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Pharmacognostical and Phytochemical studies of *Phyllanthus amarus* leaves

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Abstract

The aim of present work was to investigate the pharmacognostical and phytochemical properties of *Phyllanthus amarus* leaves. The Ethno medicine involves the use of different plant extracts or the bioactive constituents of vital importance in the health application at an affordable cost and study of such ethnomedicine keenly represents one of the best avenues in searching new economic plants for medicine. Keeping this view in mind the present investigation was carried in *Phyllanthus amarus* leaves. Morphological study and pharmacognostical properties like transverse section, palisade ratio, stomatal number, stomatal index, vein islet number, and vein termination number were studied. Physical constants like ash value, extractive value, moisture content, swelling index was also studied. Qualitative phytochemical analysis of the plants confirms the presence of various phytochemicals like carbohydrates, alkaloids, glycosides, steroids, saponins, tannins, phenols and flavanoids.

Keywords: *Phyllanthus amarus*, morphological, pharmacognostical, phytochemical

1. Introduction

The medicinal values of plants lie in the bioactive phytochemical constituents that produce definite physiological effects on the human body. Though the traditional Indian system of medicine has a long history of use, they lack adequate scientific documentation, particularly in light of modern scientific knowledge¹. These natural compounds formed the base of modern drugs as we are using today^{2,3,4}. "Phyto" is the Greek word for plant. There are many families of phytochemicals and they help the human body in a variety of ways. Phytochemicals may protect human from various diseases. Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. Phytochemicals are basically divided into two groups that are primary and secondary metabolites according to their functions in plant metabolism. Primary metabolites comprise common sugars, amino acids, proteins and chlorophyll while secondary metabolites consist of alkaloids, flavonoids, tannins, and saponins etc^{5, 6}.

World Health Organization currently encourages, recommends and promotes traditional herbal remedies as such drugs are easily available in low cost, are comparatively safe and the people have faith in such remedies⁷. It is no wonder that the world's one-fourth population i.e. 1.42 billion people, are dependent on traditional medicines for the treatment of various ailments⁸. There is a need for documentation of research work carried out on traditional medicines. With this backdrop, it becomes extremely important to make an effort towards standardization of the plant material to be used as a medicine⁹. These studies help in identification and standardization of the plant material. Correct identification and quality assurance of the starting materials is an essential prerequisite to ensure reproducible quality of herbal medicine which will contribute to its safety and efficacy.

Phyllanthus amarus Schum. & Thonn. (Euphorbiaceae) is a small weed, locally known as “Bhumyamalaki”. *P. amarus* is an important plant of Indian Ayurvedic system of medicine which is used in the problems of stomach, genitourinary system, liver, kidney and spleen. It is bitter, astringent, stomachic, diuretic, febrifuge and antiseptic. The whole plant is used in gonorrhoea, menorrhagia and other genital affections. It is useful in gastropathy, diarrhoea, dysentery, intermittent fevers, ophthalmopathy, scabies, ulcers and wounds. *P. amarus* show a wide spectrum of pharmacological activities including antiviral, antibacterial, antiplasmodial, anti-inflammatory, antimalarial, antimicrobial, anticancer, antidiabetic, hypolipidemic, antioxidant, hepatoprotective nephroprotective and diurectic properties¹⁰. The present study is designed to explore the preliminary pharmacognostical and phytochemical analysis of *Phyllanthus amarus* leaves for their pharmacological properties.

2. Materials and Methods

2.1 Plant Material

The material was purchased as aerial tender branches of *Phyllanthus amarus* from the local market of Nagpur, and authenticated by Dr. A.A. Fulzele, Dept. of Botany, S. M. Mohota College of Science, Nagpur. A sample specimen is stored for future reference in Dept. of Botany, S. M. Mohota College of Science, Nagpur. The aerial tender branches of *Phyllanthus amarus* were cleaned by removing foreign matter, dried and powdered coarsely.

2.2 Extraction

About 1000 gm of coarsely powdered *Phyllanthus amarus* leaves was taken in a 2 liter round bottom flask separately and extracted continuously with water and alcohol by soxhlet extraction. The aqueous and ethanolic extracts were then separated and filtered. The filtrate was then concentrated under reduced pressure and dried in a dessicator.

2.3. Morphology

Phyllanthus amarus is a herb that grows upto 10-60 cms tall, erect, stem terete, younger parts rough, cataphylls 1.5-1.9 mm long, deltoid acuminate; leaf 3.0-11.0 x 1.5-6.0 mm, elliptic oblong to ovate, obtuse or minutely apiculate at apex, obtuse or slightly inequilateral at base; Flowers axillary, proximal 2-3 axils with unisexual 1-3 male flowers and all succeeding axils with bisexual cymules. Male flowers -pedicel 1mm long, calyx 5, sub equal 0.7 x 0.3 mm, oblong, elliptic, apex acute, hyaline with unbranched mid rib; disc segments 5, rounded, stamens 3, filaments connate. Female flowers- pedicel 0.8-1.0 mm long, calyx lobes 5, 0.6 x 0.25 mm, ovate-oblong, acute at apex; disc flat deeply 5 lobed, lobes often toothed at apex, styles 3, free, shallowly bifid at apex. Capsule 1.8 mm in diameter, oblate and rounded, seeds about 0.9 mm long, triangular with 6-7 longitudinal ribs and many transverse striations on the back.

Fig 1. *Phyllanthus amarus* Schum. & Thonn.



2.4 Microscopical evaluation¹¹

A permanent preparation is useful for preservation of good sections for study and for preparation of standards, with which the sample can be compared. This process generally involves staining with two reagents. One of the stains imparts colour to the lignified tissues and other to the cellulose part.

In this method, safranin and fast green were used. Safranin gives red colour to the lignin and fast green gives green colour to the cellulose.

Procedure

1. To a clean watch glass containing 1% safranin solution in water, a thin uniform section was transferred for 10 min.
2. In another watch glass, containing 50% alcohol, section was kept for 5 min.
3. Then it was washed with water in another watch glass.

4. After washing with water, it was placed in the watch glass containing fast green for 2 min, and again washed with water.
5. For dehydration double stained and washed section were treated with increasing strength of alcohol for 1 min in each strength, starting with 30% alcohol, followed by 50%, 75%, 90% and 100%.
6. For permanent mounting 1mm thick glass slide and a thin cover slip was taken. The section was placed in centre and few drops of clove oil was added to remove debris. After 5 min, section was dried with blotting paper. To this section few drops of DPX was added. Slide was warmed. After it, the slide was labelled.

2.5 Qualitative studies¹¹⁻¹².

The concentrated ethanolic and aqueous extracts of *Phyllanthus amarus* and *Pachyptera hymenaea* were subjected to qualitative analytical tests for studying different constituents present in the extracts. The result of the chemical tests is shown in table 2.

Test for carbohydrates:

Molish's Tests (general test)

To 2ml of the extract solution few drops of molish's reagent and conc. sulphuric acid was added in the test tube. Violet colored ring appears at the junction of two liquid.

Test for reducing sugars

1. Fehling Test - To 2 ml of the extract solution few drops of Fehling's solution A and Fehling's solution B was added and boiled for 5 min. on boiling water bath. Yellow or red colour develops.

2. Benedict's Test - To 2 ml of the extract solution few drops of Benedict's reagent was added and the mixture was boiled on water bath for 5 min. Green colour shows the presence of reducing sugars.

Test for Proteins:

1. Biurete test (general test) - To 2 ml of the extract solution Biurete reagent was added and boiled for few mins. Violet colour develops.

2. Millons test (for Proteins) - To 2 ml of the extract solution millons reagent was added and boiled for few mins. White precipitate develops.

Test for Steroid and Triterpenoid:-

1. Salkowaski Test - To 2 ml of the extract solution 2 ml of chloroform and 2 ml of conc. H_2SO_4 was added and shake well. Chloroform layer appears red colour.

2. Liebermann-burchard test - 2 ml of the extract solution was mixed with chloroform and 1-2 ml of acetic anhydride and boiled for 2mins. Cool the content and add 2 drops of conc. H_2SO_4 . First red and then green colour appears.

3. Liebermann test - To 2 ml of the extract solution 2 ml of acetic anhydride was added and boiled for 2mins. Cool the content and add 2 drops of conc. H_2SO_4 . Blue colour develops.

Test for Glycosides:

Test for Cardiac glycosides

1. Legal Test (Test for cardenoloides) - To 2 ml of the extract solution, 1ml pyridine, 1 ml sodium nitroprusside and 1 ml ammonia was added. Pink to red colour develops.

2. Killer-killani test (test for deoxy sugars) - To 2 ml of the extract solution, glacial acetic acid 1 drop, 5% $FeCl_3$ and 2 drops of conc. H_2SO_4 . was added. Reddish brown colour appears at the junction of two liquids.

Test for Saponin glycosides

1. Foam Test - To 2 ml of the extract solution water was added and shaken well, Foam develops.

2. Hemolytic test- Drug extract was added to 1 drop of blood placed on glass slide. Hemolytic zone appears.

Test for Flavanoids

1. Shinoda Tests - To 2 ml of the extract solution, few magnesium turnings, 5 ml 95% ethanol and few drops of conc. HCl was added. Pink to red colour develops.

2. Zn-HCl Reduction test - To 2 ml of the extract solution mixture of Zn dust and conc. HCl. was added Precipitate develops.

Test for Alkaloids

Evaporate the aqueous and alcoholic extract separately. To the residue add dil. HCl. shake well and filter, perform the following tests.

1. Dragendorff Test - To 2 ml of the extract solution, Dragendorff reagent (potassium bismuth iodide solution) was added. Orange brown precipitate is formed.

2. Mayers Test - To 2 ml of the extract solution, Mayers reagent (potassium mercuric iodide solution) was added. Precipitate is observed.

3. Wagner Test - To 2 ml of the extract solution, Wagner reagent (iodine potassium iodide solution) is added. Reddish brown precipitate is observed.

4. Hager's Test - To 2 ml of the extract solution, Hager's reagent (saturated solution of picric acid) was added. Yellow precipitate is observed.

Test for Tannins and Phenolic compounds

1. To 2 ml of the extract solutions add 5% ferric chloride solution, deep blue colour is observed.

2. To 2 ml of the extract solution add few drops of 10% lead acetate solution, white precipitate is observed.

3. To 2 ml of the extract solution add few drops of gelatin solution, white precipitate is observed.

4. To 2 ml of the extract solution add few drops of potassium dichromate solution, red precipitate is observed.

5. To 2 ml of the extract solution add few drops of dil. Nitric acid solution, red to yellow colour is observed.

2.6 Florescence analysis.

The extract of *P. amarus* was examined in daylight, short UV and long UV to detect the fluorescent compounds and the observations are given in Table 3. The fluorescence colour is specific for each compound. A non fluorescent compound may fluoresce if mixed with impurities that are fluorescent. The fluorescent method is adequately sensitive and enables the precise and accurate determination of the analyte over a satisfactory concentration range without several time consuming dilution steps prior to analysis of pharmaceutical samples.

2.7 Determination of Ash value and Extractive value ¹³

Determination of Ash value

The ash of any organic material is composed of their non-volatile inorganic components. Controlled incineration of plant drugs results in an ash residue, which is composed of an inorganic mixture of metallic salts and silica. In certain drugs the percentage variation of weight of ash from sample to sample is very small and marked difference indicates a change in quality. Unwanted parts of drugs sometimes possess a character, which will raise the ash value, for eg. The scleridesin the unwanted pericarp of colocynth and the cork on liquorices, which is not required in the powder of the peeled drug. More drug contamination, such as sand or earth, is immediately detected by the ash value.

a) Total ash

Total ash involves an oxidation of the components of the products. A high ash value is indicative of contamination, substitution, adulteration or carelessness in preparing the crude drug for marketing. The total ash usually consists of carbonates, phosphates, silicates, and silica which include both physiological ash- which is derived from the plant tissue itself and non physiologic ash- which is residue of the adhering material of the plant surface, e.g. sand, soil.

Procedure

2.3 grams of powdered drug was taken in a tarred silica crucible and incinerated at temperature not exceeding 450°C until free from carbon, cooled and weighed. The percentage ash was calculated with reference to the air dried drug.

b) Acid Insoluble ash

It is the residue obtained after boiling the total ash with dil. Hydrochloric acid and igniting the remaining insoluble matter. This measures the amount of silica present especially as sand and siliceous earth.

Procedure

To the crucible containing total ash, 25 ml of dil. Hydrochloric acid was added, covered with a watch glass and boiled gently for 5 min. The insoluble matter was collected in gooch crucible, washed with hot water until the filtrate is neutral. It was dried on a hot plate and ignited to constant weight. The residue was allowed to cool in a dessicator for 30 min, and then weighed without delay.

Determination of Extractive value

This method determines the amount of active constituents in a given amount of medicinal plant material when extracted with solvents. It is employed for materials for which as yet no suitable chemical or biological assay exists.

1) Water soluble extractive value

It is applied to drugs that contain water soluble active constituents of crude drugs, e.g. Tannins, sugars, plant acids, mucilage, glycosides, etc

2) Alcohol soluble extractive value

It is employed to determine the approximate resin content of the drug. It is also used as an official method for assay in case of myrrh and asafetida.

Procedure

About 5 gm of accurately weighed powder drug material was placed in a glass stoppered conical flask. It was macerated with 100 ml of solvent for 6 hours, shaking frequently and then was allowed to stand for 18 hours. Extract was filtered rapidly taking care not to lose any solvent. 25 ml of this filtrate was transferred to a tarred flat bottom dish and evaporated to dryness on a water bath. The residue was dried at 105^o C till its weight become constant, cooled in dessicator for 30 min. and weighed without delay.

2.8 Determination of moisture content¹¹⁻¹²

A very useful form of dish for the determination of moisture is a thin flat porcelain dish. The burning of powder should proceed slowly and the material must not be allowed to catch fire or to give off smoke as dense fumes. The most common method for the determination of moisture is to heat and dry till one get constant weight at 100^oC However many substance loose other volatile content or some of their constituents undergo change with constant loss of weight at a temperature 100^oC. Hence other method is use for such type of substances.

Procedure:

Weigh accurately 10 gm of crude drug in a china dish. Dry the dish at 105^oC in the oven for 5 hr. and again weigh. Continue the drying and weighing for every 1 hr. interval. The process is continuing till the difference between the two successive weights should not be more than 0.01 gm. The loss in weight is usually recorded as moisture. The percentage of loss drying (LOD) is carried out by the following formula.

$$\% \text{ LOD} = \frac{\text{Final weight of china dish} + \text{extract} - \text{Weight of china dish}}{\text{weight of sample}} \times 100$$

2.9 Determination of swelling index¹¹⁻¹²

Swelling index reflects the mucilage content of drug especially seeds. This technique has been accepted as an official method for evaluating seeds by different pharmacopoeias.

Procedure:

Transfer 1 gm. of drug to a 25 ml of stoppered measuring cylinder. Fill up-to 20 ml mark with water. Agitate gently and occasionally during 24 hr. and allow to stand. Measure the volume occupied by swollen drug.

3. Result and Discussion

3.1 Microscopy of the Leaf:

Epidermal walls wavy, stomata anisocytic, which is distributed mainly on the lower epidermis. Upper epidermis has a thin cuticle. Stomata are followed by respiratory cavities beneath. There is a single layer of palisade cells, which occupy nearly half of the space between the two epidermis. Below the palisade there is a row of broad collecting cells, each of which occur in relation to 3 or 4 palisade cells. Reduced vascular elements are clearly seen running on long stretch beneath the collecting cells. The vascular bundle containing xylem and phloem is present in the centre.

Fig.2. Upper Epidermis & Lower Epidermis

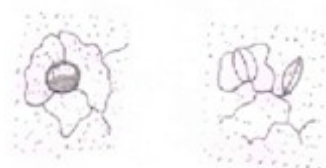


Fig.3. Palisade cells

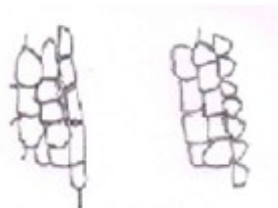


Fig.4. Transverse section of leaf

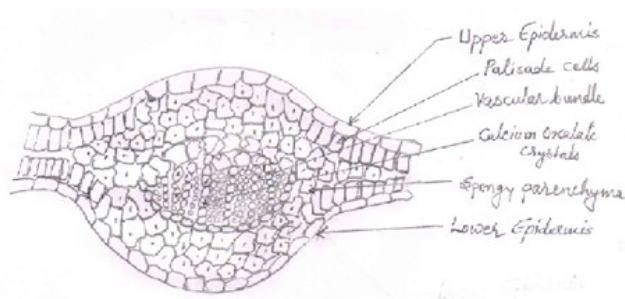


Table 1: Leaf measurements of *Phyllanthus amarus*

Sr. No	Leaf measurements		Values
1	Stomatal number	Adaxial side	236.47 ± 5.62 mm
		Abaxial side	419.04 ± 4.36 mm
2	Stomatal index	Adaxial side	29.28 ± 0.76
		Abaxial side	16.33 ± 1.53
3	Palisade ratio		14.56 ± 0.59
4	Vein islet number		18.11 ± 0.79
5	Vein termination number		44.82 ± 3.61 mm

Leaves measurement value of *Phyllanthus amarus* Schum.&Thonn. were found to be stomatal number are 236.47 ± 5.62 mm on adaxial side and 419.04 ± 4.36 mm on abaxial side; stomatal index are 29.28 ± 0.76 and 16.33 ± 1.53 on adaxial and abaxial side, respectively; palisade ratio, vein islet and vein termination number are 14.56 ± 0.59, 18.11 ± 0.79 and 44.82 ± 3.61 mm, respectively.

Table 2: Qualitative photochemical analysis

Sr. No.	Tests	<i>P. amarus</i>	
		Ethanolic extract	Aqueous extract
1	Carbohydrates	+	+
2	Alkaloids	+	+
3	Steroids and sterols	+	+
4	Glycosides	+	+
5	Proteins and amino acids	-	-
6	Tannins and Phenols	+	+
7	Saponin	+	+
8	Flavanoids	+	+
9	Gums and mucillages	-	-
10	Fixed oils	-	-
11.	Volatile oils	-	-

All the results of qualitative phytochemical analysis are shown in Table 2 and + ve sign represents the presence and -ve sign represents the absence of particular constituent in the extracts of *Phyllanthus amarus*.

The present study carried out on the plant samples revealed the presence of medicinally important bioactive compounds. The phytochemical characters of all the plants investigated are summarized in the Table: 2. The Et. and Aq. extract of *Phyllanthus amarus* reveals the presence of carbohydrates, alkaloids, glycosides, steroids, saponins, tannins and phenols, and flavanoids.

Table 3: Florescence analysis.

Sr. No	Extracts	Color of solvent layer under		
		UV 254 nm	UV 356 nm	Day light
1	<i>P. amarus</i> Et.	Brown	Light green	Yellow
2	<i>P. amarus</i> Aq.	Brown	Light green	Greenish

The Florescence analysis of *Phyllanthus amarus* ethanolic and aqueous extract exhibit different colours (Table 3). From (Table 4) the physical constants of *Phyllanthus amarus* leaf total ash and acid insoluble ash was found to be 7.5 and 4.9 %w/w respectively, and Water soluble extractive and Alcohol soluble extractive was found to be 9-12 %w/w and 13-18 %w/w respectively. The percentage loss on drying was found to be 25.6 %w/w and Swelling index was found to be 3.4 ml/kg.

Table 4: Physical constants of *Phyllanthus amarus* leaf

Sr. No	Parameters Evaluated	Result
1	Total ash	7.5 %w/w
2	Acid insoluble ash	4.9 %w/w
3	Water soluble extractive	9-12 %w/w
4	Alcohol soluble extractive	13-18 %w/w
5	Loss on drying	25.6 %w/w
6	Swelling index	3.4 ml/kg

3. Conclusion

The microscopic diagnostic characters and phytochemical studies drawn from the present investigation by using simple techniques will help to authenticate genuine sample of *Phyllanthus amarus* from its five different species found in India. This is the first such report on microscopic diagnostic characters and phytochemical studies of Bhumyamalaki.

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