Chapter 3 Phyto analytical processing

3.1 Introduction

Traditional and folklore medicines play an important role in health services around the globe. About three quarters of the world’s population relies on plants and their extracts for health care (Gabhe et al., 2006). The plants are distinct not only in their therapeutic properties but also in a variety of morphological characters including those of roots, stems, leaves, flower, pollen etc. The main limitation in the use of traditional remedies is the lack of standardization of raw material, manufacturing process and the final product. A biomarker on the other hand is a group of chemical compounds which are not only unique for the plant material but also correlates with biological efficacy. So the need arises to lay standards by which the right material could be selected and incorporated into the formulation. Many scientific documentations are available on crude drug extracts, but promoting these herbal drugs in international/national market is difficult due to lack of reproducible biological reports, selection of wrong plants, lack of data on time, area of collection and identity of the source material is confused, because the origin of particular drug is assigned to more than one plant and sometimes has difficult morphological and taxonomical characters. Development and validation of analytical methods play important role in the discovery, development and manufacture of pharmaceuticals.

3.2 Collection and authentication of plant materials

The plant specimens for the proposed study Eclipta alba (L), Hassk and Lippia nodiflora Linn., were collected from the paddy fields and other irrigated fields in and around Madurai District, Tamil Nadu, India during the month of October 2009. The herbarium of these plants was identified and authenticated by Dr. D. Stephen, Assistant Professor, Department of Botany, American college of Arts and Science, Madurai, Tamil Nadu and the specimen (Herbarium No. UCP / PC / H – 14 / 2009) was deposited in Department of Pharmaceutical Chemistry, Ultra College of Pharmacy, Madurai, Tamil Nadu, India.
3.3 Determination of physicochemical parameters (Muherjee PK, 2008)

Determination of loss on drying

The loss on drying is the loss of weight in percentage w/w resulting from water and volatile matter of any kind that can be driven off under specified conditions. The test was carried out on well mixed sample of the substance.

One gram of aerial parts of *Eclipta alba* and *Lippia nodiflora* were transferred into a petridish plate and the contents were distributed evenly to a depth not exceeding 10 mm. The loaded plate was heated at 105°C in hot air oven for 1 hr and then cooled in desiccators, loss in weight was recorded as moisture content. Respective moisture content percentage of the samples was calculated.

Determination of ash values

The ash content of a crude drug is generally taken to be the residue remaining after incineration. It usually represents the inorganic salt naturally occurring in the drug and adhering to it, but it may also involve the inorganic matter added for the purpose of adulteration. There is a considerable difference which varies within narrow limits in the case of some individual drug. Hence an ash determination furnishes a basis for judging the identity and cleanliness of a drug and gives information related to its adulteration with inorganic matter. Ash standards have been established for a number of official drugs. Usually these standards set a maximum limit on the total ash or on the acid insoluble ash permitted, the total ash is the residue remaining after incineration. The acid insoluble ash is a part of the total ash, which is insoluble in dilute HCl.

The ash or residue yielded by an organic chemical compound is a rule to measure the amount of inorganic matter which is present as impurity. In most cases the inorganic matter is present in small amount which are difficult to remove in the purification process and which are not objectionable if only traces are present. Ash values are helpful in determining the quality and purity of the crude drug in powdered form.
Determination of total ash value of *E. alba* and *L. nodiflora*

The total ash was determined by incinerating 2 gm of air – dried coarsely powdered drug in a tarred silica crucible which was previously ignited and cooled before weighing. The drug was incinerated by gradually increasing the heat in a muffle furnace at 450°C for 4 hrs. The ignition was repeated until constant weight was obtained. After complete incineration, it was cooled in a desiccator. Then the percentage of the total ash with reference to air – dried drug was calculated.

**Determination of acid – insoluble ash**

The ash was washed from the crucible into 100 ml beaker using 25 ml of 2 N HCl. It was then boiled for 5 minutes over a Bunsen burner and filtered through an ashless filter paper (Whatman No: 42). The residue was washed with hot water twice, ignited to ash, cooled in desiccators and weighed. The residue was weighed and the acid insoluble ash of the drug was calculated with reference to the air dried sample of crude drug.

Acid insoluble ash value is frequently necessary to evaluate the crude drugs. This ash value indicates contamination with siliceous material e.g. earth and sand. The comparison of this with the total ash value of the sample will differentiate between contaminating minerals and variations of the natural ash of the drug.

**Determination of water – soluble ash**

Water soluble ash is a good indicator of either previous extraction of the water soluble salts in the drug or incorrect preparation. The ash was washed from the crucible into 100 ml beaker using 25 ml of chloroform water and it was boiled for 5 minutes over a bunsen burner and filtered through ash less filter paper (Whatman No: 42). The residue was washed with hot water twice, ignited to ash cooled and weighed. The weight of insoluble matter was subtracted from the weight of ash. The weight of insoluble matter was subtracted from the weight of ash. The difference in weight represents the water soluble ash. The percentage of water - soluble ash was calculated with reference to air – dried drug.
Determination of extractive values of *E. alba* and *L. nodiflora*

Extractive values of crude drugs are useful for their evaluation, especially when the constituents of a drug cannot be readily estimated by any other means. It gives an idea about the nature of the chemical constituents present in a crude drug. It is useful for the estimation of chemical constituents, soluble in that particular solvent used for extraction. It is employed for which no chemical or biological assay method exists.

**Determination of alcohol soluble extractive value of *E. alba* and *L. nodiflora***

Alcohol is an ideal solvent for extraction of various chemicals like tannins, resins. Therefore this method is frequently employed to determine the approximate resin content of drug. Generally 95 per cent ethyl alcohol is used for determination of alcohol soluble extracts. Alcohol soluble extracts are one of the tools for standardization of crude drug.

5 gm of dried coarse powder of plant material was macerated with 100 ml of 90 per cent ethanol in a closed flask for 24 hrs, shaking frequently during 6 hrs and allowing to stand for 18 hrs. It was filtered immediately taking precaution against loss of alcohol and 25 ml of filtrate was evaporated to dryness in a tarred flat bottomed shallow dish and dried at 105°C and weighed. The percentage of alcohol soluble extractive was calculated with reference to air dried drug.

**Determination of water soluble extractive value of *E. alba* and *L. nodiflora***

Determination of water soluble extractive value is used for evaluating crude drugs which are not readily estimated by other means. The extracts obtained by exhausting crude drugs are indicative of approximate measure of their chemical constituents. This method is applied to drugs which contain water soluble active constituents of crude drugs such as tannins, sugars, plant acids, mucilage and glycosides. The water soluble extractive value can be used to indicate poor quality, adulteration with any unwanted material or incorrect processing of the crude drug during the process of drying and storage.

About 5 gm of powdered plant material was added to 50 ml of water at 80°C in a stoppered flask. It was shaken well and allowed to stand for 10 minutes. It was cooled to
$15^\circ$ C, 2 gm of kieselghur was added into it and filtered. 5 ml of the filtrate was transferred to a tarred evaporating basin and evaporated on a water bath and the residue was weighed. The percentage of water soluble extractive was calculated with reference to air dried drug.

### 3.3.1 Results and Discussion

Physicochemical parameters of powder as shown in the Table 3.1 were in compliance with those mentioned in Ayurvedic Pharmacopoeia of India. The percentage of loss of weight on drying, total ash, acid insoluble-ash, water-soluble ash and sulphated ash were obtained by employing standard methods of analysis. The percentage of alcohol soluble extractive value and water soluble extractive value were also determined and the results are depicted in Table 3.1.

The determination of physicochemical parameter is important in determination of adulterants and improper handling of drugs. Ash values are important quantitative standards (Rajesh et al., 2010) and criterion to analyze the identity and purity of crude drugs especially in the powder form (Patnia et al., 2005). Moreover the total ash of a crude drug also reflects the care taken in drug preservation and the purity of crude and the prepared drug (Purohit et al., 2005). Acid insoluble ash is a part of total ash and measures the amount of silica present, especially as sand and siliceous earth. Water soluble ash is the water soluble portion of the total ash. The total ash content of *E. alba* is 15.91 % and *L. nodiflora* is 13.97 %. Water soluble ash for *E. alba* and *L. nodiflora* are 16.42 % and 8.25 % respectively. More water soluble ash value appears in *E. alba* denoting that this plant powder ash is more soluble to water compared to the other ashes, whereas in *L. nodiflora* water soluble ash value is less than the acid insoluble ash value. Percent weight loss on drying or moisture content was found to be 8.73 % for *E. alba* and 10.78 % for *L. nodiflora* (Figure 3.1).

Extractive values obtained from *E. alba* and *L. nodiflora* using water and alcohol were recorded in Table 3.1. It is useful for the evaluation of a crude drug as it gives an idea about the nature of chemical constituents present in it and is useful for estimation of chemical constituents, soluble in that particular solvent used for extraction (Joseph and George, 2011). The water soluble extractive value was indicating the presence of sugar,
acids and inorganic compounds and alcohol soluble extractive values indicated the presence of polar constituents like phenols, alkaloids, steroids, glycosides, flavonoids and secondary metabolites present in the *E. alba* plant sample. *E. alba* water extractive value of 17.56 % showed that water permeates the cells of the aerial parts and thus, a better extractive compared to alcohol with extractive value of 10.27 %. Whereas in *L. nodiflora* water soluble extractive value is less (9.42 %) compared to alcohol soluble extractive value of 18.63 % which shows water that much not permeates the cells of the aerial parts of *L. nodiflora* compared to *E. alba*. The result of percentage extractive yield for *L. nodiflora* indicates that crude powder was highly soluble in alcohol than water.

### Table 3.1. Physicochemical constants of *Eclipta alba* and *Lippia nodiflora*

<table>
<thead>
<tr>
<th>S. No</th>
<th>Physicochemical constants</th>
<th><em>E. alba</em></th>
<th><em>L. nodiflora</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Percentage of loss on drying</td>
<td>8.73 % ± 0.32 w/w</td>
<td>10.78 % ± 0.34 w/w</td>
</tr>
<tr>
<td>2.</td>
<td>Percentage of ash content</td>
<td>15.91 % ± 0.21 w/w</td>
<td>13.97 % ± 0.22 w/w</td>
</tr>
<tr>
<td>3.</td>
<td>Percentage of acid insoluble ash</td>
<td>8.66 % ± 0.77 w/w</td>
<td>9.72 % ± 0.37 w/w</td>
</tr>
<tr>
<td>4.</td>
<td>Percentage of water soluble ash</td>
<td>7.42 % ± 0.08 w/w</td>
<td>8.25 % ± 0.04 w/w</td>
</tr>
<tr>
<td>5.</td>
<td>Sulphated ash</td>
<td>2.62 % ± 0.65 w/w</td>
<td>1.27 % ± 0.15 w/w</td>
</tr>
<tr>
<td>6.</td>
<td>Percentage of alcohol soluble extractive value</td>
<td>10.27 % ± 0.71 w/v</td>
<td>18.63 % ± 0.28 w/v</td>
</tr>
<tr>
<td>7.</td>
<td>Percentage of water soluble extractive value</td>
<td>17.56 % ± 0.92 w/v</td>
<td>9.42 % ± 0.76 w/v</td>
</tr>
</tbody>
</table>
Fig. 3.1. Physicochemical constants of *Eclipta alba* and *Lippia nodiflora*

3.4 Determination of mineral content

Glass stoppered shallow bottle was weighed that had been dried in the same conditions to be employed in the determination. 1.0 gm of the sample powder was transferred to the bottle. The loaded bottle was placed in a drying chamber. The sample was dried at a temperature 105°C to a constant weight. The drying chamber was opened and bottle was allowed to cool. The bottle and contents were weighed. The process was repeated until the successive weights differed not more than 0.5 mg.

The glasswares were sterilized in an oven at 150-160°C for 2 hrs. Plant material was kept at 150°C to a constant weight for the preparation of sample for mineral analysis. Dried plant material ground to fine powder and used for dry ashing. Precleaned silica crucible was heated at 600°C until the weight of the crucible became constant. Five gram plant material was taken in the silica crucible and heated in a muffle furnace at 400°C till there was no evolution of smoke. The crucible was cooled at room temperature in a dessicators and ash was moistened with concentrated H₂SO₄ (0.5 ml). Crucible was
placed on hot plate and heated until fumes of H$_2$SO$_4$ ceased to evolve. The crucible with sulphated ash was then heated in a muffle furnace at 600$^0$C till the weight of the content become constant (Indrayan et al., 2005). pH value was determined as per method of Sharma and Kaur (1998) and chloride sulphate and nitrate was determined as per (Trivedi and Goel, 1986). Among the five inorganic elements in ash, Na and K were determined by using flame photometric method. Ca and Mg were estimated by titration method and phosphorus was determined by UV spectrophotometer.

The inorganic elements in powder sample were determined for eight elements such as Na, K, Ca, Pb, Cd, Hg, As and Fe. Na and K were determined by using flame photometer and Ca, Pb, Cd, Hg, As and Fe were determined by Atomic Absorption Spectrometry.

0.25 gm of each of the powdered plant samples digested in 6.5 ml of acid solution (HNO$_3$, H$_2$SO$_4$, HClO$_4$ in ratio of 5: 1: 0.5). The corresponding solution was heated until white fumes had appeared. The clear solution was diluted upto 50 ml with distilled water and filtered with whatman filter paper No.1. The standard working solutions of elements of interest were prepared to make the standard calibration curve. Absorption for a sample solution uses the calibration curves to determine the concentration of particular element in that sample. A varian AA240FS Atomic Absorption Spectrometer (AAS) was used for the determination of six metals that is Ca, Pb, Cd, Hg, As and Fe, cathode lamp used as radiation source. Air acetylene gas was used for all the experiments. This method provides both sensitivity and selectivity since other elements in the sample will not generally absorb the chosen wave length and thus, will not interfere with the measurement.

**Results and Discussion**

The selected two plants, *Eclipta alba* and *Lippia nodiflora* were subjected to studies on determination of mineral content. The analysis of inorganic elements has been detected in two plants by AAS and by flame photo meter and results were tabulated in Table 3.2. Sodium and potassium concentration were found to be higher in *Eclipta alba* than *Lippia nodiflora*. Elemental composition of *E. alba* plant powder results showed that Na, K, Ca, Si, Cd, Mg, Mn, Fe, Zn and Cu viz., 6.98 %, 13.42 %, 9.87 %, 10.51 %,
1.84 %, 7.14 %, 0.79 %, 1.89 %, 8.82 % and 17.46 % respectively. Elemental composition of *L. nodiflora* plant powder results showed that Na, K, Ca, Si, Cd, Mg, Mn, Fe, Zn and Cu viz., 4.24 %, 11.23 %, 16.77 %, 12.11 %, 0.91 %, 9.08 %, 0.16 %, 7.21 %, 5.17 % and 2.65 % respectively.

**Table 3.2. Elemental composition of plant powder of *E. alba* and *L. nodiflora***

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Elements</th>
<th><em>E. alba</em></th>
<th><em>L. nodiflora</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sodium (Na)</td>
<td>6.98 %</td>
<td>4.24 %</td>
</tr>
<tr>
<td>2.</td>
<td>Potassium (K)</td>
<td>13.42 %</td>
<td>11.23 %</td>
</tr>
<tr>
<td>3.</td>
<td>Calcium (Ca)</td>
<td>9.87 %</td>
<td>16.77 %</td>
</tr>
<tr>
<td>4.</td>
<td>Silicon (Si)</td>
<td>10.51 %</td>
<td>12.11 %</td>
</tr>
<tr>
<td>5.</td>
<td>Cadmium (Cd)</td>
<td>1.84 %</td>
<td>0.91 %</td>
</tr>
<tr>
<td>6.</td>
<td>Magnesium (Mg)</td>
<td>7.14 %</td>
<td>9.08 %</td>
</tr>
<tr>
<td>7.</td>
<td>Manganese (Mn)</td>
<td>0.79 %</td>
<td>0.16 %</td>
</tr>
<tr>
<td>8.</td>
<td>Iron (Fe)</td>
<td>1.89 %</td>
<td>7.21 %</td>
</tr>
<tr>
<td>9.</td>
<td>Zinc (Zn)</td>
<td>8.82 %</td>
<td>5.17 %</td>
</tr>
<tr>
<td>10.</td>
<td>Copper (Cu)</td>
<td>17.46 %</td>
<td>2.65 %</td>
</tr>
</tbody>
</table>

In terms of healthy hair growth, the most important vitamins and minerals are iron, zinc, copper, selenium, biotin, pyridoxine, vitamin B$_{12}$ (Joy *et al.*, 2001). Protein, phosphorus, iodine, zinc and vitamins A, B, C and E as well as dietary excesses of selenium, iodine, copper and cobalt are essential for hair growth (Scott, 1988). Zinc is an essential element to many metallo enzymes and metabolic processes, including keratogenesis. Copper deficiency results in fibre depigmentation and loss of hair tensile strength and elasticity leading to breakage. Protein deficiency arising through starvation, low protein diet, or chronic catabolic disease results in hair production of abnormal texture and decreased length and diameter (Mark Dunnett, 2010). It was observed from the mineral analyses that the extract produced an increase in mineral concentrations which are essential for hair growth.
Fig. 3. 2. Elemental composition of plant powder of *E. alba* and *L. nodiflora*

### 3.5 Fluorescence analysis

Many phyto drugs when suitably illuminated emit light of different wavelength or colour from that which falls on them. The fluorescence analysis of drug extract helps to identify the drug with specific fluorescent colours and to find out the fluorescent impurities. The study of fluorescence analysis can be used as a diagnostic tool for testing adulteration.

The plant powder of *E. alba* and *L. nodiflora* and the extracts of the powder in various solvents were examined under ordinary visible light and UV light (365 nm). The plant powder was also treated with various chemical reagents and the changes in colour were recorded (Thomas *et al.*, 2008) and reported in Table 3.3 and 3.4.
Table 3.3. Fluorescence analysis of *E. alba* plant powder with various reagents

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Treatments</th>
<th>Visible Light</th>
<th>UV Light (365nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Powdered drug alone</td>
<td>Dark green</td>
<td>Light brown</td>
</tr>
<tr>
<td>2</td>
<td>Powder + H$_2$O</td>
<td>Dark yellowish green</td>
<td>Light brown</td>
</tr>
<tr>
<td>3</td>
<td>Powder + 1 N HCl</td>
<td>Dull green</td>
<td>Brown</td>
</tr>
<tr>
<td>4</td>
<td>Powder + 10 % NaOH</td>
<td>Dark Yellow</td>
<td>Dark Greenish brown</td>
</tr>
<tr>
<td>5</td>
<td>Powder + NH$_3$</td>
<td>Yellow</td>
<td>Green</td>
</tr>
<tr>
<td>6</td>
<td>Powder + HNO$_3$</td>
<td>Dark yellow</td>
<td>Dark greenish yellow</td>
</tr>
<tr>
<td>7</td>
<td>Powder + 10 % KOH</td>
<td>Light yellow</td>
<td>Light green</td>
</tr>
<tr>
<td>8</td>
<td>Powder + acetone</td>
<td>Green</td>
<td>Dark brown</td>
</tr>
<tr>
<td>9</td>
<td>Powder + ethyl acetate</td>
<td>Dark green</td>
<td>Dark brown</td>
</tr>
<tr>
<td>10</td>
<td>Powder + methanol</td>
<td>Green</td>
<td>Brown</td>
</tr>
</tbody>
</table>
Table 3.4. Fluorescence analysis of *L. nodiflora* plant powder with various reagents

<table>
<thead>
<tr>
<th>S.No</th>
<th>Treatments</th>
<th>Visible Light</th>
<th>UV Light (365nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Powdered drug alone</td>
<td>Light green</td>
<td>Brown</td>
</tr>
<tr>
<td>2</td>
<td>Powder + H₂O</td>
<td>Light yellow</td>
<td>Dark brown</td>
</tr>
<tr>
<td>3</td>
<td>Powder + 1 N HCl</td>
<td>Light yellow</td>
<td>Light green</td>
</tr>
<tr>
<td>4</td>
<td>Powder + 10 % NaOH</td>
<td>Very dark yellow</td>
<td>Dark brown</td>
</tr>
<tr>
<td>5</td>
<td>Powder + NH₃</td>
<td>Light yellow</td>
<td>Dark green</td>
</tr>
<tr>
<td>6</td>
<td>Powder + HNO₃</td>
<td>Dark orange</td>
<td>Dark brown</td>
</tr>
<tr>
<td>7</td>
<td>Powder + 10 % KOH</td>
<td>Light yellow</td>
<td>Light green</td>
</tr>
<tr>
<td>8</td>
<td>Powder + acetone</td>
<td>Yellowish green</td>
<td>Dark brown</td>
</tr>
<tr>
<td>9</td>
<td>Powder + ethyl acetate</td>
<td>Dark green</td>
<td>Dark brown</td>
</tr>
<tr>
<td>10</td>
<td>Powder + methanol</td>
<td>Dark green</td>
<td>Dark brown</td>
</tr>
</tbody>
</table>

3.6 Phytochemical studies

3.6.1 Preparation of *E. alba* and *L. nodiflora* extracts

The fresh whole plant of *E. alba* and *L. nodiflora* was washed with distilled water to remove unwanted foreign materials like soil and dusts. After, washed plant material was dried under shade at room temperature without direct exposure of sunrays. It was
then coarsely grounded by using mechanical device. The powdered plant material was passed through sieve no 40 and stored in an airtight container for further use.

The coarsely powdered plant materials of *E. alba* (2000 gm) and *L. nodiflora* (2000 gm) were extracted separately to exhaustion in a soxhlet apparatus for 72 hours by using petroleum ether (60 - 80° C), chloroform and ethanol (95 %) solvent (Merk and Spectrum Chemicals, India) systems. All the extracts were filtered through a cotton plug followed by Whatman filter paper (No.1) and then concentrated by using a rotary evaporator at low temperature (40 - 50° C) and reduced pressure. The extracts were preserved in airtight containers and kept at 4° C until further use.

![Preparation of Extracts](image)

*Fig. 3.3. Flow chart for preparation of extracts from *E. alba* and *L. nodiflora*"
Results

The extracts obtained by successive extraction of dried powder of *Eclipta alba* using petroleum ether, chloroform, ethyl acetate and ethanol respectively were 7.2, 4.9, 10.3 and 25.5 % on the basis of air dried crude drug. The extracts obtained by successive extraction of dried powder of *Lippia nodiflora* using petroleum ether, chloroform, ethyl acetate and ethanol respectively were 8.1, 5.4, 16.0 and 35.2 % on the basis of air dried crude drug.

3.6.2 Qualitative phytochemical investigation of *E. alba* and *L. nodiflora*

Materials and methods

Plants used in traditional medicine contain a wide range of bioactive compounds that can be used to treat infectious diseases and cosmeceutics (Anpin raja *et al.*, 2010; Tirupathi Rao *et al.*, 2011; Jeeva *et al.*, 2006). The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds (Balakumar *et al.*, 2011).

Purification of solvents (Beckett *et al.*, 1986; Furniss *et al.*, 1998)

Commercially available grades of organic solvents are of adequate purity for use in many reactions provided that the presence of small quantities of water is not harmful to the course of the reaction, and also that the presence of other impurities is unlikely to cause undesirable side reactions. The commercially available grades for general use are often accompanied by specifications indicating the amount and nature of any impurities present. When the levels of impurities, including moisture are unacceptable for particular reactions and when large volume of such solvents are likely to be required, it is frequently more economic to purify the commercial grade than to purchase the more expensive AR grades. Solvents of the appropriate grade of purity should also be used in isolation and purification processes, particularly in the later stages immediately prior to spectroscopic and / or elemental analysis. These purified solvent have been used if specific grade are not mentioned otherwise. Purification procedure for various solvents was given below.
Hexane

The hexane was distilled and used for extraction and chromatographic purposes.

Benzene

The analytical reagent grade benzene is satisfactory for most purposes. It was distilled and the fraction boiling at 80 – 81°C was collected and stored.

Toluene

The toluene was dried by treatment with anhydrous calcium chloride and filtered, distilled and the fraction boiling at 112 – 113°C was collected and stored.

Chloroform

The chloroform was shaken well with equal volume of distilled water twice to remove water soluble impurities and separated using a separating funnel. It was dried over anhydrous calcium chloride for 24 hrs, filtered and dried again over anhydrous potassium carbonate for 24 hrs. This was decanted, distilled and the fraction boiling at 64°C was collected and stored in a dark brown bottle. Absolute alcohol of 1 ml was added as a preservative.

Ethyl acetate

Ethyl acetate was refluxed for 4 hrs and distilled. The distillate was shaken with sufficient amount of anhydrous potassium carbonate, filtered and redistilled. The fraction boiling at 77°C was collected and used.

Methanol

Methanol was lime distilled and used for extraction and chromatographic purposes.

Ethanol

Ethanol was lime distilled and used for extraction and chromatographic purposes.
3.6.3 Phytochemical screening

The powdered drugs of *Eclipta alba* and *Lippia nodiflora* were subjected to preliminary phytochemical screening for the detection of various plant constituents. The extracts obtained were then subjected to qualitative tests for their identification of various plant constituents like alkaloids, carbohydrates, glycosides, proteins, amino acids, steroids *etc.* The various tests are performed and results obtained are as under. Reagents were prepared for this tests based on the research of Kokate *et al.* (2007); Evans (2002) and Handa *et al.* (2001).

Detection of carbohydrates

1 gm of extract was dissolved with mother solvent and filtered. The filtrate was used to perform the following tests for detecting the presence of carbohydrates.

a) Molisch’s test (general test)

2 - 3 ml of filtrate was treated with 2 – 3 drops of α - naphthol solution in an alcohol, shaked well and then 1 ml of conc. H$_2$SO$_4$ was added carefully along the sides of the test tube. Formation of violet ring at the junction of two liquid indicated the presence of carbohydrates.

b) Fehling’s test

1 ml of Fehling’s A and 1 ml of Fehling’s B solutions were mixed and boiled for 1 min then equal volume of extract was added. It was heated in a boiling water bath for 5 - 10 min. Formation of brick red precipitate indicated the presence of carbohydrates.

c) Benedict’s test

Equal volume of Benedict’s reagent and extract were mixed in a test tube and it was heated in a boiling water bath for 5 min. Presence of reducing sugar was indicated by red precipitate.

d) Barfoed’s test

Equal volume of Barfoed’s reagent and extract were mixed and it was heated for 1 - 2 min in a boiling water bath and then cooled. Presence of carbohydrate was indicated by red cuprous oxide.
Detection of alkaloids

The small residue of extract was added with small volume of dil HCl. It was then shaken well and filtered. The filtrate was used to perform following tests.

a) Dragondorff’s test (14 gms of KI with 5.2 gms of bismuth carbonate in 50 ml of glacial acetic acid)

Few drops of Dragondorff’s reagent were added to 2 – 3 ml of the filtrate. Presence of alkaloids was indicated by formation of orange brown precipitate.

b) Mayer’s test (1.36 gms of HgCl$_2$ in 60 ml of distilled water + 5 gms of KI in 20 ml of distilled water, make up the volume to 100 ml)

Few drops of Mayer’s reagent were added to 2 – 3 ml of the filtrate. Presence of alkaloids was indicated by formation of cream colour precipitate.

c) Hager’s test (Saturated solution of picric acid)

Few drops of Hager’s reagent were added to 2 – 3 ml of the filtrate. Presence of alkaloids was indicated by formation of yellow colour precipitate.

d) Wagner’s test (1.27 gms of I$_2$ and 2 gms of KI in 5 ml of distilled water and make up the volume to 100 ml of distilled water)

Few drops of Wagner’s reagent were added to 2 - 3 ml of the filtrate. Presence of alkaloids was indicated by formation of reddish brown precipitate.

e) Muroxide’s test

Few drops of conc. HNO$_3$ were added to 3 – 4 ml of filtrate solution and it was evaporated to dryness. Then the content was cooled and then 2 drops of NH$_4$OH solution was added. Formation of purple colour indicated the presence of alkaloids.

Detection of glycosides

Detection of cardiac glycosides

a) Baljets test

A thick section showed yellow to orange colour under the microscope or mixed with sodium picrate.
b) **Legal’s test**

Extracts were treated with sodium nitro prusside in pyridine and methanolic alkali. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

c) **Keller-Killiani test**

One drop of 5 % ferric chloride solution was added to 2 ml of ethanolic extract and 3 ml of glacial acetic acid and this solution was transferred carefully to the surface of 2 ml of conc. H₂SO₄. Formation of reddish brown colour at the junction of the two liquid layers and bluish green colour appeared in upper layer which indicated the presence of cardiac glycosides.

### Detection of anthroquinone glycosides

a) **Modified Borntrager’s test**

Extracts were treated with ferric chloride solution and boiled on water bath for about 5 min. The mixture was cooled and shaken with an equal volume of benzene. The benzene layer was separated and treated with ammonia solution. Formation of pink colour in the ammoniacal layer indicated the presence of anthranol glycosides.

### Detection of saponins

a) **Foam or frothing test**

Few grams of extracts were diluted with 20 ml of distilled water and shaken in a graduated cylinder for 15 min. Formation of 1 dm layer of foam indicated the presence of saponins.

### Detection of steroids

a) **Salkowski test**

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of conc. H₂SO₄, shaken and allowed to stand. Formation of red in the chloroform layer and acid layer showed greenish yellow fluorescence which indicated the presence of steroids.
b) **Libermann Burchard’s test**

About 1 mg of extract was dissolved in 1 ml of CHCl₃ and filtered. The filtrate was treated with few drops of acetic anhydride then boiled and cooled. Conc. H₂SO₄ was added from the side of the test tube. Formation of green colour indicated the presence of steroids.

**Detection of tannins**

a) **Gelatin test**

Extracts were treated with 1 % gelatin solution containing sodium chloride. Formation of white precipitate indicates the presence of tannins.

b) The crude extract or dry powder was treated with alcoholic FeCl₃ (Ferric Chloride) reagent and formation of blue colour indicated the presence of tannins.

**Detection of flavonoids**

a) **Shinoda’s test**

Small quantity of extract was dissolved in 5 ml of ethanol (95 %) and treated with few drops of conc. HCl and 0.5 gm magnesium turnings. Presence of flavanoids was indicated by magenta colour.

b) **Alkaline reagent test**

Extracts were treated with few drops of NaOH. Formation of intense yellow colour, which became colourless on addition of dilute acid, indicated the presence of flavanoids.

c) **Lead acetate test**

Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicated the presence of flavanoids.

**Detection of amino acids**

a) **Ninhydrin test**

To the extract, 0.25 % ninhydrin reagent was added and boiled for few min. Formation of blue colour indicated the presence of amino acids.
Detection of proteins

a) Biuret test
The extracts were treated with 1 ml of 10 % NaOH and heated. To this a drop of 0.7 % copper sulphate solution was added. Formation of purplish violet colour indicated the presence of proteins.

b) Millon’s test
2 to 3 ml of extract was mixed with 5 ml of Millon’s reagent and formation of white precipitate indicated the presence of proteins.

c) Xanthoprotein test
The extracts were treated with few drops of conc. HNO₃ solution. Formation of yellow colour indicated the presence of proteins.

Results and discussions

The extracts were subjected to preliminary phytochemical screening to find the chemical constituents present. The results of preliminary phytochemical investigation of petroleum ether and ethyl acetate fraction of ethanolic extracts of *Eclipta alba* and *Lippia nodiflora* are shown in Table 3.5. It found the presence of carbohydrates, aminoacids, steroids, saponins and proteins in petroleum ether extract of *Eclipta alba* and alkaloids, carbohydrates, flavanoids, amino acids, cardiac glycosides, steroids, saponins, proteins and tannins in ethyl acetate fraction of ethanolic extract of *Eclipta alba*. Qualitative phytochemical screening revealed the presence of alkaloids, aminoacids, steroids and proteins in petroleum ether extract of *Lippia nodiflora* and alkaloids, carbohydrates, flavanoids, amino acids, saponins, proteins and tannins in ethyl acetate fraction of ethanolic extract of *Lippia nodiflora*. Alkaloids were present in ethyl acetate fraction of ethanolic extracts of *Eclipta alba* and *Lippia nodiflora*. Amino acids, flavonoids, cardiac glycosides, steroids, saponins, proteins and tannins were present and carbohydrates and anthraquinone glycosides were absent in ethyl acetate fraction of ethanolic extracts of *E. alba*. Carbohydrates, flavonoids, saponins and tannins were present in ethyl acetate fraction of ethanolic extracts of *L. nodiflora* and absent in petroleum ether extracts of *L. nodiflora*. Cardiac glycosides and anthraquinone glycosides were present in ethyl
acetate fraction of ethanolic extracts of *E. alba* and *L. nodiflora* and absent in petroleum ether extract.

### Table 3.5. Qualitative Phytochemical screening of *E. alba* and *L. nodiflora* extracts

<table>
<thead>
<tr>
<th>S.No</th>
<th>Plant constituents</th>
<th><em>E. alba</em></th>
<th><em>L. nodiflora</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Petroleum ether extract</td>
<td>Ethyl acetate fraction of ethanolic extract</td>
</tr>
<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>Flavonoids</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4.</td>
<td>Amino acids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5.</td>
<td>Glycosides</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1. Cardiac glycosides</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2. Anthraquinone glycosides</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6.</td>
<td>Steroids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7.</td>
<td>Saponins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8.</td>
<td>Proteins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9.</td>
<td>Tannins</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = Showed colour reaction

- = Did not showed colour reaction
3.6.4 Quantitative phytochemical studies

Flavonoids are considered as one of the most varied and prevalent group of natural compounds and probably they are one of the most important natural phenolics. From literature point of view, it has been recognized that many flavonoid compounds demonstrated a wide range of activities including antioxidant properties (Mohamed et al., 2010) and their effects on human nutrition and health were also significant. Hence it was essential to calculate the total amounts of phenolics and flavonoids in specific extract.

Quantification of total phenolic content

Preparation of gallic acid standard solution (1mg/ml)

10 ml of gallic acid was taken in a standard flask and mixed with 10 ml of methanol solution (1 mg / ml). From this 20 ml of mixed solution, 2 ml was taken and then mixed with 18 ml of methanol (0.1 mg / ml). From this 5, 4, 3, 2, 1 ml of solution was taken in separate test tubes and made upto 10 ml with methanol.

Preparation of Folin Ciocalteau reagent

5 ml of Folin Ciocalteau solution was taken in a 50 ml amber colour standard flask and was mixed with 45 ml of methanol.

Preparation of 7.5 % sodium carbonate

3.25 g of sodium carbonate was taken in a 50 ml standard flask and mixed with 50 ml of distilled water.

Preparation of test sample (1 mg / ml)

10 mg of extract was taken in a standard flask and was mixed with 10 ml of methanol.

Determination of total phenolic content

1 mg / ml of extract samples (Eclipta alba and Lippia nodiflora) were prepared and then 0.1 ml of sample, 1.9 ml distilled water and 0.1 ml of Folin Ciocalteau reagent were added in a tube, and then 1 ml of 20 % Na₂CO₃ was added. The reaction mixture was incubated at room temperature in dark for 2 hrs and the absorbance of the blue colour
sample was recorded at 765 nm in UV visible spectrophotometer. The blank consist of all reagents and solvents excluding sample.

The sample was tested in triplicate and a calibration curve for gallic acid was obtained. The results were compared to gallic acid calibration curve and the total phenolic content of extracts was expressed as µg of gallic acid equivalents (GAE) per mg of dry extracts.

**Quantification of total flavonoid content**

**Preparation of quercetin standard solution**

Ten mg of quercetin was taken in a standard flask and made up to 10 ml with methanol. From this 5, 4.5, 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.5 ml of the solutions were taken in separate test tubes and made upto 10 ml volume with methanol.

**Preparation of 5 % NaNO₂ solution**

Five ml of NaNO₂ was taken in 100 ml standard flask and made upto 100 ml with distilled water.

**Preparation of 10 % AlCl₃ solution**

One gram of AlCl₃ was taken in 100 ml of standard flask and made up to 100 ml.

**Preparation of 1M NaOH**

Four grams of NaOH was taken in 100 ml standard flask and made up to 100 ml.

**Preparation of sample solution**

Ten mg of extract was taken and mixed with 10 ml of methanol.

**Determination of total flavanoid content**

One mg / ml of extracts samples (*Eclipta alba* and *Lippia nodiflora*) were prepared and then extracts or standard solution (0.1 ml) were mixed with 4 ml distilled water and 0.3 ml of 5 % NaNO₂. After 5 min, 0.3 ml of 10 % AlCl₃ was added. After 6 min, 2.0 ml of 1M NaOH was added to the mixture and total volume was made up to 10
ml with distilled water. Immediately the absorbance of the mixture was determined at 420 nm in UV - Visible spectrophotometer. The blank consist of all reagents except sample.

The sample was tested in triplicate and a calibration curve for quercetin was obtained. Total flavanoid content was expressed as µg quercetin equivalents (QE) per mg dry extract. Drug used was quercetin which was obtained from Sigma Aldrich, USA.

**Results and Discussion**

The total phenolic content and total flavanoid contents of the hexane, chloroform, ethyl acetate and ethanol extracts of *Eclipta alba* and *Lippia nodiflora* are shown in Table 3.8. The different extracts of whole plant of *E. alba* and *L. nodiflora* were evaluated for the detection of its total phenolic and total flavanoidal content. Measurement of total phenolic content by Folin-Ciocalteu assay in hexane, chloroform, ethyl acetate and ethanolic extract of *Eclipta alba* was found to be 0.0, 6.02 ± 0.18, 12.23 ± 0.05 and 6.05 ± 0.15 mE GAE/mg of the extract respectively and in hexane, chloroform, ethyl acetate and ethanolic extract of *Lippia nodiflora* was found to be 5.82 ± 0.09, 6.10 ± 0.08, 15.77 ± 0.12 and 13.71 ± 0.05 mE GAE/mg of the extract respectively [with the equation *y* = 0.025*x* + 0.0580 (*r*² = 0.9952)].

Flavanoid content in hexane, chloroform, ethyl acetate and ethanolic extract of *Eclipta alba* was found to be 2.85 ± 0.11, 4.3 ± 0.063, 5.5 ± 0.051 and 6.15 ± 0.058 mE Quercetin/mg of the extract respectively and in hexane, chloroform, ethyl acetate and ethanolic extract of *Lippia nodiflora* was found to be 3.89 ± 0.032, 4.15 ± 0.15, 7.09 ± 0.032 and 5.05 ± 0.15 mE Quercetin/mg of the extract respectively [with the equation *y* = 0.0391*x* + 0.040 (*r*² = 0.9830)].

The *E. alba* and *L. nodiflora* plant extracts were tested in triplicate and a calibration curve for gallic acid and quercetin are showed in Figure 3.4 and 3.5. The absorbance of gallic acid and quercetin are showed in Table 3.6 and 3.7. The total phenolic content of extracts was expressed as µg of equivalents (GAE) per mg of dry extracts and the total flavanoid content was expressed as µg quercetin equivalents (QE) per mg dry extract and values are shown in Table 3.8.
Table 3.6. Absorbance value of gallic acid (765 nm)

<table>
<thead>
<tr>
<th>S.No</th>
<th>Concentration (µg/ml)</th>
<th>Absorbance of gallic acid (765 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2</td>
<td>0.11</td>
</tr>
<tr>
<td>2.</td>
<td>4</td>
<td>0.26</td>
</tr>
<tr>
<td>3.</td>
<td>6</td>
<td>0.34</td>
</tr>
<tr>
<td>4.</td>
<td>8</td>
<td>0.42</td>
</tr>
<tr>
<td>5.</td>
<td>10</td>
<td>0.50</td>
</tr>
<tr>
<td>6.</td>
<td>12</td>
<td>0.59</td>
</tr>
<tr>
<td>7.</td>
<td>14</td>
<td>0.62</td>
</tr>
<tr>
<td>8.</td>
<td>16</td>
<td>0.76</td>
</tr>
<tr>
<td>9.</td>
<td>18</td>
<td>0.81</td>
</tr>
<tr>
<td>10.</td>
<td>20</td>
<td>0.95</td>
</tr>
</tbody>
</table>

\[ y = 0.025x + 0.0580 \]
\[ r^2 = 0.9952 \]

Fig. 3.4. Calibration curve of gallic acid
### Table 3.7. Absorbance value of quercetin (420 nm)

<table>
<thead>
<tr>
<th>S.No</th>
<th>Concentration (µg/ml)</th>
<th>Absorbance of quercetin (420 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1</td>
<td>0.09</td>
</tr>
<tr>
<td>2.</td>
<td>2</td>
<td>0.12</td>
</tr>
<tr>
<td>3.</td>
<td>3</td>
<td>0.15</td>
</tr>
<tr>
<td>4.</td>
<td>4</td>
<td>0.18</td>
</tr>
<tr>
<td>5.</td>
<td>5</td>
<td>0.21</td>
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<td>6.</td>
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<td>0.25</td>
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<td>7.</td>
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<td>0.29</td>
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<td>0.37</td>
</tr>
<tr>
<td>10.</td>
<td>10</td>
<td>0.41</td>
</tr>
</tbody>
</table>

\[ y = 0.0391x + 0.040 \]

\[ r^2 = 0.9830 \]

### Fig. 3.5. Calibration curve of quercetin
Ethyl acetate extracts of *E. alba* and *L. nodiflora* showed higher phenolic content and flavanoid content than the other extracts. So the ethyl acetate extract of both plant for the biological (antimicrobial activity, antidandruff activity and antioxidant activity) and pharmacological studies (dermal irritancy study and hair growth promoting activity) were selected. Further the active constituent was isolated and characterized and the antimicrobial activity and antidandruff activity of isolated compounds were studied.

### Table 3.8. Total phenolic and flavonoid content

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of the extract</th>
<th>Total phenolic content (mE GA/mg dry extract)</th>
<th>Total flavonoid content (mE quercetin/mg dry extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Eclipta alba Hassk</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Hexane</td>
<td>-</td>
<td>2.85 ± 0.11</td>
</tr>
<tr>
<td>2.</td>
<td>Chloroform</td>
<td>6.02 ± 0.18</td>
<td>4.3 ± 0.063</td>
</tr>
<tr>
<td>3.</td>
<td>Ethyl acetate</td>
<td>12.23 ± 0.05</td>
<td>5.5 ± 0.051</td>
</tr>
<tr>
<td>4.</td>
<td>Ethanol</td>
<td>6.05 ± 0.15</td>
<td>6.15 ± 0.058</td>
</tr>
<tr>
<td></td>
<td><strong>Lippia nodiflora Linn</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Hexane</td>
<td>5.82 ± 0.09</td>
<td>3.89 ± 0.032</td>
</tr>
<tr>
<td>2.</td>
<td>Chloroform</td>
<td>6.10 ± 0.08</td>
<td>4.15 ± 0.15</td>
</tr>
<tr>
<td>3.</td>
<td>Ethyl acetate</td>
<td>15.77 ± 0.12</td>
<td>7.09 ± 0.032</td>
</tr>
<tr>
<td>4.</td>
<td>Ethanol</td>
<td>13.72 ± 0.05</td>
<td>5.05 ± 0.15</td>
</tr>
</tbody>
</table>
Chapter 3

Phyto analytical processing

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