Chapter III

Preliminary Phytochemical Investigations
3. PHYTOCHEMICAL INVESTIGATIONS ON AERIAL PARTS OF 
*PHYLLANTHUS AMARUS* Schum & Thonn.

3.1 Introduction

3.1.1 Soxhlet extraction

Though various methods are available for the extraction of secondary metabolites from plants, effective extraction is influenced by the method and selection of solvents. Soxhlet extraction method is convenient and widely used for extraction because of its continuous process, less time and less solvent-consumption than maceration and percolation. The powdered plant material is placed in a Soxhlet apparatus, which is fitted on top of a collecting flask beneath a reflux condenser. A suitable solvent is added to flask and the set up is heated under reflux below the boiling point of the solvent. The steam of the solvent, which contacts with the material will dissolve metabolites and bring it back to round bottomed flask.

3.1.2 Qualitative Phytochemical Tests

In addition to the carbohydrates, proteins and lipids many other compounds like lignans, terpenoids, alkaloids, glycosides, tannins, flavonoids, essential oils and other similar secondary metabolites which exert physiological activity are synthesized in the plant. A systematic and complete study of crude drugs should include a thorough investigation of both primary and secondary metabolites derived as a result of plant metabolism. Different qualitative chemical tests are to be performed for establishing profile of a given extract for its nature of chemical composition.

3.2 Materials and Methods

3.2.1 Instruments used

1. Soxhelt apparatus (Borosil Glass Works Ltd, Mumbai)
2. Rotary evaporator (Buchi R-210, Switzerland)
3. Digital balance-AX220 (Schimazdu, Japan)
4. UV cabinet – 254 & 366 nm (Remi equipment pvt. Ltd, Mumbai)
3.2.2 Chemicals and reagents

- All chemicals, reagents and solvents used were of analytical grade obtained from Qualigens Fine Chemicals Pvt. Ltd, Merck Chemicals Pvt. Ltd., Sigma Chemical Company, U.S.A.
- Hexane, ethylacetate and methanolic extracts of Phyllanthus amarus aerial parts.
- Distilled water from Milli-Q RO system was used
- Pre-coated TLC aluminum silica gel 60 F\textsubscript{254} plates from Merck Co., Mumbai.

3.3 Collection, extraction and preliminary phytochemical screening

3.3.1 Collection of the plants

The aerial parts of Phyllanthus amarus were collected from Paderu region, Visakhapatnam District, Andhra Pradesh during the month of October, 2010. The authentication of the above plant was done by Dr. M. Venkaiah, Taxonomist, Andhra University and the voucher specimen (BG/PMK/PA-10-10) deposited in the herbarium, University College of Pharmaceutical Sciences, Andhra University.

3.3.2 Preparation of the plant extracts

Freshly collected plant material was shade dried and coarsely powdered. The dried powdered material (5 kg) was extracted in Soxhlet apparatus successively with hexane, ethyl acetate and methanol. The solvent thus obtained was concentrated under vacuum at temperature of 40ºC by using rotary evaporator (Buchi R-210, Switzerland) to obtain thick mass of the respective extract.

The extracts were collected and stored in dessicator for further phytochemical and pharmacological studies.
3.3.3 Qualitative phytochemical evaluation

In the present study, the hexane, ethyl acetate, methanol extracts of *P. amarus*, were subjected to qualitative chemical tests using standard procedures to detect various phytoconstituents present in them.

The following chemical tests were carried out on the extracts by following standard procedures reported in various books and research articles. (Hawk, 1954; Kokate, 1991; Middelton, 1956; Peach and Tracey, 1955; Rosenthaler, 1930; Shah and Quadry, 1980; Wagner *et al.*, 1984; Wallis, 1965).
3.3.3.1. Detection of Alkaloids

About 50 mg of solvent – free extract was stirred with little quantity of dilute hydrochloric acid and filtered. The filtrate was tested with various alkaloidal reagents as follows:

1) Mayer’s Test

To a few mL of filtrate, two drops of Mayer’s reagent was added along with the sides of the test tube. If the test is positive, it gives white or creamy precipitate.

2) Wagner’s Test

To a few mL of the filtrate, few drops of Wagner’s reagent were added along with the sides of the test tube. Formation of reddish brown precipitate indicates test as positive.

3) Hager’s Test

To a few mL of filtrate 1 or 2 mL of Hager’s reagent was added. A prominent yellow precipitate indicates positive test.

4) Dragendroff’s Test

To a few mL of filtrate, 1 or 2 mL of Dragendroff’s reagent was added. A prominent reddish brown precipitate indicates positive test.

3.3.3.2. Detection of Carbohydrates

About 100mg of the extract was dissolved in 5 mL of distilled water and filtered. The filtrate was subjected to the following tests.

1) Molisch’s Test

To 2 mL of filtrate, two drops of alcoholic solution of $\alpha$ – napthol was added. The mixture was shaken well and 1 mL of concentrated sulphuric acid was added slowly along the sides of the test tube, the test tube was cooled in ice water and allowed to stand. A violet ring at the junction of two liquids indicates the presence of carbohydrates.
2) **Fehling’s Test**

1 mL of filtrate was boiled on a water bath with 1 mL each of Fehling’s solution A and B. Formation of red precipitate indicates the presence of sugar.

3) **Barfoed’s Test**

To 1 mL of the filtrate, 1 mL of Barfoed’s reagent was added and heated on a boiling water bath for 2 minutes. Red precipitate indicates the presence of sugar.

4) **Benedict’s test**

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

### 3.3.3.3. Detection of Glycosides

For detection of glycosides, about 50 mg of extract was hydrolyzed with concentrated hydrochloric acid for 2 hrs on a water bath, filtered and the hydrolysate was subjected to the following test.

1) **Borntrager’s Test**

To 2 mL of filtrate hydrolysate, 3mL of ethylacetate was added and shaken, ethylacetate layer was separated and 10% ammonia solution was added to it. Formation of pink color indicates the presence of anthroquinone glycosides.

2) **Legal’s Test**

About 20 mg of the extract was dissolved in pyridine. Sodium nitroprusside solution was added and made alkaline using 10% sodium hydroxide solution. Presence of glycoside is indicated by a characteristic pink color.

### 3.3.3.4. Detection of Saponins

**Foam or Froth Test**

A small quantity of the extract was diluted with distilled water to 20 mL. The suspension was shaken in a graduated cylinder for 15 minutes. A two centimeter layer of foam or froth which is stable for 10 minutes indicates the presence of saponins.
3.3.3.5. Detection of Phytosterols and Triterpenoids

1) Libermann – Burchard’s test
The extract was dissolved in acetic anhydride, heated to boiling, cooled and then 1 mL of concentrated sulphuric acid was added along the side of the test tube. Red, pink or violet color at the junction of the liquids indicates the presence of steroids / triterpenoids and their glycosides.

2) Salkowski test
Few drops of concentrated sulphuric acid was added to the ethylacetate extract, shaken on standing, red colour in the lower layer indicates the presence of steroids and golden yellow color indicates the presence of triterpenoids.

3.3.3.6. Detection of Phenolic Compounds and Tannins
1) Ferric chloride test
About 50 mg of extract was dissolved in distilled water and to this few drops of neutral 5% ferric chloride solution was added. Formation of blue, green and violet color indicates the presence of phenolic compounds.

2) Gelatin test
A little quantity of extract was dissolved in distilled water and 2 mL of 1% solution of gelatin containing 10% sodium chloride was added to it. Development of white precipitate indicates the presence of phenolic compounds.

3) Lead acetate test
A small quantity of extract was dissolved in distilled water and to this; 3 mL of 10% lead acetate solution was added. A bulky white precipitate indicates the presence of phenolic compounds.

4) Alkaline reagents
An aqueous solution of extract was treated with 10% ammonium hydroxide solution – yellow fluorescence indicates the presence of flavonoids.
5) Shinoda test or Magnesium – Hydrochloric acid reduction

A little quantity of extract was dissolved in alcohol and few fragments of magnesium turnings and con.hydrochloric acid (drop wise) were added. If any pink or crimson – red color develops, presence of flavonol glycoside is inferred.

3.3.3.7. Test for lignans

0.5mL of aqueous solution of extract was added to 2mL of 2% (V/V) furfuraldehyde in a test tube– Red color indicates the presence of flavonoids.

3.4 Results

3.4.1 Percentage Yield of extracts

The yield and % yield of different extracts of the selected plant is given below.

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Type of extract</th>
<th>Yield (gm)</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hexane extract</td>
<td>220</td>
<td>4.4%</td>
</tr>
<tr>
<td>2</td>
<td>Ethylacetate extract</td>
<td>360</td>
<td>7.2%</td>
</tr>
<tr>
<td>3</td>
<td>Methanol extract</td>
<td>540</td>
<td>10.8%</td>
</tr>
</tbody>
</table>

3.4.2 Results:

Qualitative phytochemical screening of hexane extract revealed the presence of sterols, lignans, terpenoids, flavonoids, tannins and saponins. Ethylacetate extract of P.amarus aerial parts revealed the presence of alkaloids, carbohydrates, flavonoids, tannins, triterpenes, sterols and saponins. Methanolic extract of P.amarus aerial parts revealed the presence of carbohydrates, alkaloids, flavonoids, glycosides, tannins and saponins. The presence and absence of different phytoconstituents were summarized in the Table 3.02.
### Table 3.02. Nature of phytoconstituents present in hexane, ethylacetate and methanol extracts of *P. amarus* aerial parts

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Phytochemicals</th>
<th>Hexane extract</th>
<th>Ethyl acetate extract</th>
<th>Methanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sterols</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Lignans</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Carbohydrates</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Terpenoids</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Glycosides</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Saponins</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Tannins</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Alkaloids</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ Presence  - Absence

**3.5 Discussion**

Successive Soxhlet extraction of *P. amarus* aerial parts yielded 4.4% of hexane extract, 7.2% of ethylacetate extract and 10.8% of methanol extract. Qualitative phytochemical screening of hexane, ethylacetate and methanolic extracts of *P. amarus* aerial parts revealed the presence of sterols, lignans, terpenoids, carbohydrates, glycosides, alkaloids, tannins, flavonoids and saponins. These laboratory tests may indicate the presence of lignans like phyllanthin, hypophyllanthin, niranthin, phyltetralin, flavonoids like rutin, quercetin, tannins like gallic acid, ellagic acid, geraniin, sterols like β-sitosterol, stigmasterol, amarosterol A & B, alkaloids like securinol, epibubbialine, terpenes like lupeol, phyllantheol, oleanolic acid, ursolic acid which were reported from the Phyllanthus genus, in the literature.