate.

**B. Phenol test**

Ethanolic solution of plant extract was added to ferric chloride solution. Formation of intense colour indicates the reaction as positive for presence of phenolics.

**Detection of flavonoids**

**A. Shinoda test**

The extract (50 mg) was dissolved in 5 ml of alcohol and few fragments of magnesium ribbon and concentrated hydrochloric acid (Drop wise) were added. Presence of flavanol glycosides was inferred by the development of pink to crimson colour.
B. Lead acetate test

All the four extracts taken in different test tubes were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

C. Alkaline reagent test

All the four extracts of *Feronia limonia* were taken in a test tube and treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid which points out the presence of flavonoids.

Detection of gum and mucilage

The extract (100mg) was dissolved in 10 ml of distilled water and 25 ml of absolute alcohol was added with constant stirring. White or cloudy precipitate designates the presence of gum and mucilage.

Detection of terpenes

Copper acetate Test

Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour infers the presence of terpenes.

3.2.4 Quantitative phytochemical screening

Determination of carbohydrates

The determination of carbohydrates present in various samples of *Feronia limonia* was carried out according to the procedure of Krishnaveni et al., (1984).

5 ml of 2.5 N HCl was added to 100 mg of sample in a test tube and hydrolyzed in a boiling water bath for 3 hours; cooled to room temperature and sodium carbonate was
added till the effervescence stops. The tube with contents was centrifuged, supernatant was collected and final volume made upto 100 ml by using distilled water. 0.2 ml of sample was taken out and the volume was made upto 1 ml using distilled water. Further 1 ml of phenol reagent was added immediately followed by 5.0 ml of sulphuric acid. The tubes were kept for 20 min at 25-30°C and the absorbance was recorded at 490 nm.

**Determination of proteins**

The determination of proteins present in various samples of *Feronia limonia* was estimated by the method of Lowry et al., (1957). To 50 ml of 50% methanol (1:5 w/v), the dried & powdered samples were added and stirred continuously for 24 h by at 25 °C; then centrifuged at 7,000 rpm for 10 min. 0.2 ml was pipetted out to test tube and the volume was made to 1 ml with water. Alkaline copper reagent (5 ml) was added, allowed to stand for 10 min and finally Folin Ciocalteau reagent (5 ml) was added. The tubes were incubated in dark for 30 min and the intensity of the colour developed was recorded at 660 nm by using UV-Vis spectrophotometer.

**Estimation of total lipid content**

25 g of sample was used to extract lipids with 175 ml of petroleum ether at a solvent condensation rate of 2–3 drops/sec for 16 hr period by using soxhlet extraction. The extract was concentrated and evaporated at room temperature to dryness. The difference in weight of the extract gives the total lipid content which was expressed as mg/g dry matter.

**Determination of total phenolic content (TPC)**

The amount of total phenolics present in *Feronia limonia* plant extracts was estimated by using spectrophotometric method as described by Singleton et al., (1999).
1 mg/ml concentration of methanolic solution of the extract of all samples were used. To prepare the reaction mixture, 2.5 ml of 10% Folin-Ciocalteu’s reagent dissolved in water; 2.5 ml 7.5% of NaHCO₃ and 0.5 ml methanolic solution of extracts were added. Blank solution was prepared by adding 2.5 ml 10% Folin-Ciocalteu’s reagent dissolved in water, 2.5 ml of 7.5% of NaHCO₃ and 0.5 ml methanol. All the samples were incubated for 45 min in a thermostat at 45°C and the absorbance was read at 765 nm by using spectrophotometer. The procedure was done in triplicate and the mean value of absorbance was taken. The standard solution of gallic acid was also prepared by following same procedure and the calibration curve was determined. The concentration of total phenolics was recorded in mg/ml from the calibration curve based on the absorbance and was expressed as gallic acid equivalent (mg of GA/g of extract).

**Determination of total flavonoid content (TFC)**

The concentration of total flavonoids in the *Feronia limonia* plant extracts was determined by using spectrophotometric method of Quettier et al., (2000). The reaction mixture was prepared by adding 1 ml of 2% AlCl₃ prepared in methanol and 1 ml of 1 mg/ml methanolic solution of extract. The mixture was incubated at room temperature for 1 hour and the absorbance was read at 415 nm using spectrophotometer. The procedure was repeated for three times and the mean value of absorbance was noted. The standard solution of rutin was also prepared by following same procedure and the calibration curve was constructed. On the basis of the measured absorbance, concentration of total flavonoids was read in mg/ml on the calibration line and was expressed in terms of rutin equivalent (mg of RE/g of extract).
3.2.5 Determination of ash

The ash formed by ignition of herbal materials is determined by three different methods such as total ash, water-soluble ash and acid-insoluble ash (WHO, 2002).

**Total ash:** The total ash method is followed to measure the total amount of material remained after ignition which includes both physiological ash and non-physiological ash. The physiological ash is derived from the plant tissue itself whereas non-physiological ash is the residue of the extraneous matter such as sand and soil that is adhering to the plant surface.

About 3 g of accurately weighed ground air-dried material was taken in a previously ignited suitable crucible such as of silica or platinum, cooled and weighed. The material was spread in an even layer and the material was incinerated by gradually increasing the heat, not exceeding 450°C, until it is white and free from carbon. Then it was cooled in a desiccator and weighed. To obtain a carbon-free ash the charred mass was exhausted with 2ml of hot water or saturated solution of ammonium nitrate and residue was collected on an ashless filter-paper. The residue along with the filter-paper was incinerated; filtrate was added, evaporated to dryness and ignited at a temperature not exceeding 450 °C. The content was calculated as mg of ash per g of air-dried material.

\[
\text{Weight of residue} \times 100 \]
\[
\text{Weight of sample}
\]

**Acid-insoluble ash:** Acid-insoluble ash is the residue achieved when the total ash is boiled with dilute hydrochloric acid and then the remaining insoluble matter is ignited. The method commonly determines the amount of silica present especially sand and
siliceous matter. To the crucible containing the total ash, 25 ml of hydrochloric acid (~70 g/l) TS was added, covered with a watch-glass and boiled gently for 5 minutes. The watch-glass was rinsed with 5 ml of hot water and this liquid was added to the crucible. The insoluble matter collected on an ashless filter-paper and washed with hot water until the filtrate was neutral. Filter-paper containing the insoluble matter was transferred to the original crucible, dried on a hotplate and ignited to constant weight. The residue was allowed to cool in a desiccator for 30 minutes, then weighed without delay. The content of acid-insoluble ash was calculated in mg per g of air-dried material.

\[
\text{Weight of residue} \\
\% \text{ Acid insoluble ash} = \frac{\text{Weight of residue}}{\text{Weight of ash taken}} \times 100
\]

**Water-soluble ash**

Water-soluble ash is obtained by difference in weight of total ash and the residue remained after treatment of the total ash with water.

25 ml of water was added to the crucible containing the total ash and was boiled for 5 minutes. The insoluble matter was collected in a sintered-glass crucible or ashless filter paper; washed with hot water and was ignited for 15 minutes at a temperature in a crucible not exceeding 450 °C. The weight of the residue was subtracted from the weight of total ash. The content of water-soluble ash was calculated in mg per g of air-dried material.

\[
\text{Weight of residue} \\
\% \text{ Water soluble ash} = \frac{\text{Weight of residue}}{\text{Weight of ash taken}} \times 100
\]
3.2.6 Determination of extractive value

The determination of alcohol and water soluble extractive value serves as a way to evaluate the quality and purity of drugs. Extraction of the drug is done either by maceration or by a continuous extraction in a Soxhlet extractor.

The extractive values were recorded in alcohol and water with a view to study the distribution of various constituents of *Feronia limonia* Linn. Accurately weighed 4.0 g of coarsely air-dried powdered material was placed in a glass-stoppered conical flask; macerated for 6 h with 100 ml of the solvent with frequent shaking and then allowed to stand for 18 h. The mixture was filtered taking care not to lose any solvent. 25 ml of the filtrate was transferred to a flat-bottomed dish and evaporated to dryness on a water bath. The residue was dried at 105\(^\circ\)C for 6 h, cooled in a desiccator for 30 min and then weighed without delay. The Percentage of Water soluble/Alcohol soluble extractive value was calculated using formula: \[
\frac{(B - A) \times 4 \times 100}{W}.
\]

where, A is Empty weight of the dish (g); B is Weight of dish + residue (g); W is Weight of plant material taken (g).

3.2.7 Moisture content

For determination of moisture content, the sample materials were taken in a flat-bottomed dish which is weighed, then kept overnight in hot air oven at 100–110\(^\circ\)C and again weighed. The loss in weight was regarded as a measure of moisture content.

3.2.8 Mineral analysis

Accurately weighed 2.0 g of plant sample was transferred to a silica crucible and kept in a muffle furnace for ashing for 3 hours at 450\(^\circ\)C. 5 ml of 6M HCL was added to the crucible; kept on a hot plate and digested to get a clear solution. 0.1 M HNO\(_3\) solution
was used to dissolve the final residue and volume was made up to 50 ml. Standard working solutions were prepared by further diluting the prepared stock solutions. Calcium, Phosphorous, Iron, Copper, Cadmium, Lead, Potassium and Chromium in *Feronia limonia* plant samples were analyzed using atomic absorption spectrophotometer. The absorption wavelength for the determination of each metal together with its linear working range and correlation coefficient of calibration graphs were determined.

### 3.2.9 Fluorescence analysis

The fluorescence analysis was performed according to Chase and Pratt (1949). The samples were added in a test tube, treated with reagents such as Ammonia, Hydrochloric acid, Hexane, Sodium hydroxide, Acetic acid, Benzene, Petroleum ether and Chloroform and finally observed for development of colour within 1-2 minutes under both daylight and UV light in order to avoid drying and resultant colour change.
3.3 RESULTS

3.3.1 Extraction (Table 3.1)

Successive solvent extraction method was carried out for isolation of phytochemical compounds present in *Feronia limonia* Linn. Solvents from non polar to polar such as petroleum ether, chloroform, methanol and water were carefully chosen to get maximum yield with minimum toxicity. The standardization of extraction process was done with respect to type of solvent, time period, ratio of plant material to solvent used and temperature.

3.3.2 Qualitative phytochemical screening (Table 3.2)

The extracts from different parts such as leaf, whole fruit and fruit pulp of the plant *Feronia limonia* Linn. were subjected to qualitative phytochemical analysis. The results revealed the presence of major constituents in all the tested samples. All the four extracts of pulp, whole fruit and leaf samples have responded positively for alkaloids by exhibiting colour formation in Mayer’s test, Wagner’s test, Hager’s test and Dragendorff’s test; the results for carbohydrate tests such as in Molisch’s test, Fehling’s test, Barfoed’s test and Benedict’s test are shown positive; presence of proteins was confirmed by Millon’s test, Biuret test, Ninhydrin test and Xanthoproteic test.

**Tannins:** The methanol and aqueous extracts of all the three samples namely pulp, whole fruit and leaf have shown presence of tannins verified by tests like Ferric chloride test and Gelatin test by formation of green colour and white precipitate respectively. The other extracts petroleum ether and chloroform have shown absence of tannins.
**Phenolics:** Presence of phenolics by development of intense muddy brown colour in Phenol test and Ellagic test was revealed in methanol and aqueous extract whereas negative for Petroleum extract & chloroform extract of both whole fruit and fruit pulp samples. All the extracts of leaf sample have yielded negative results for phenolics.

**Flavonoids:** The methanol and aqueous extracts of fruit pulp and whole fruit responded positively for presence of flavonoids in Lead acetate and Alkaline reagent test by yielding white precipitate and yellow fluorescence respectively. However, the petroleum ether and chloroform extracts showed negative results. Only the aqueous extract of leaf sample indicated the presence of flavonoids and other remaining three extracts gave negative response.

**Glycosides:** Presence of glycosides by production of pink colour in both Borntrager’s test and Legal’s test was exhibited in all the extracts of whole fruit. The petroleum ether extract of fruit pulp showed absence of glycosides, whereas, the other three extracts are responded positively. The chloroform extract and petroleum ether extract of leaf have shown absence of glycosides and the other two methanol and aqueous extracts were shown positive result.

**Steroids:** Presence of steroids was revealed by checking yellow and brown colour in Salkowiski’s test and Libermann Burchard’s test. In the petroleum and aqueous extracts of fruit pulp there was no presence of steroids but chloroform and methanol extracts indicated its occurrence. All the extracts of whole fruit specifies the presence of steroids except aqueous extract. The petroleum and aqueous extracts of leaf sample revealed the positive results, whereas remaining two extracts chloroform and methanol responded negatively.
Lipids and fats: Formation of oil stain in Spot test and Formation of soap in Saponification test was used as means for evaluating the presence of lipids. The petroleum ether extract of all the samples have shown positive findings whereas all the other extracts indicated absence of lipids and fats in the specific tests.

Saponins: Chloroform extract and petroleum ether extract of both whole fruit and fruit pulp revealed the negative outcome in Froth test and Foam test while methanol and aqueous extracts have shown presence of saponins. The methanol extract of leaf has positive results and all other remaining extracts responded negatively for the saponins.

Terpenoids: Only the petroleum ether extract of whole fruit and fruit pulp samples signified the occurrence of terpenoids in Copper acetate test by exhibiting emerald green colour, however all other extracts have showed its absence. Chloroform extract and petroleum ether extract of leaf gave positive response but methanol and aqueous extracts gave negative response.

Gums and mucilage: Complete absence of gums and mucilage was seen in all the extracts of leaf. Petroleum and aqueous extracts of fruit pulp have revealed their existence which was confirmed by Alcohol test yielding white precipitate whereas the other two extracts chloroform and methanol exhibited negative results.

3.3.3 Quantitative phytochemical screening

3.3.3.1 Primary metabolites (Table 3.3)

The results of quantitative screening of primary metabolites such as proteins, carbohydrates and fats of Feronia limonia whole fruit, fruit pulp and leaf are represented in table 3.3. It was observed that the amount of protein was higher in the whole fruit of about 41.70 ± 0.83 mg/g when compared to fruit pulp (29.16 ± 0.14 mg/g) and leaf
(10.03 ± 0.17 mg/g). Considerably less amount of protein was noted in the leaf. The carbohydrate content was higher in whole fruit of about 26.82 ± 0.02 mg/g followed by pulp and leaves corresponding to 20.31 ± 0.41 mg/g and 9.11 ± 0.06 mg/g respectively. The total fat content was comparatively higher in whole fruit is about 1.91 ± 0.13 mg/g, in fruit pulp 1.89 ± 0.33 mg/g and lowest in the leaf of 0.18 ± 0.33 mg/g.

3.3.3.2 Secondary metabolites (Table 3.4)

The total phenol content (TPC) was determined in comparison with standard gallic acid and the results are expressed in terms of mg GAE/g dry sample. From table 3.4 it is seen that the total phenolic content of whole fruit sample is the highest of about 78.14 ± 0.61 mg GAE/g. The pulp also has shown good amount of phenolics of about 74.57 ± 0.44 mg GAE/g in comparison to whole fruit whereas leaf has moderate amount of 26.18 ± 0.33 mg GAE/g of phenolics.

The total flavonoid content (TFC) was determined in comparison with standard rutin and the results are expressed in terms of mg RE/g dry sample. TFC was found almost similar in both whole fruit and fruit pulp samples of about 30.22 ± 0.08 mg RE/g and 29.11 ± 0.26 mg RE/g respectively. The amount of TFC in leaf was 10.18 ± 1.04 mg RE/g which was quite low compared to other two samples (Table 3.4).

3.3.3.3 Physicochemical analysis (Table 3.5)

Physicochemical constituents like total ash value, acid insoluble ash value and water soluble ash value were analyzed and is presented in the table 3.5. Highest total ash content was observed in case of whole fruit (10.13%), moderate amount in fruit pulp (7.79%) and low in leaf (58%). The values for acid insoluble ash fruit pulp, whole fruit
and leaf are 0.82%, 0.98% and 0.55% respectively. The range for water soluble ash values are 4.96%, 5.43% and 3.75% corresponding to fruit pulp, whole fruit and leaf.

Also water soluble extractive value and alcohol soluble extractive value were analyzed for all the three samples. The water soluble extractive value of fruit pulp, whole fruit and leaf obtained was 8.15%, 8.16% and 6.10%. The range for alcohol soluble extractive value was 11.17%, 14.25% and 4.05% respectively.

3.3.3.4 Percentage yield (Table 3.6)

Percentage yield of successive extracts of various solvents like pet. ether, chloroform, methanol and water were also taken into consideration and which yielded 6.1%, 6.3% and 2.10% for petroleum ether extract; 3.8%, 4.5% and 0.65% for chloroform extract; 10.7%, 13.3% and 3.91% for methanol extract; 14%, 15.8% and 7.45% for aqueous extract of fruit pulp, whole fruit and leaf (Table 3.6).

From the above phytochemical and physicochemical analysis it was observed that there was no much difference in the phytochemicals of whole fruit and pulp when compared. The leaves also possess comparatively less amount of phytochemicals. Hence keeping these aspects in view we have selected only fruit pulp for further studies.

3.3.3.5 Fluorescence analysis (Figure 3.1)

Behaviour of powder of fruit pulp of Feronia limonia with different chemical reagents in visible and ultraviolet light was observed. It is exhibited yellow, orange and brown in visible light; blue and yellow in UV light in distilled water. Colorless, light blue and brown at daylight; blue and brown in UV for ammonia. Light blue to straw yellow in visible light; light blue and orange in UV with HCl. Treatment with hexane resulted in cream to ash for daylight and blue to purple for UV light. Similarly for NaOH and
NaOH: Methanol cream to straw yellow for visible and cream to green for ultraviolet. Acetic acid solution has shown cream to green in daylight and blue to orange color range in UV. Behaviour of extracts with benzene treatment indicated green to purple for daylight and yellow to ash for UV light. Petroleum ether exhibited green to cream at daylight and blue to pink in UV. The extracts in chloroform appeared cream to yellow and blue to cream at visible and UV respectively.

3.3.3.6 Mineral analysis (Table 3.7)

Analysis of minerals such as calcium, phosphorous, iron, copper, cadmium, lead, potassium and chromium were performed using fruit pulp ash and expressed in parts per million (ppm) which yielded 130, 110, 60, 740, 7, 10, 1800 and 15 ppm respectively.

From the above results it was observed that *Feronia limonia* is a rich source of nutrients which is evident from primary metabolite screening and also high in mineral content evident from ash and mineral analysis.
3.4 Discussion

3.4.1 Plant material

The herbaria are used by the researchers in a particular study to exhibit correctly the source of their data by preserving the voucher specimens and samples of plants used. For studying the evolutionary relationships among plants belonging to a particular group, to provide information on morphology and as a source of DNA material for molecular analysis; herbarium specimens are the primary source of these data. A significant function of herbaria in the scientific research is to provide a permanent repository of specimens that helps to find the identity of species sampled in ethnobotanical, pharmacological or ecological studies. *Feronia limonia* is sometimes confused with the plant *Aegle mermelos* and other citrus species, thus to distinguish the plant and as a source of data we have submitted the herbarium specimen and got authenticated.

3.4.2 Extraction

The separation of fraction from plant tissues which is medicinally active through standard extraction procedure by using selective solvents. The complex mixtures of metabolites obtained from plants either in liquid, semisolid or dry powder form that are anticipated in oral or external use. These preparations include infusions, decoctions, tinctures, pilular (semisolid) extracts, powdered extracts and fluid extracts which contains complex mixture of plant metabolites such as glycosides, alkaloids, terpenoids, lignans and flavonoids (Remington and Remington, 2008; Handa et al., 2008). Solvents diffuse into the plant material and solubilize compounds with similar polarity during extraction. The objective of standardization in extraction procedures for crude drugs is to achieve the fraction having therapeutical potential and also to remove unwanted components by the
use of solvent. Thus based on the careful literature study, we have selected and used successive solvent soxhlet extraction method in order to achieve the maximum yield from the plant.

3.4.3 Qualitative phytochemical screening

Phytochemical screening is very important in the discovery of new therapeutical and industrially beneficial compounds from medicinal plants (Ambasta et al., 1986). The phytochemical substances possess definite physiological functions to act in the human body and have high medicinal importance. A wide range of medicinal properties which help to protect against various diseases are due to the presence of phytochemicals in plants such as saponins which protect against hypercholesterolemia; steroids & triterpenoids reveal the analgesic properties and alkaloids against chronic diseases.

All the parts of *Feronia limonia* have shown to be rich in primary and secondary metabolites. The preliminary qualitative chemical tests of *Feronia limonia* exhibited the presence of saponins, glycosides, alkaloids, steroids, phenolic compounds, fixed oils and fats, terpenoids, tannins and gum & mucilage which are responsible for various biological activities such as immuno-modulator, antiulcer and antioxidant activity. The results are in accordance with the findings of Sheeja and Kuttan (2005) who reported the comparative phytochemical studies on the leaves of *Aegle marmelos*.

3.4.4 Quantitative phytochemical screening

In India, the fruit was traditionally a “poor man’s food”. This is evident by our phytochemical screening and mineral analysis which has shown to possess high amount of carbohydrates, proteins and minerals.
The quantitative estimation of primary metabolites such as carbohydrates & proteins were high and low levels of lipid in the plant. Earlier workers have observed that the carbohydrate content is a very good source of energy (Murray et al., 1986; Ozcan et al., 2009). The presence of higher protein level in the fruit of *Feronia limonia* indicates the possible increase of use in food value and as a protein based bioactive compound. The low level of lipid is an indication that it would have merely little or no cholesterol. Thus, the results obtained in the present study indicated that *Feronia limonia* has the potential to act as a useful drug due to the presence of various phytochemical components such as protein, carbohydrate, lipids, flavonoids, phenols and tannins. The results are very much encouraging but further scientific validation is necessary before being put into clinical practice.

Investigation on secondary metabolites is necessary for further extraction, separation, purification, identification and crystallization of various novel phytoconstituents. The fruit sample has shown higher level of total phenol content (78.14 ± 0.61 mg/g dw). The high levels of phenol is utmost important in regulation of plant growth, development and resistance to diseases. The level of flavonoid content was 30.22 ± 0.08 mg/g dw in fruit sample. These amounts were comparable with results shown by Sachin and Arya (2012) who observed TPC of wood apple which has shown 38.61 mg (GAE) /g DW sample. The difference in values could be due to variation in the assay procedures for solvent extractions and variety of fruit used by the researchers.

Fu *et al.* (2010) have reported the difference in total phenolic content of different vegetables and fruits are due to non-phenolic reducing compounds such as sugars and organic acids that interferes with the determination of total phenolic contents by the
Folin-Ciocalteu method leading to an over-evaluation of the phenolic content. However, different phenolics show different responses with the Folin Ciocalteu reagent. The vanillin test for the detection of flavonoid in wood apple, ambadi and ambat chukka showed a negative TFC indicating that there is absence of condensed tannin in these fruits and vegetables. The difference in the TFC values is due to the inherent variability of the raw material, as well as differences in methodology or standards used.

Further efforts were made to analyze the waste portion called rind of *Feronia limonia* which also found to possess few important phytochemicals and minerals. Thus the waste portions also can be considered for utilization in value added nutraceutical food products and cosmetics.

### 3.4.5 Physicochemical analysis

The total ash value can be used as an indicative to detect impurities present in the drug since it is constant for a given drug and is used as a valuable diagnostic parameter. *Feronia limonia* has more water soluble ash than acid insoluble ash. The extractive value of alcohol was more than water. The results for various types of ash and extractive values provide a basis to identify the quality and purity of the drug.

Most of the phytochemicals produce fluorescence when illuminated suitably. The color produced by fluorescence is specific to each phytocompound. However, even non fluorescent compounds also fluoresce when it is mixed with impurities which are fluorescent. The fluorescence method is sensitive and gives the precise determination over a range of concentration without much time consumption.

The phytoconstituents which include glycosides, phenolics, tannins, alkaloids and amines are responsible for the medicinal as well as toxic properties. In the formation of
these phytocompounds, trace elements play an important role (Rajurkar and Damame, 1998). Trace elements possess the healing power against various ailments and disorders. To understand the pharmacological activity of the medicinal herbs, it is fundamental to know the trace elemental contents present in plants. Minerals comprise of only 4-6% of the human body but are of critical importance in the diet. Essential trace elements include manganese, chromium, iron, zinc, silicon, iodine, copper and fluoride. Major minerals which include potassium, phosphorus, calcium, magnesium, sodium chloride and sulphur that exist in body fluids or tissues are required in greater amounts of 100 mg per day and they signify 1% or less of body weight. Standard recommended doses for some trace elements are set and their deficiency leads to disease, but deficiency of other elements other than trace elements do not cause any problems (Archana, 2013). Mineral analysis of *Feronia limonia* fruit pulp suggests that it is a good source of nutrient due to the presence of minerals such Ca, P, Fe, Cu, Cd, Pb, K and Cr which are essential in maintenance of homeostasis of the body and good health.

There are no reports available so far on any parameter of whole fruit as per our knowledge. Our investigations provide a source to compare different edible parts such as fruit and leaf. The present study also supports the contention that the plant *Feronia limonia* Linn can be exploited as a source of “Poor man’s food” and it is important to plant these trees along roadsides or in plantations to conserve it as an ancient treasure and also it is valuable resource in the discovery of natural pharmaceutical products.