Chapter IV

STANDARDIZATION OF EXTRACTS OF EMBELIA RIBES
INTRODUCTION

Herbal drugs constitute a major part in all the tradition systems of medicine. Herbal medicine is a triumph of popular therapeutic diversity. Plants have been used for medicine from time immemorial because they have fitted the immediate personal need and are easily accessible and inexpensive.

With the passage of time, there has been more demand of herbal medicines due to a general disillusionment with conventional medicine. The desire for a "natural" lifestyle has resulted in an increasing utilization of alternative or complementary therapies with the natural products in general and herbal medicines in particular.

The demand is high but there is a shortage of supply of genuine herbal medicines. Herbal products represent a number of unique problems when quality aspects are considered. These are because of the nature of the herbal ingredients present therein, which are complex mixtures of different secondary metabolites that can vary considerably depending on environment and genetic factors.

In almost all the traditional systems of medicine, the quality control aspects have been considered from its inception itself by the Rishis and later by the Vaidyas and Hakims. However, in modern concept, it requires necessary changes in their approach.

For the quality control of traditional medicines, the traditional methods are procured, studied, documented and then the traditional information about identification and quality assessment is interpreted properly in terms of modern assessment.

Quality assurance is an integral part of traditional medicine, which ensures that it delivers the required quantity of quality medicament.

The fingerprinting and marker compound analyses are nowadays getting momentum for the standardization of traditional medicinal formulations. Here, the concentration of the secondary metabolites, which are the major constituents of herbal drugs, is studied, which provides valued scientific standardization procedures. This technique not only helps in establishing the correct botanical identity but also helps in regulating the chemical sanctity of the herbs.
For standardization and quality assurance, three attributes viz. authenticity, purity and assay are desirable.

Authenticity relates to proving that the material is true i.e., it corresponds to the right identity. Authentication itself involves many parameters including gross morphology, microscopy, chemical analysis etc.

Purity pertains to evaluating that there are no adulterants present in the plant material. Purity depends upon the absence of foreign matter, whether organic or inorganic, while quality refers essentially to the concentration of the active constituents or components, the product is used and it's economic and commercial value is estimated. Based on the concentration and nature of the constituents though, crude drug may conform to all the official standards of purity and be of good quality (Mukherjee, 2002).

QUALITY CONTROL AND QUALITY ASSURANCE OF HERBAL DRUGS
The quality control of plant products is a general requirement to be fulfilled. Good quality assurance is necessary when dealing with the plant products, intended to be released in market as drug constituents or as test substances in basic pharmacological experiments. Therefore, efforts should be made to obtain and maintain the high quality of these plant products.

Quality refers to the intrinsic value of the drug i.e. the amount of medicinal principles or active constituents present. These constituents are classified into groups of non-protoplasmic cell contents. These groups include carbohydrates, glycosides, neutral principles, acids alkaloids, volatile oils, lipids, oleoresins, balsam, steroids, amino acids, hormones etc.

Solubility profile, starting from petroleum ether to water, shows the wide range of non-polar and that it may be a good source of active ingredients for pharmacological evaluation. Ash values, on the other hand, are one of the best physicochemical parameters for the evaluation of plant drug for its purity, quality and strength. To check any adulteration or non-deliberate mixing in the commercial batches specifications must be laid down for each herb. To check any adulteration or non-deliberate mixing in the commercial batches specification must be laid down for each herb.
AIMS AND OBJECTIVES

The present study was designed with the following aims and objectives:

(a) To prepare aqueous and ethanolic extracts of *Embelia ribes* Burm fruits.

(b) To standardize aqueous and ethanolic extracts of *Embelia ribes* Burm fruits.
EXPERIMENTAL DESIGN

1. Preparation of Extracts of *Embelia ribes* Burm fruits

The dried fruits of *Embelia ribes* Burm were purchased from the local market, New Delhi, India in October, 2005 and the botanical authentication was carried out by Dr. M. P. Sharma, Department of Botany, Faculty of Science, Hamdard University, New Delhi, India (voucher specimen no. UB 2).

The dried and coarsely powdered drug (100 g) was packed in a Soxhlet apparatus and was subjected to extraction with water and ethanol separately for 72 h. The filtrate was evaporated under vacuum drier and brown mass residue obtained was stored at 4°C for further use. The average yield of aqueous and ethanolic *Embelia ribes* extracts was approximately 5.26 and 7.9% respectively. For experimental study, the weighed amount of aqueous and ethanolic *Embelia ribes* extracts (100 and 200 mg/kg) were suspended in 1% Tween 80 in distilled water and administered to adult male Wistar albino rats by oral route.

2. Standardization of *Embelia ribes* Extracts (IP, 1996; WHO, 1998; Mukherjee, 2002)

The following parameters were taken into consideration:

1. Extractive value
   Hot Extraction
2. Ash values
   a) Total ash
   b) Acid insoluble ash
   c) Water soluble ash
3. Determination of pH
4. Foaming Index
5. Preliminary Phytochemical Screening
6. Microbial Contamination
7. TLC Profile
8. HPTLC Profile
METHODS

1. EXTRACTIVE VALUE

The amount of an extract that a drug yields in a particular solvent is often an approximate measure of the amount of certain constituents that the drug contains. The drug should be extracted with different solvents in order of their increasing polarity to get the correct and dependable values. Generally petroleum ether, alcohol and water extractives are taken into consideration for fixing the standard of a drug. The petroleum ether extract contains fixed oil, resins and volatile substances, but when the extract is heated at 105°C until constant weight, the volatile substances are volatilized leaving only resin, coloring matter and fixed oil. Alcohol can dissolve almost all the substances, but is generally used for determining the extractive index for those drugs which contain glycosides, resins, alkaloids etc. Water is used for the drugs containing water-soluble substances as chief constituents. The extractive values were determined according to the method described in Pharmacopoeia (Indian Pharmacopoeia 1996, Appendix 3.37).

HOT EXTRACTION

The dried and coarsely powdered drug (10 g) was packed in a Soxhlet apparatus and was subjected to extraction with different solvents like ethanol, petroleum ether, chloroform, acetone, methanol and water over 72 hours. The total volume of extract was readjusted with the same solvent to 100 ml. The extract was divided into 4 parts each of 25 ml. Then each part of 25 ml of extract was transferred to a tared bottom dish and was evaporated to dryness on a water bath, then weighed without delay and their constant extractive values with different solvents were calculated.

\[
\text{Weight of extractable matter} = \frac{\text{Weight of Drug}}{X 100}
\]

2. ASH VALUES

Ash value is an important parameter for the purpose of determination of inorganic materials, such as carbonates, silicates, oxalates and phosphates. Heating causes the loss of organic material in the form of \( \text{CO}_2 \) leaving behind the inorganic components. Ash value is an important characteristic of a drug and with the help of this parameter we can detect the extent of adulteration as well as establish the quality and purity of
the drug. There is a considerable difference in the ash values of different drugs but mostly the difference varies within narrow limits in case of the same drug. The ash remaining following ignition of medicinal plant materials is determined by three different methods which measure total ash, acid insoluble ash and water soluble ash. The acid insoluble ash consists mainly of silica and high acid insoluble ash thereby indicating the contamination with earthly material. The water soluble ash is used to estimate the amount the amount of inorganic elements.

\[
\text{Weight of Ash} = \frac{\text{Weight of Ash}}{\text{Weight of extract}} \times 100
\]

**a) Total ash**

The total ash is designed to measure the total amount of material remaining after ignition. Indian Pharmacopoeia 1996 and WHO prescribes suitable methods for determination of ash values.

The extract (1 g) was placed in the tared platinum or silica crucible and was incinerated at a temperature not exceeding 450°C until free from carbon. It is then cooled and weighed to get the total ash content.

**b) Acid insoluble ash**

Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid (HCl) and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth.

Ash was boiled with 25 ml of dilute HCl (6N) for five minutes. The insoluble matter collected on an ash less filter paper, washed with hot water and ignited at a temperature not exceeding 450°C to a constant weight.

**c) Water soluble ash**

Water soluble ash is the difference in weight between the total ash and the residue after treatment of total ash with water. It is a good indicator of either previous extraction of water soluble salts in the extracts.

Ash was dissolved in distilled water and the insoluble part collected on an ash less filter paper and ignited at 450°C to constant weight. By subtracting the weight of insoluble part from that of the ash, the weight of soluble part of ash is obtained.
3. DETERMINATION OF pH

pH 1% solution:
An accurately weighed 1 g of the drug was dissolved in accurately measured 100 ml of distilled water, filtered and checked pH of the filtrate with a standardized glass electrode.

pH 10% solution:
An accurately weighed 1 g of the drug was dissolved in accurately measured 100 ml of distilled water, filtered and checked pH of the filtrate with a standardized glass electrode.

4. FOAMING INDEX

Many medicinal plant materials contain saponins that can cause persistent foam when an aqueous decoction is shaken. The foaming ability of an aqueous decoction of plant material and their extract is measured in terms of foaming index.

About 1gm of extract weighed accurately and transferred to 500ml conical flask containing 100ml of boiling water. Maintained at moderate boiling for 30 minutes. Cooled and filtered into 100ml volumetric flask. The decoction was poured into 10ml stopped test tube (height 16cm, diameter 16mm) in successive portions of 1ml, 2ml, 3ml etc. upto 10ml and adjusted the volume of liquid in each tube with water to 10ml. Stoppered the tubes and was shaken them in a lengthwise motion for 15 sec, two shakes per second. Allowed to stand for 15 minutes and the height of foam was measured.

The results assessed as follows:

- If the height of foam in every tube is less than 10mm the foaming index is less than 100.
- If the height of foam in any tube is 1cm, the volume of plant material decoction in the tube is used to determine the index. If this tube is the first or second tube in a series, prepare an intermediate dilution in a similar manner to obtain a more precise result.
- If the height of the foam is more than 1cm in every tube, the foaming index is over 1000. In this case repeat the determination using a new series of decoction of detection in order to obtain a result.
Calculate the foaming index using the following formula:

$$\frac{1000}{a}$$

Where \( a \) is the volume in ml of decoction used for preparing the dilution in the tube where foaming to a height of 1cm is observed.

5. PRELIMINARY PHYTOCHEMICAL SCREENING

The preliminary phytochemical screening was carried out using plant extracts for their content of different classes of compounds. The extract obtained then subjected to qualitative chemical tests for identification of various plant constituents present in the crude drug. The extracts should be subjected to preliminary phytochemical investigation for detection of following (Evans, 2002; Ali, 1998):

1. Alkaloids
2. Carbohydrates
3. Cardiac Glycosides
4. Phenolic Compounds
5. Flavonoids
6. Protein
7. Saponins
8. Sterols
9. Resins
10. Lipids/Fats

6. MICROBIAL DETERMINATION

Medicinal plant materials generally carry a great number of bacteria and moulds, often originating in the soil, while a large range of bacteria and fungi form the naturally occurring microflora of herbs, aerobic spore forming bacteria frequently predominate. Current practice of harvesting, handling and production may cause additional contamination and microbial growth. The determination of *Escherichia coli* and moulds can indicate the quality of production and harvesting practices. There are some microbes seen in the plant materials that are pathogenic to the human beings, e.g. *Escherichia coli*, *Salmonella*, *Pseudomonas*, *Staphylococcus aureus* and other type of yeast and moulds. Determination of microbial counts explained as per WHO
guidelines.

One gram of extract was taken and suspended in 50 ml of sterile distilled water. The suspension was shaken for sufficient period of time so as to allow maximum mixing. After this the suspension was filtered by using a disposable sterilized filter paper. The filtrate was used as stock solution. Series dilution (1:1, 1:100, 1:10000) of this stock solution were made and 1 ml of different diluted solution was separately inoculated (with spreading method) on a nutrient agar medium and incubated at 37°C for 24 hours. After 24 hours, the Petri plates with most clearly visible colonies were taken and number of colonies determined by using colony counter.

The microbial load per gram of sample was then calculated by using dilution factor.

**Composition of nutrient agar medium**

- a. Agar 15.0%
- b. Peptic Digest of Animal Tissue 5.0%
- c. Sodium Chloride 5.0%
- d. Beef Extract 1.5%
- e. Yeast Extract 1.5%
- f. pH 7.4+/- 0.2 at 25°C
- g. Distilled Water 1000 ml

The medium was autoclaved at 15 lbs per square inch pressure at 121°C.

7. **THIN LAYER CHROMATOGRAPHY (TLC) PROFILE**

Two extracts, namely aqueous and ethanolic extracts obtained from the crude drugs were subjected to thin layer chromatography to find out the number of compounds present in them. The details of the procedure followed were as follows:

**Preparation of the plates**

The adsorbent used for thin layer chromatography was silica gel G. About 25 g of silica gel G was taken in a glass mortar and about 35 ml of distilled water was added to it. This mixture was stirred with glass rod until it became homogeneous. This mixture was then allowed to swell for about 15 minutes. Then an additional 15 ml of distilled water was added to it with stirring. The suspension was then transferred to a 150 ml flask fitted with a plastic stopper, and was shaken vigorously for about 2 minutes. This suspension was then spread immediately on thin layer chromatographic...
plates with the help of a thin layer chromatography (TLC) applicator (SUPERFIT), of Continental Instruments, Bombay was used.

Drying and storage of the plates
The freshly coated plates were then air dried until the transparence of the layer had disappeared. The plates were then stacked in a drying rack and were heated in an oven for 30 min. at 121°C. The activated plates were kept in a desiccator, till required for further use.

Application of the sample
For applying test samples on TLC plate, glass capillaries were used. The spots were applied with the help of a transparent template, keeping a minimum distance of 1 cm between the two adjacent spots. The spots of the samples were marked on the top of the plate to know their identity.

Chromatographic chamber, conditions of saturation and the development of TLC plates
Chromatographic rectangular glass chamber (16.5 cm x 29.5 cm) was used in the experiments. To avoid insufficient chamber saturation and the undesirable edge effect, a smooth sheet of filter paper approximately of 15 x 40 cm size was placed in the chromatographic chamber in a ‘U’ shape and was allowed to be soaked in the developing solvent. After being thus moistened, the paper was then pressed against the walls of the chamber, so that it adhered to the walls. The chamber was allowed to saturate for 2 hours before use.

Developing Solvent System
A number of developing solvent systems were tried, for each extract.

Spraying Equipment
Compressed air sprayer with a fine nozzle was used to detect the different constituents present on TLC plates. Air compressor was attached to a glass sprayer. The sprayer was filled with about 50 ml of the detecting reagent (indicated in tables concerned) and then used. After each spray, the sprayer was washed separately with water, chromic acid, distilled water and then with acetone.
8. HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC) PROFILE

Thin layer chromatography (TLC) is a technique where the components of mixtures separate by differential migration through a planar bed of a stationary phase, the mobile phase flowing by virtue of capillary forces. The solutes are detected in-situ on the surface of the thin layer plates by visualizing reagents after the chromatography has been completed. High Performance Thin Layer Chromatography (HPTLC) is a technique of simultaneous processing of the sample under similar conditions leading to better analytical precision and accuracy. For development of HPTLC fingerprint, extracts (20 mg) were suspended in 4 ml volume of two different solvents viz., distilled water and ethanol separately. The extraction was done by soxhlet apparatus. The extracted material along with solvent was filtered through whatman No. 1 filter paper.

The TLC was done for each extract on a pre-coated silica gel 60 F254 plates (E. Merck, 0.20 mm thickness, 10 X 10 cm) using different mobile phases. Chromatograms were viewed under long wavelength UV light (520 and 365). The Rf values for different spots were recorded by HPTLC Apparatus (Reprostar Chromatography Documentation Apparatus i.e. CAMAG Switzerland).

Test solution
20 mg of aqueous extract was dissolved in 4 ml of distilled water and apply 1, 2, and 5 µl of these solutions on TLC plate as reference marker for TLC profiling.
4 mg of ethanolic extract was dissolved in 4 ml of ethyl acetate and apply 15 µl of this solution on TLC plate as reference marker for TLC profiling.

Standard solution
8 mg of reference compound Embelin was dissolved in 4 ml of ethyl acetate (2 µg/µl). From this solution the following concentrations were prepared 0.25, 0.50, 0.75, 1.0 and 1.25 µg/µl.

Solvent system
Benzene: Ethyl Acetate (6: 4) for aqueous extract
Ethyl Acetate : Methanol (9 : 1) for ethanolic extract

Procedure
Aqueous extract was applied on one pre-coated silica gel Aluminum TLC plate and ethanolic extract and standard solution on another pre-coated silica gel Aluminum...
TLC plate of uniform thickness of 0.2 mm. After 10 min drying at room temperature the plate was developed in a twin trough TLC chamber (20 X 10 cm, CAMAG) saturated with respective solvent system. Solvent was allowed to run upto 75% of the plate height i.e. 75 mm above the line of band application. Again the plate was dried at room temperature for 30 min and then scanned in TLC scanner – 3 (CAMAG) at 520 and 365 nm respectively.

Visualization
The results were visualized using winCATS software. Chromatograms were viewed under wavelength UV light (520 and 365 nm). The Rf values for different spots were recorded by HPTLC Apparatus (Reprostar Chromatography Documentation Apparatus i.e. CAMAG, Switzerland).
Chapter IV  Standardization of Embelia ribes Extracts

RESULTS

Standardization of Embelia Ribes Burm Extracts

1. EXTRACTIVE VALUES
The average yield of Embelia ribes extracts in water, ethanol, Petroleum ether, chloroform, acetone and methanol were found to be 5.261, 7.908, 4.084, 3.506, 6.196 and 3.228 respectively.

2. ASH VALUES
Total ash value, acid insoluble ash value and water soluble ash value of aqueous extract of Embelia ribes were found to be 5.250, 1.216 and 3.776 respectively.
Total ash value, acid insoluble ash value and water soluble ash value of ethanolic extract of Embelia ribes were found to be 4.356, 1.040 and 2.606 respectively.

3. DETERMINATION OF pH
pH values of 1% and 10 % solution of aqueous extract of Embelia ribes were found to be 9.17 and 9.13 respectively.
pH values of 1% and 10 % solution of ethanolic extract of Embelia ribes were found to be 5.52 and 5.15 respectively.

4. FOAMING INDEX
Foaming Index of aqueous and ethanolic extracts of Embelia ribes was found to be 200 and 555.56 respectively.

5. PHYTOCHEMICAL SCREENING
Table 4: Aqueous and Ethanol Extracts of Embelia ribes

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Constituents</th>
<th>Aqueous Extract</th>
<th>Ethanol Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>Carbohydrates</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Cardiac Glycosides</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
6. MICROBIAL DETERMINATION

Table 5A: AQUEOUS EXTRACT

<table>
<thead>
<tr>
<th>Dilution of stock</th>
<th>Number of colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>Uncountable</td>
</tr>
<tr>
<td>1:100</td>
<td>Uncountable</td>
</tr>
<tr>
<td>1:10000</td>
<td>90 Lacks per gm</td>
</tr>
</tbody>
</table>

Table 5B: ETHANOLIC EXTRACT

<table>
<thead>
<tr>
<th>Dilution of stock</th>
<th>Number of colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>Uncountable</td>
</tr>
<tr>
<td>1:100</td>
<td>Uncountable</td>
</tr>
<tr>
<td>1:10000</td>
<td>127 Billion per gm</td>
</tr>
</tbody>
</table>

7. THIN LAYER CHROMATOGRAPHY (TLC) PROFILE

Table 6A: AQUEOUS EXTRACT

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>Observational Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene : Ethyl Acetate (6 : 4)</td>
<td>520 nm</td>
</tr>
</tbody>
</table>

Table 6B: ETHANOLIC EXTRACT

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>Observational Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl Acetate : Methanol (9 : 1)</td>
<td>365 nm</td>
</tr>
</tbody>
</table>
8. HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC) fingerprint profiling and Embelin estimation in Embelia ribes fruits

Table 7A: AQUEOUS EXTRACT: (520 nm)

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>No. of Spots</th>
<th>Max R&lt;sub&gt;f&lt;/sub&gt; values of spots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene : Ethyl Acetate</td>
<td>7</td>
<td>0.32, 0.34, 0.42, 0.45, 0.52, 0.65 and 0.78</td>
</tr>
<tr>
<td>(6 : 4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7B: ETHANOLIC EXTRACT: (365 nm)

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>No. of Spots</th>
<th>Max R&lt;sub&gt;f&lt;/sub&gt; values of spots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl Acetate : Methanol</td>
<td>7</td>
<td>0.06, 0.19, 0.25, 0.41, 0.45, 0.51, 0.61</td>
</tr>
<tr>
<td>(9 : 1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HPTLC Fingerprint and Embelin Estimation in Ethanolic Embelia ribes Fruits Residue Dissolved in Ethyl Acetate

Embelin, the main phyto-constituent of Embelia ribes fruits is soluble in ethanol and insoluble in water. Though, aqueous and ethanolic extracts of Embelia ribes fruits tested positive for phenolic compounds (Table 10, Page 119), it was thought worthwhile, to screen the ethanolic extract for presence / absence of embelin, using the HPTLC separation.

Standard stock solution (2 µg/µl) of embelin was prepared in ethyl acetate. From this solution, the following concentrations were prepared: 0.25, 0.50, 0.75, 1.0 and 1.25 µg/µl.

Ethanolic extract residue of Embelia ribes Burm was dissolved in ethyl acetate to get a concentration of 1 µg/µl. 15 µl of this extract were spotted on the pre-coated silica gel GF<sub>254</sub> HPTLC plate (10 X 10 cm, 0.20 mm thickness, E. Merck, Germany) along with 1 µl of each of the standard embelin concentrations using LINOMAT - V apparatus (CAMAG, Switzerland). After 10 min of drying at room temperature, the plate was developed in a twin trough TLC chamber (20 X 10 cm, CAMAG, Switzerland) saturated with ethyl acetate : methanol (9 : 1). Solvent was allowed to run up to 75% of the plate height i.e. 75 mm above the line of band application. Again, the plate was dried at room temperature for 30 min and then scanning was
performed on TLC scanner – 3 (CAMAG, Switzerland) in the absorbance mode at 365 nm and the results were visualized using winCATS software.

The results were visualized using winCATS software. The representative chromatograms of embelin and ethanolic extract are given as figures 17 and 18.

Extract of fruits was found to contain 7 compounds corresponding to max Rf values (0.06, 0.19, 0.25, 0.41, 0.45, 0.51, 0.61) at 365 nm. Out of these compounds, band with max Rf 0.06 matched exactly with that of embelin (max Rf 0.06). A positive correlation was found between embelin concentration and area under the embelin peak. The regression equation was found to be Y=98.088X ± 100.06. The correlation coefficient (r) was found to be 0.8964. p-value was found to be 0.0394 (significant). The total content of embelin in Embelia ribes fruits was estimated to be 0.0337176%.

![Figure 17: Representative HPTLC chromatogram of Embelin in Ethyl Acetate](image1)

![Figure 18: Representative HPTLC chromatogram of ethanolic Embelia ribes Burm Fruits Residue Dissolved in Ethyl Acetate](image2)