4. Standardization of Selected Medicinal Plants

4.1 Introduction

On the earth herbal drugs have been used since the beginning of human beings era and as a result are almost as old as human beings life itself. About 70-95% population of developing countries depends on herbal medicine for primary health care mainly because of high cost or inaccessibility to modern conventional drugs. In India, over 80% population depends upon herbal drugs. Many of the herbal formulations are used for the treatment of diabetes, liver diseases, arthritis, memory enhancers and as adaptogens (Chawla et al., 2013). A survey from Australian state of Victoria on 2526 adults showed that about a quarter of the adult population used some form of herbal drugs during 2006-2007. Similarly, about one in five or an estimated 38.2 million adults in the United States used herbs and supplements in 2002 (Zhang et al., 2011). Even with such wide acceptability, the number of standardized herbal drugs is less due to lack of regulatory standards and implementation protocols (Chawla et al., 2013). Standardization of botanicals is a process of evaluating the quality and purity of crude drugs by means of various parameters like morphological, microscopical, physical, chemical and biological observations (Agrawal SS, Paridhavi M. 2012). Due to the growing interest of people in herbal drugs, regulatory agencies are coming up with guidelines that will help to standardize the herbal drugs. Moreover, World Health Organization (WHO) has come up with series of technical guidelines which are helping in ensuring the quality of medicinal products as well as safety for the consumers.

The present study is hence an effort to standardize the selected plant materials. The dried leaves of *Clitoria ternatea* (*C. ternatea*), *Tephrosia purpurea* (*T. purpurea*) and *Malvastrum coromandelianum* (*M. coromandelianum*) were standardized as per the WHO guidelines.
4.2 Experimental

4.2.1 Collection and authentication

The leaves of the plant *C. ternatea* and *T. purpurea* were collected from areas in and around Akola, Maharashtra, India during the months of October 2010. The leaves of the plant *M. coromandelianum* were obtained from areas in and around Tumkur, Karnataka, India during the month of October 2010. The plant material was authenticated by Dr. Gopalakrishna Bhat, former Professor, Department of Botany, Poorna Prajna College, Udupi, Karnataka. Voucher specimens of the plant (Nos. 596, 597, 598) have been deposited in the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal, India.

4.2.2 Morphology and microscopy

Morphological characters of the plant material are based on size, shape, colour, surface characteristics and texture. For the microscopical studies, free hand sections of the leaves of the selected medicinal plants were taken and stained with phloroglucinol and hydrochloric acid (1:1). Slides were observed under Olympus System Microscope, Model BX41 fitted with Olympus DP20 camera. Images were captured with the help of Cell A software (Kokate 1991).

4.2.3 Powder analysis

Dried plant material was powdered and passed through sieve no.60 and used for the powder analysis. Small amount of powder was stained with phloroglucinol: HCl (1:1) and observed under microscope (Khandelwal et al., 2001).

4.2.4 Physicochemical standardization

4.2.4.1 Foreign matter

The plant material (250 g) was spread uniformly in the form of a thin layer without overlapping. The sample was inspected using magnifying lens (6x). The foreign matter
was separated manually. After complete separation, the foreign matter was weighed and percentage w/w present in the sample was determined (WHO 1998).

4.2.4.2 Total ash

About 2 g of the air-dried material was placed in a tared silica crucible. The material was spread as an even layer and the crucible was placed in muffle furnace and ignited by gradually increasing the heat to 500-600 °C until it is white, indicating the absence of carbon. Crucible was cooled in a desiccator and weighed. The percentage of total ash was calculated with reference to the air dried material (WHO 1998).

4.2.4.3 Acid insoluble ash

To the crucible containing the total ash, 25 ml of hydrochloric acid was added and it was allowed to boil gently for 5 minutes. The insoluble matter was collected on an ashless filter-paper and it was washed with hot water until it becomes free from acid. Filter paper containing the insoluble matter was transferred to the original crucible and ignited to constant weight. The crucible was allowed to cool in a desiccator for 30 minutes and then weighed. The percentage of acid insoluble ash was calculated with reference to the air dried material (WHO 1998).

4.2.4.4 Water soluble ash

To the crucible containing the total ash, 25 ml of water was added and boiled for 5 minutes. The insoluble matter was collected on an ashless filter-paper and it was washed with hot water. Filter paper containing the insoluble matter was transferred to the original crucible and ignited to constant weight. Weight of this residue was subtracted from the weight of total ash to get weight of water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried material (WHO 1998).
4.2.4.5 Alcohol soluble extractive value
Accurately weighed 4 g of coarsely powdered air-dried material was transferred to a 250 ml glass-stoppered conical flask. To this 100 ml of the ethanol (95%) was transferred. The plant material was allowed to macerate for 24 h with frequent shaking for first 6 h and then allowed to stand for 18 h. It was then filtered rapidly taking care not to lose any solvent. About 25 ml of the filtrate was transferred to a tared flat-bottom dish and evaporated to dryness on a water bath. It was then dried at 105 °C to constant weight, cooled in a desiccator for 30 min and weighed. The percentage of alcohol soluble extractive value was calculated with reference to the air dried material (WHO 1998).

4.2.4.6 Water soluble extractive value
Accurately weighed 4 g of coarsely powdered air-dried material was transferred to a 250 ml glass-stoppered conical flask. To this 100 ml of the water was transferred. The plant material was allowed to macerate for 24 h with frequent shaking for first 6 h and then allowed to stand for 18 h. It was then filtered rapidly taking care not to lose any solvent. About 25 ml of the filtrate was transferred to a tared flat-bottom dish and evaporated to dryness on a water bath. It was then dried at 105 °C to constant weight, cooled in a desiccator for 30 min and weighed. The percentage of alcohol soluble extractive value was calculated with reference to the air dried material (WHO 1998).

4.2.4.7 Moisture content by loss on drying
About 2-5 g of accurately weighed drug was dried at 100-105 °C in a hot air oven till constant weight. Loss on drying was calculated with reference to the initial weight of crude drug (WHO 1998).

4.2.4.8 Fluorescence analysis of the powdered crude drugs
Fluorescence of the powdered crude drugs has been studied as a pharmacognostic character to distinguish between plants and their species. The powdered crude drugs were
observed as such under day light and UV and fluorescent light and then again after treatment with different reagents as explained by Chase et al (1949) and Kokoski et al (1958).

4.2.5 Phytochemical evaluation

4.2.5.1 Preparation of extracts

The dried coarsely powdered leaves of the plant (50 g) was successively extracted with the solvents of increasing order of polarity (petroleum ether, chloroform, acetone, and methanol) by the hot extraction process using a Soxhlet apparatus. The marc left after each extraction process, was dried in order to remove the solvent. Marc remaining after methanol extraction was macerated with chloroform water for 24 h in order to get aqueous extract. After completion of extraction process the solvent was removed by distillation under reduced pressure and the prepared extract was stored in vacuum desiccator till further use.

4.2.5.2 Preliminary phytochemical screening

Each of the extract was subjected to various phytochemical tests to detect the presence of various phytoconstituents as listed below (Kokate 1991).

- **Test for alkaloids**
  Mayer’s test, Dragendorff’s test, Wagner’s test, Hager’s test

- **Test for saponins**
  Foam test

- **Test for carbohydrates**
  Molisch’s test, Fehling’s test, Benedict’s test

- **Test for Anthracene glycosides**
  Borntrager’s test, Modified Borntrager’s test

- **Test for steroids**
  Liebermann Burchard test
- **Test for fixed oils and fats**
  Spot test, Saponification test

- **Test for tannins and phenolic compounds**
  Ferric chloride test, Lead acetate test

- **Test for proteins and amino acids**
  Biuret test, Ninhydrin test

- **Test for gums and mucilage**
  Precipitation with absolute alcohol, Molisch’s test

- **Test for flavonoids**
  Shinoda test

### 4.2.5.3 Estimation of total phenolic content

For the estimation of total phenolic content of the leaf extract, calibration curve was prepared by mixing 1 ml methanolic solution of gallic acid (10-100 µg/ml) with 5 ml Folin-Ciocalteu reagent (diluted ten-fold) and 4 ml sodium carbonate (0.7 M). Absorbance was measured at 765 nm and calibration curve was plotted for concentration against absorbance. Same procedure was repeated with plant extracts (1 ml; 100 µg/ml) and absorbance was measured to determine total plant phenolic contents (Adesegun et al., 2009). The total content of phenolic compounds in the extract in gallic acid equivalents (GAE) was calculated by the following formula:

\[
T = \frac{C \times V}{M}
\]

Where,

- \(T\) = Total phenolic compounds (mg/g of plant extract) in GAE
- \(C\) = Concentration of gallic acid established from the calibration curve (mg/ml)
- \(V\) = Volume of extract (ml)
- \(M\) = Weight of plant extract (g)
4.2.5.4 Estimation of total flavonoid content

For the estimation of total flavonoid content quercetin was used as a standard. Briefly, 10 mg of quercetin was dissolved in 10 ml methanol to get 1000 µg/ml solution. It was further diluted to get concentration ranging from 10-100 µg/ml. The diluted standard solutions (0.5 ml) were separately mixed with 1.5 mL of methanol, 0.1 ml aluminum chloride (10%), 0.1 ml potassium acetate (1M) and 2.8 ml of distilled water. Reaction mixture was incubated at room temperature for 30 min. Absorbance was measured at 415 nm; calibration curve was plotted for concentration against absorbance. Same procedure was followed for the extracts also (0.5 ml; 100 µg/ml). In the blank solution the volume of 10% aluminum chloride was substituted with the same volume of distilled water (Chang et al., 2002). The total flavonoid content in the extract in quercetin equivalents (QE) was calculated by the same formula as given in estimation of total phenolic content.

4.2.5.5 Estimation of tannin content

Tannin content in crude plant material was estimated as per the procedure explained by Killedar et al (2010) by using gallic acid (10-100 µg/ml) as standard. For the preparation of standard curve 1ml working standard gallic acid solutions, 7.5 ml water, 0.5ml Folin-Denis reagent and 1ml sodium carbonate (35% w/v) were mixed and the absorbance of so formed blue colour solution was measured at 700 nm within 30 min of the reaction by using UV spectrophotometer against blank (i.e replacing 0.5 mL Folin-Denis reagent with 0.5mL distilled water). Calibration curve was plotted for absorbance against concentration. Sample for the determination of non-hydrolysable tannins was prepared by refluxing 2g each of air dried leaf powder sample with 75 ml of double distilled water for 30 min for complete extraction of tannins. Whole the mixture after cooling was filtered through Whatmann filter paper no. 41. Filtrate was centrifuged at 2000 rpm for 20 min.
Supernatant was collected in 100 ml volumetric flask and volume was made to 100 ml by distilled water. Sample for the determination of hydrolysable tannins was prepared as explained above but with the addition of 0.1 ml of hydrochloric acid while refluxing. Tannin content in the sample was estimated as explained in the preparation of standard gallic acid curve. Content of tannic acid in crude drug was expressed as % w/w.

### 4.2.6 Marker based standardization of selected medicinal plant extracts by High Performance Thin Layer Chromatography

#### 4.2.6.1 Estimation of β-sitosterol in *C. ternatea, T. purpurea* and *M. coromandelianum*

**Chromatographic conditions**

<table>
<thead>
<tr>
<th>Stationary phase:</th>
<th>Aluminum backed TLC Silica Gel GF$_{254}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase:</td>
<td>Benzene: Ethyl acetate (9.5: 0.5)</td>
</tr>
<tr>
<td>Development chamber:</td>
<td>Camag Twin Trough</td>
</tr>
<tr>
<td>Chamber saturation:</td>
<td>20 min</td>
</tr>
<tr>
<td>Standard:</td>
<td>β-sitosterol in methanol (100 µg/ml)</td>
</tr>
</tbody>
</table>
| Sample:           | a) Pet. ether extract of *C. ternatea* (1000 µg/ml) in methanol  
b) Pet. ether extract of *T. purpurea* (1000 µg/ml) in methanol  
c) Pet. ether extract of *M. coromandelianum* (1000 µg/ml) in methanol |
| Sample volume/track: | 10 µl                                   |
| Derivatization:   | Methanolic sulphuric acid (10%) spray reagent |
| Detection wavelength: | 366 nm                              |
4.2.6.2 HPTLC estimation of kaempferol in *C. ternatea*

Chromatographic conditions

**Stationary phase:** Aluminum backed TLC Silica Gel GF$_{254}$

**Mobile phase:** Toluene: Ethyl acetate: Formic acid (7.8: 1.8:0.4)

**Development chamber:** Camag Twin Trough

**Chamber saturation:** 20 min

**Standard:** Kaempferol in methanol (100 µg/ml)

**Sample:** Methanol extract of *C. ternatea* (1000 µg/ml) in methanol

**Sample volume/track:** 10 µl

**Detection wavelength:** 366 nm

4.2.6.3 HPTLC estimation of lupeol in *T. purpurea*

**Stationary phase:** Aluminum backed TLC Silica Gel GF$_{254}$

**Mobile phase:** Benzene: Ethyl acetate (9.5: 0.5)

**Development chamber:** Camag Twin Trough

**Chamber saturation:** 20 min

**Standard:** Lupeol in n-hexane (100 µg/ml)

**Sample:** Pet. ether extract of *T. purpurea* (1000 µg/ml) in methanol

**Sample volume/track:** 10 µl

**Derivatization:** Methanolic sulphuric acid (10%) spray reagent

**Detection wavelength:** 400 nm
4.2.6.4 HPTLC estimation of quercetin in *T. purpurea*

Stationary phase: Aluminum backed TLC Silica Gel GF<sub>254</sub>
Mobile phase: Toluene: Ethyl acetate: Formic acid (7: 3: 1)
Development chamber: Camag Twin Trough
Chamber saturation: 20 min
Standard: Quercetin in methanol (100 µg/ml)
Sample: Chloroform extract of *T. purpurea* (1000 µg/ml) in methanol
Sample volume/track: 10 µl
Detection wavelength: 400 nm

4.2.6.5 HPTLC estimation of rutin in *T. purpurea*

Stationary phase: Aluminum backed TLC Silica Gel GF<sub>254</sub>
Development chamber: Camag Twin Trough
Chamber saturation: 20 min
Standard: Rutin in methanol (100 µg/ml)
Sample: a) Acetone extract of *C. ternatea* (1000 µg/ml) in methanol
    b) Methanol extract of *C. ternatea* (1000 µg/ml) in methanol
Sample volume/track: 10 µl
Detection wavelength: 366 nm

After each chromatography the percentage content of each marker in respective plant extract was calculated as per the following formula.

\[
\text{% content} = \frac{AUC \text{ of sample} \times \text{Conc. of std} \times \text{% purity}}{AUC \text{ of std} \times \text{Conc. of sample}}
\]
4.3 Results and Discussion

4.3.1 Morphology of *C. ternatea*

Leaf is unipinnate, with single terminal leaflet (imparipinnate). Leaflets arranged on rachis 7.5 to 12.5 cm long, with 2 to 3 pairs of opposite leaflets. Leaflet is elliptic to oblong in shape 2.5 to 6.3 cm long and 2 to 3.8 cm broad. Terminal leaflet is bigger in size, with entire margin, glabrous. Veins more prominent at the lower side. Base of the leaf is obtuse or acute, with short petiole, (2 to 3 mm long). Stipule is filament shaped two at the base of the terminal leaflet. Colour of the leaf is pale yellowish green, with astringent and slight bitter taste (Fig. 4.1)

4.3.2 Microscopy of *C. ternatea*

The histology of the leaf can be best studied as lamina and midrib region. Lamina of the leaf exhibits dorsiventral nature and shows the layer of upper epidermis with polygonal tabular cells covered with smooth cuticle. Mesophyll consists of single layer of palisade parenchyma which has radially elongated cells with cuticle. Spongy parenchyma of mesophyll consists of loosely arranged parenchyma cells traversed by vascular elements and has prismatic calcium oxalate crystals. Cells of lower epidermis are similar to upper epidermis and have unicellular covering trichomes. Midrib shows broadly convex shape at the lower side and elevated shape at upper side. Upper epidermal cells of midrib consist of palisade cells and a narrow band of collenchyma under both the epidermis of midrib region (2 to 3 cell layer thick under upper and 1 to 2 cell layer thick under lower epidermis). Well-developed, collateral vascular bundle is seen at the center of the midrib
which is dorsiventral encircled by lignified sclerenchymatous sheath of pericyclic fibres (Fig. 4.2). Powder microscopy of the leaf shows the presence of paracytic stomata and unicellular, thick walled simple trichomes with pointed end along with prismatic calcium oxalate crystals (Fig 4.3).

![Fig. 4.2: TS of leaf of C. ternatea PAL: Palisade, T: Trichome, UE: Upper epidermis, LE: Lower epidermis, CO: Collenchyma, PF: Pericyclic fibre, XY: Xylem, PH: Phloem](image)

![Fig. 4.3: Powder microscopy of C. ternatea leaf a: Paracytic stomata; b and c: unicellular covering trichomes; d: Prismatic calcium oxalate crystals](image)
4.3.3 Morphology of *T. purpurea*

*T. purpurea* is abundantly branched semi-erect herbaceous perennial plant. Leaves are imparipinnate about 6.5 cm to 12.5 cm long containing 13 to 21 leaflets with short petiole. Leaflet is green in colour with rounded apex and narrow base. Upper surface of leaf is glabrous while lower surface is obscurely silky (Fig. 4.4).

4.3.4 Microscopy of *T. purpurea*

Transverse section of the leaflet shows isobilateral nature of the leaf. The lamina portion consists upper epidermis, mesophyll tissue and lower epidermis. Upper epidermis of lamina consists of a single layer of cells which is tangentially elongated, covered with cuticle. Upper palisade has 3 to 5 layers of palisade parenchyma while the lower palisade has 2 to 3 layers. Spongy parenchyma lies in between the upper and lower palisade and composed of loosely arranged parenchyma cells with vascular elements and prisms of calcium oxalate crystals. The lower epidermis shows the presence of bicellular, uniseriate covering trichomes. The lower epidermal portion of midrib is more convex than the upper epidermis. The midrib portion shows the presence of large, collateral type of vascular bundle surrounded by spongy parenchyma cells. The vascular bundle is arc shaped showing the presence of well-developed spiral xylem vessels (Fig 4.5).

Powder microscopy shows the presence of bi-cellular uniseriate covering trichomes, fragments of leaf epidermis with paracytic stomata, xylem vessel and prisms of calcium oxalate crystals (Fig. 4.6).
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Fig. 4.5: TS of leaf of *T. purpurea* UP: Upper palisade; LP: Lower palisade; XY: Xylem; UE: Upper epidermis; LE: Lower epidermis; SC: Sclerenchyma

Fig. 4.6: Powder microscopy of *T. purpurea* leaf a) Uniseriate covering trichomes; b) Anisocytic stomata; c) Xylem vessel; d) Prismatic calcium oxalate crystals
4.3.5 Morphology of M. coromandelianum

*M. coromandelianum* is a strong-stemmed, woody-rooted herb, grows upto 1 m in height. Leaves are ovate or ovate-elliptic, 4.5 cm long, 3.5 cm wide, with sharp or blunt apex, prominent midrib, margins serrated, 3-nerved from base. Leaf stalks are 1.5-4 cm long (Fig. 4.7).

4.3.6 Microscopy of M. coromandelianum

The histology of the leaf can be best studied as lamina and midrib region (Fig. 4.8a). The transverse section of leaf lamina was dorsiventral with single layered lower and upper epidermis, compactly arranged and cuticulized (Fig. 4.8b). The epidermis showed two types of modifications i.e. trichomes and stomata. The two types of trichomes that are unicellular, uniseriate, lignified covering trichomes which are more on lower epidermis than upper one; (Fig. 4.8c) while bi-cellular head, sessile, non-lignified glandular trichomes were found on both epidermis (Fig. 4.8d). The three celled unequal anisocytic type stomata were well distributed in lamina region (Fig. 4.8e). The spongy parenchyma of mesophyll shows the absence of ergastic cell content. The midrib region shows the similar type of epidermis which is devoid of stomata. Dorsal surface of midrib shows concave shape. Below and above the upper and lower epidermis the thick cellulosic cell walled compactly arranged two to three layered collenchyma was present. Vascular bundle was present at the center of the midrib by spongy parenchyma. The vascular bundle was arc shaped, bi-collateral type; the phloem are surrounds the xylem. The phloem shows the presence of sieve tube and companion tubes; phloem fibers are absent. The xylem shows well developed spiral xylem vessels (Fig. 4.8f). Powder microscopy of
the leaf showed the presence of unicellular, lignified covering trichome with smooth cuticle (Fig. 4.9a and b), spiral xylem vessels slightly lignified (Fig. 4.9c) and anisocytic stomata (Fig. 4.9d).

Fig. 4.8: TS of leaf of *M. coromandelianum* Ct: Cuticle, T1: Covering trichome, T2: Bi-cellular sessile glandular trichome, St: Anisocytic stomata, Xy: Xylem, Ph: Phloem, Sp: Spongy parenchyma, Co: Collenchyma.

Fig. 4.9: Powder microscopy of *M. coromandelianum* leaf T: Unicellular, lignified, covering trichome, XyV: Xylem vessel, St: Anisocytic stomata
4.3.7 Physicochemical standardization

4.3.7.1 Foreign matter

Herbal drugs should be devoid of insects, molds, animal excreta and other contaminants like soil, stone, dust and other extraneous matter like glass pieces, metal parts etc. Parts of the plant other than those which are intended to be used are also considered as foreign matter and should be determined and it should not cross the limits set by WHO (Mukherjee, 2002). Foreign matter in selected plant material was separated by observing first with naked eye and then with magnifying lens. It was then weighed and percentage content was determined. Since all the plant materials were collected personally, utmost care was taken to avoid the entry of foreign material and hence the content of foreign matter was very negligible. Results are shown in Table 4.1.

| Table 4.1: Content of foreign matter in C. ternatea, T. purpurea and M. coromandelianum (% w/w) |
|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| C. ternatea | T. purpurea | M. coromandelianum |
| 0.23 ± 0.01 | 0.31 ± 0.03 | 0.19 ± 0.005 |

Values are expressed as Mean ± SEM of three determinations

4.3.7.2 Ash values

Ash value determination is a very important tool to access the quality of herbal raw material since higher ash value is an indication of adulteration and or improper processing of raw material. The percentage variation of the weight of ash in certain drugs from sample to sample is very small and any marked difference indicates a change in quality. Total ash is designed to determine the amount of material that remains after incineration. Total ash can be classified as physiological which is derived from plant itself and non-physiological ash which derives from extraneous matter like sand and soil. Acid insoluble ash is the residue obtained after boiling the total ash with hydrochloric acid and incinerating the washed insoluble matter left on the filter paper. This determination
determines the presence of silica, especially from sand and soil. Water soluble ash is the difference in weight between the total ash and the residue obtained after boiling the total ash with water (Mukherjee, 2002). Results for the percentages of total ash, acid-insoluble ash and water-soluble ash are shown in Table 4.2. Results are expressed as mean ± SEM.

| Table 4.2: Ash values for C. ternatea, T. purpurea, M. coromandelianum (% w/w) |
|---------------------------------|-----------------|-----------------|-----------------|
| Parameter                      | C. ternatea     | T. purpurea     | M. coromandelianum |
| Total ash                      | 9.74 ± 0.29     | 7.15 ± 0.01     | 15.27 ± 0.10     |
| Acid insoluble ash             | 0.94 ± 0.07     | 0.93 ± 0.04     | 0.59 ± 0.03      |
| Water soluble ash              | 4.34 ± 0.20     | 2.58 ± 0.04     | 5.62 ± 0.05      |

Values are expressed as Mean ± SEM of three determinations

4.3.7.3 Extractive values

Extractive value determinations tell us the amount of phytoconstituents in a given amount of medicinal plant material when extracted with a particular solvent. Under a given set of conditions these values varies within a narrow limit and hence can be set as an in-house standard for routinely used drugs. These values can also tell us about the adulteration of crude drug with already exhausted drug as it will yield low extractive values. The results of alcohol soluble and water soluble extractive values are shown in Table 4.3. All the three plants have showed higher water soluble extractive value which may be due the presence of carbohydrates, proteins and amino acids.

| Table 4.3: Extractive values for C. ternatea, T. purpurea, M. coromandelianum (% w/w) |
|---------------------------------|----------------|----------------|----------------|
| Parameter                      | C. ternatea    | T. purpurea    | M. coromandelianum |
| Alcohol soluble                 | 6.36           | 11.89          | 6.25           |
| Water soluble                   | 15.35          | 19.1           | 16.58          |
4.3.7.4 Moisture content

Moisture is an unavoidable component of the crude drugs, and it must be reduced as much as possible. Drying of the crude drug will help in their preservation, it will stop enzymatic or hydrolytic reactions which might alter the nature of the phytoconstituents of the drugs; will help in subsequent grinding and will reduce the weight and bulk of the drug. To decide the upper limit for the moisture content is impracticable as it will vary from drug to drug. Moisture content in the selected crude drug was determined by loss on drying which is nothing but loss of mass expressed as percent w/w (British Herbal Pharmacopoeia, 1990) and the results are shown in Table D. *C. ternatea* and *T. purpurea* showed the % LOD of 8.84 and 8.65 respectively while *M. coromandelianum* showed the % LOD of 4.29. Results are shown in Table 4.4 and values are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th><strong>Table 4.4:</strong> % Loss on drying (LOD) for leaves of <em>C. ternatea</em>, <em>T. purpurea</em> and <em>M. coromandelianum</em> (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. ternatea</strong></td>
</tr>
<tr>
<td>8.84 ± 0.07</td>
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</tbody>
</table>

Values are expressed as Mean ± SEM of three determinations

4.3.7.5 Fluorescence analysis of the powdered crude drugs

The powdered crude drugs were observed under day light, short wavelength (254 nm) and long wavelength (366 nm) after treatment with various reagents as per the procedure given by Chase et al (1949) and Kokoski et al (1958). Changes in colour were observed and recorded. This technique of observing plant material under fluorescence light has been used as a pharmacognostic tool to distinguish between plants and their species. The results are tabulated in Table 4.5.
**Standardization of Selected Medicinal Plants**

4.3.8 Phytochemical evaluation

Plants are being used for their therapeutic potential from ancient times by the simple process without the isolation of single entity i.e. in the form of crude drugs or the galenicals prepared from them. Pharmacological action of any crude drug is solely governed by the nature of its phytoconstituents. Plants are known to contain various primary metabolites like carbohydrates, proteins and fats that are consumed as food by animals and humans. They also contain various secondary metabolites like alkaloids, terpenoids, flavonoids, glycosides, tannins etc. which shows certain physiological effects.

4.3.8.1 Preparation of extracts

Successive extracts of the selected medicinal plants were prepared by extracting successively with petroleum ether (60-80 °), chloroform, acetone, methanol and water. The percentage yield, colour and consistency of respective extract are shown in Table 4.6. All the three plants have showed higher % yield in methanol and aqueous showing the presence of more amount of polar compounds followed by less polar (chloroform) and lastly non-polar (petroleum ether). Acetone which is a ketone class having the same polarity as of methanol showed less percentage yield in all the selected plants.
### Table 4.5: Fluorescence analysis of leaf powder of *C. ternatea, T. purpurea* and *M. coromandelianum*

<table>
<thead>
<tr>
<th>Drug + Reagent</th>
<th><em>C. ternatea</em></th>
<th></th>
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<th></th>
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<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>254 nm</td>
<td>366 nm</td>
<td>Visible</td>
<td>254 nm</td>
<td>366 nm</td>
<td>Visible</td>
<td>254 nm</td>
<td>366 nm</td>
<td>Visible</td>
<td>254 nm</td>
<td>366 nm</td>
<td>Visible</td>
<td>254 nm</td>
<td>366 nm</td>
</tr>
<tr>
<td>Hydrochloric acid (1N)</td>
<td>Dark brown</td>
<td>Light brown</td>
<td>Green</td>
<td>Dark brown</td>
<td>Dark violet</td>
<td>Light green</td>
<td>Dark brown</td>
<td>Dark black</td>
<td>Green</td>
<td>Blackish brown</td>
<td>Green</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfuric acid (1N)</td>
<td>Black</td>
<td>Dark brown</td>
<td>Green</td>
<td>Dark brown</td>
<td>Dark green</td>
<td>Black</td>
<td>Dark brown</td>
<td>Dark green</td>
<td>Yellowish green</td>
<td>Blackish brown</td>
<td>Green</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HNO₃ (1N)</td>
<td>Black</td>
<td>Dark brown</td>
<td>Green</td>
<td>Dark brown</td>
<td>Dark green</td>
<td>Black</td>
<td>Dark brown</td>
<td>Dark green</td>
<td>Yellowish green</td>
<td>Blackish brown</td>
<td>Green</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Picric acid</td>
<td>Dark yellow</td>
<td>Light green</td>
<td>Yellowish green</td>
<td>Dark yellow</td>
<td>Dark brown</td>
<td>Yellowish green</td>
<td>Dark brown</td>
<td>Blackish green</td>
<td>Dark brown</td>
<td>Yellowish brown</td>
<td>Dark green</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>Dark brown</td>
<td>Dark black</td>
<td>Dark green</td>
<td>Dark green</td>
<td>Dark violet</td>
<td>Green</td>
<td>Dark brown</td>
<td>Dark green</td>
<td>Blackish green</td>
<td>Dark brown</td>
<td>Dark green</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iodine</td>
<td>Dark brown</td>
<td>Light green</td>
<td>Green</td>
<td>Dark green</td>
<td>Violet</td>
<td>Green</td>
<td>Dark green</td>
<td>Dark green</td>
<td>Blackish green</td>
<td>Dark brown</td>
<td>Dark green</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% Ferric chloride</td>
<td>Dark black</td>
<td>Dark brown</td>
<td>Brownish green</td>
<td>Dark green</td>
<td>Yellowish green</td>
<td>Dark brown</td>
<td>Dark green</td>
<td>Dark brown</td>
<td>Brownish green</td>
<td>Dark brown</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note: Visible column indicates the color observed under UV light.*
Table 4.6: Colour, consistency and % yield for *C. ternatea* (CT), *T. purpurea* (TP) and *M. coromandelianum* (MC)

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Colour</th>
<th>Consistency</th>
<th>Yield (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CT</td>
<td>TP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT</td>
<td>TP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT</td>
<td>TP</td>
</tr>
<tr>
<td>Pet. Ether</td>
<td>Green</td>
<td>Green</td>
<td>Waxy semisolid</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Dark green</td>
<td>Green</td>
<td>Semi-solid</td>
</tr>
<tr>
<td>Acetone</td>
<td>Green</td>
<td>Green</td>
<td>Semi-solid</td>
</tr>
<tr>
<td>Methanol</td>
<td>Dark brown</td>
<td>Brown</td>
<td>Brown</td>
</tr>
<tr>
<td>Aqueous</td>
<td>Brown</td>
<td>Brown</td>
<td>Dry powder</td>
</tr>
</tbody>
</table>
4.3.8.2 Preliminary phytochemical screening

All the extracts of selected medicinal plant were screened for the presence of different phytoconstituents and results are depicted in Table 4.7.

Table 4.7: Phytochemical screening of various extracts of *C. ternatea* (CT), *T. purpurea* (TP) and *M. coromandelianum* (MC)

<table>
<thead>
<tr>
<th>Test</th>
<th>Pet. Ether</th>
<th>Chloroform</th>
<th>Acetone</th>
<th>Methanol</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CT</td>
<td>TP</td>
<td>MC</td>
<td>CT</td>
<td>TP</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anthroquinone Glycosides</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fixed oils and fats</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenolic and tannins</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proteins and amino acids</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gums</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

+ Indicates present, - Indicates absent

4.3.8.3 Estimation of total phenolic content

Polyphenols, isolated from higher plants are well known for their antioxidant properties. More than 4000 phenolic and polyphenolic compounds have been identified like phenolic acids, tannins, coumarins, anthraquinones and flavonoids (Trease and Evans 2002). Phenolic compounds show antioxidant activity because of their redox properties, which allow them to act as reducing agents; they also act as hydrogen donors and quenches singlet oxygen (Tawaha et al., 2007). Total phenolic content was estimated by using gallic
acid as standard (Fig. 4.10) as per the procedure of Adesegun et al., (2009) and results are shown in Table 4.8. Results are expressed as mean ± SEM.

![Figure 4.10: Standard plot of gallic acid for total phenolic content]

**Table 4.8: Total phenolic content in different extracts of *C. ternatea*, *T. purpurea* and *M. coromandelianum* (mg/g GAE)**

<table>
<thead>
<tr>
<th>Extract</th>
<th><em>C. ternatea</em></th>
<th><em>T. purpurea</em></th>
<th><em>M. coromandelianum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pet. ether</td>
<td>20.66 ± 0.82</td>
<td>69.43 ± 2.46</td>
<td>85 ± 0.41</td>
</tr>
<tr>
<td>Chloroform</td>
<td>46.07 ± 2.05</td>
<td>107.95 ± 6.15</td>
<td>107.54 ± 4.10</td>
</tr>
<tr>
<td>Acetone</td>
<td>98.11 ± 6.97</td>
<td>440.74 ± 17.62</td>
<td>93.61 ± 3.28</td>
</tr>
<tr>
<td>Methanol</td>
<td>97.30 ± 1.23</td>
<td>235 ± 11.07</td>
<td>145.25 ± 2.46</td>
</tr>
<tr>
<td>Aqueous</td>
<td>70.25 ± 6.15</td>
<td>97.71 ± 2.46</td>
<td>16.15 ± 2.05</td>
</tr>
</tbody>
</table>

**4.3.8.4 Estimation of total flavonoid content**

Over the past decade flavonoids have received much consideration because of many prospective beneficial effects have been reported. As many diets are rich in polyphenolic compounds, flavonoids gained much importance as therapeutic class compared to others (Agrawal, 2011). Chang et al (2002) advocated the use of estimation of content of flavonoids as a quality control tool for herbal raw materials. Total flavonoid content was
estimated as per the procedure of Chang et al., (2002) by using quercetin as standard (Fig 4.11) and results are shown in Table 4.9. Results are expressed as mean ± SEM.

![Total Flavonoid](image)

**Fig. 4.11: Standard plot of quercetin for total flavonoid content**

<table>
<thead>
<tr>
<th>Extract</th>
<th><em>C. ternatea</em></th>
<th><em>T. purpurea</em></th>
<th><em>M. coromandelianum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pet. ether</td>
<td>47.33 ± 1.11</td>
<td>63.44 ± 0.56</td>
<td>36.78 ± 0.56</td>
</tr>
<tr>
<td>Chloroform</td>
<td>22.89 ± 1.11</td>
<td>34 ± 1.11</td>
<td>95.67 ± 0.56</td>
</tr>
<tr>
<td>Acetone</td>
<td>43.44 ± 1.67</td>
<td>135.67 ± 0.56</td>
<td>29 ± 1.67</td>
</tr>
<tr>
<td>Methanol</td>
<td>36.78 ± 0.56</td>
<td>19 ± 0.56</td>
<td>41.78 ± 5.56</td>
</tr>
<tr>
<td>Aqueous</td>
<td>12.33 ± 0.56</td>
<td>21.78 ± 2.22</td>
<td>6.22 ± 1.11</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM of three determinations

### 4.3.8.5 Estimation of tannins

Tannins are “water-soluble phenolic compounds having molecular weights between 500 and 3000; besides giving the usual phenolic reactions, they possess special properties such as the ability to precipitate alkaloids, gelatin and other proteins” (Bate-Smith EC, Swein T, 1962). Tannins acts as primary antioxidants in which they donate hydrogen atom or electrons; they also function as secondary antioxidants in which they chelate
metal ions such as Fe(II) and interfere with one of the reaction steps in the Fenton reaction and thereby retard oxidation (Karamac M, 2009, Amarowicz R, 2007). Tannins were estimated as per the method of Killedar and More (2010) by using gallic acid as standard (Fig. 4.12). Results are expressed as mean ± SEM and data are shown in Table 4.10.

![Tannin content graph](image)

**Fig. 4.12: Standard plot of gallic acid for tannin content estimation**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Tannin content (%w/w)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non hydrolysable</td>
<td>Hydrolysable</td>
<td></td>
</tr>
<tr>
<td><strong>C. ternatea</strong></td>
<td>0.404 ± 0.001</td>
<td>0.310 ± 0.001</td>
<td></td>
</tr>
<tr>
<td><strong>T. purpurea</strong></td>
<td>0.589 ± 0.004</td>
<td>0.662 ± 0.001</td>
<td></td>
</tr>
<tr>
<td><strong>M. coromandelianum</strong></td>
<td>0.575 ± 0.001</td>
<td>0.499 ± 0.001</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM of three determinations.
4.3.9 Marker based standardization of selected medicinal plant extracts by high performance thin layer chromatography

With the emergence of new terminologies like nutraceuticals, food supplements, botanicals, dietary supplements, multifunctional foods, etc., the market of natural substances has intensely changed. There is a need of adequate analytical tool which will ensure the quality and purity of these herbal products. These analytical tools should be such that they can withstand with the complexity of natural product mixture (Nicoletti M, 2011). Marker based standardization by using high performance thin layer chromatography (HPTLC) or planar chromatography as it is commonly known as; is such an analytical tool which allows us to analyze substance under test along with a marker which is a biochemical constituent from either primary or secondary metabolite. Because of simplicity, HPTLC has become a very important tool to check quality, purity, stability and identity of the complex botanical entities. In the present study extracts of the selected medicinal plants were standardized by using this HPTLC technique.

4.3.9.1 HPTLC Estimation of \( \beta \)-sitosterol in *C. ternatea*, *T. purpurea* and *M. coromandelianum*

\( \beta \)-sitosterol is one of the phytosterol which is reported for its analgesic, anthelminthic and antimutagenic (Villasenor et al., 2002), anti-inflammatory (Prieto et al., 2006) and antihepatotoxic activities (Gawade et al., 2012). \( \beta \)-sitosterol was estimated in all the three plants (Fig. 4.13) and the results are shown in Table 4.11.

4.3.9.2 HPTLC estimation of kaempferol in *C. ternatea*

Kaempferol is a flavonoid an important phytoconstituent present in *C. ternatea*. It is also found in apples, strawberries, beans, tea and broccoli (Somerset and Johannot, 2008, Chen and Chen, 2013). Wojdylo et al. (2007) showed that plants containing kaempferol showed higher antioxidant activity and the role of antioxidants and hepatoprotection is
well established. Therefore the content of kaempferol (Fig. 4.14) was estimated in the methanolic extract of *C. ternatea*. Results are shown in Table 4.12.

![3D chromatogram of β-sitosterol](image)

**Fig. 4.13: Chromatogram of β-sitosterol and pet ether extracts of *C. ternatea*, *T. purpurea* and *M. coromandelianum***
Table 4.11: Estimation of β-sitosterol in C. ternatea, T. purpurea and M. coromandelianum

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Extract</th>
<th>Rf</th>
<th>AUC</th>
<th>Content (%w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Standard</td>
<td>0.26</td>
<td>1068.1</td>
<td>98</td>
</tr>
<tr>
<td>2</td>
<td>Pet. Ether C. ternatea</td>
<td>0.26</td>
<td>830.5</td>
<td>7.61</td>
</tr>
<tr>
<td>3</td>
<td>Pet. Ether T. purpurea</td>
<td>0.26</td>
<td>424.9</td>
<td>3.89</td>
</tr>
<tr>
<td>4</td>
<td>Pet. Ether M. Coromandelianum</td>
<td>0.26</td>
<td>445</td>
<td>4.08</td>
</tr>
</tbody>
</table>

3D chromatogram of Kaempferol

Chromatogram of standard kaempferol

Chromatogram of C. ternatea methanolic extract

Fig. 4.14: Chromatogram of kaempferol and methanolic extracts of C. ternatea
Standardization of Selected Medicinal Plants

4.3.9.3 HPTLC estimation of lupeol in *T. purpurea*

Lupeol is a pentacyclic triterpene found in various flowering plants. Prasad et al (2008) reported the protective effect of lupeol against androgen induced oxidative stress in Swiss albino mice while the anti-inflammatory activity of lupeol has been reported by Geetha and Varalakshmi (2001). It is well known fact that drug induced hepatotoxicity is characterized by higher oxidative stress and inflammatory responses. *T. purpurea* is reported to contain lupeol and hence it was quantified by HPTLC (Fig 4.15). Results are shown in Table 4.13.

Table 4.12: Estimation of Kaempferol in *C. ternatea* in methanol extract

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Extract</th>
<th><em>R</em>&lt;sub&gt;f&lt;/sub&gt;</th>
<th>AUC</th>
<th>Content (%w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Standard</td>
<td>0.39</td>
<td>18863.6</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>Methanol <em>C. ternatea</em></td>
<td>0.37</td>
<td>2453</td>
<td>1.23</td>
</tr>
</tbody>
</table>

---
Table 4.13: Estimation of Lupeol in *T. purpurea* petroleum ether extract

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Extract</th>
<th>R&lt;sub&gt;f&lt;/sub&gt;</th>
<th>AUC</th>
<th>Content (%w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Standard</td>
<td>0.33</td>
<td>5748.8</td>
<td>98</td>
</tr>
<tr>
<td>2</td>
<td>Pet. Ether <em>T. purpurea</em></td>
<td>0.33</td>
<td>1474.5</td>
<td>2.51</td>
</tr>
</tbody>
</table>
4.3.9.4 HPTLC estimation of quercetin in *T. purpurea*

Quercetin is a flavonoid and is reported for its antioxidant activity (Nacaia et al., 1999) and hepatoprotective activity (Janbaz et al., 2004 and Lekic et al., 2013). Content of quercetin (Fig. 4.16) was estimated in the chloroform extract of *T. purpurea* and results are shown in Table 4.14.

![3D chromatogram of Quercetin](image)

**Fig. 4.16: Chromatogram of quercetin and chloroform extract of *T. purpurea***
Table 4.14: Estimation of Quercetin in \textit{T. purpurea} chloroform extract

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Extract</th>
<th>(R_f)</th>
<th>AUC</th>
<th>Content (%w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Standard</td>
<td>0.43</td>
<td>17508.9</td>
<td>98</td>
</tr>
<tr>
<td>2</td>
<td>Chloroform \textit{T. purpurea}</td>
<td>0.43</td>
<td>1030.2</td>
<td>0.57</td>
</tr>
</tbody>
</table>

4.3.9.5 HPTLC estimation of rutin in \textit{T. purpurea}

Rutin is a flavonoid glycoside present in many plants including \textit{T. Purpurea}. It is well known for its antioxidant activity (Iacopini et al., 2008), antihyperglycemic (Kamalakkannan et al., 2006), antiprotozoal, antitumor, anti-inflammatory and hepatoprotective activity (Janbaz et al., 2002). The results are shown in Fig. 4.17 and Table 4.15.
Table 4.15: Estimation of Rutin in *T. purpurea* acetone and methanol extract

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Extract</th>
<th>( R_f )</th>
<th>AUC</th>
<th>Content (%/w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Standard</td>
<td>0.42</td>
<td>11706.1</td>
<td>98</td>
</tr>
<tr>
<td>2</td>
<td>Acetone <em>T. purpurea</em></td>
<td>0.42</td>
<td>10460.5</td>
<td>8.75</td>
</tr>
<tr>
<td>3</td>
<td>Methanol <em>T. purpurea</em></td>
<td>0.42</td>
<td>7733.2</td>
<td>6.47</td>
</tr>
</tbody>
</table>
4.4 Conclusion

Standardization now a day has become an integral part of herbal medicine. Since the popularity and use of herbal medicine is increasing day by day especially in western countries, more stress is given on the standardization of the raw material as quality and efficacy of the finished product is much more dependent on the quality of raw material. Dried leaf of *C. ternatea*, *T. purpurea* and *M. coromandelianum* were standardized in terms of their morphology, microscopy and powder characteristic followed physicochemical standardization as per the WHO guidelines. Further, different extracts of the selected medicinal plant were prepared and evaluated for the detection various secondary metabolites. Total phenolic and flavonoid content was determined as per the established methods since these compounds are well known for their wide spectrum of therapeutic activity. HPTLC is a technique which gives clearer picture about the quality of the extracts. Different extracts of the selected medicinal plants were standardized by using different markers like β-sitosterol, kaempferol, lupeol, quercetin and rutin. The results obtained can be considered as standard for further research studies in these plants.
4.5 References


