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PHARMACOGNOSTIC STUDIES OF *Phyllanthus amarus*

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ABSTRACT

Phyllanthus amarus (Family- Euphorbiaceae) is a widely used important medicinal plant in various diseases. However, available literature revealed that there is a need to summarize the pharmacognostic characters in a simple form with high specification hence the present investigations performed. In the present study evaluation of various pharmacognostical parameters such as macroscopic, microscopy, physicochemical and in detail phytochemical studies of the *P. amarus* performed. Additionally anatomical sections of the leaf stem and root carried out using standard methods, for the purpose of its monograph preparation. The findings of the present study will help to summarize the important diagnostic indices that can use for identification and preparation of monograph of *P. amarus*.

Keywords: *Phyllanthus amarus*, pharmacognostic, phytochemical, monograph

INTRODUCTION

The species of genus Phyllanthus long been used in folk medicine for liver protection and other various diseases **[1, 2]**. *Phyllanthus amarus* Schumm and Thonn (PA) (Family: Euphorbiaceae) is a perennial annual herb, growing as a weed throughout India, commonly known as Jamgli amli, Jaramla, or Bhuiamla **[1, 2]**. The extracts and the compounds isolated from *P. amarus* have shown a wide spectrum of pharmacological activities including antiviral, antibacterial, antiplasmodial, antimalarial, antimicrobial, anticancer, antidiabetic, hypolipidemic, antioxidant, hepatoprotective, nephro-protective, and diurectic properties. A number of preclinical and clinical studies have confirmed the medicinal properties of

various *P. amarus* species that have been mentioned in traditional system of medicine **[1-5]**.

Pharmacognostical studies are important for quality control of herbal drugs along with validate genuineness of the crude drugs of plant origin. As the histological studies of herbal drugs are not only to study the adulterants but also are indispensable in the accurate identification. Review of literature revealed that only few of them have studied the structural details of *P. amarus*. Saha and Krishna Murthy studied the structural details **[6]**. Khatoon *et al*., studied three species of Phyllanthus including *P. amarus* **[7]**. Yelene *et al*., carried out the leaf structural studies **[8]**. Sharma and Sheela have studied distinguishing characters of various Phyllanthus species by simple microscopic techniques **[9]**. Sen *et al*., have studied it as the source of Tamalaki used in the treatment of Tamaka-svasa (Bronchial asthma) and other respiratory disorders by analyzing therapeutic uses, actions, properties, taste, synonyms as well as pharmacognostical characters **[10]**.

The present study was an attempt to distinguish pharmacognostic features of *P. amarus* on basis of its morphologic, microscopic and phytochemical characteristics. The selected standardized extracts of *P. amarus* were in-depth analyzed for their HPTLC data including finger print analysis, detection of class of compounds like Lignans, tannins and flavonoids. The study includes highlighting the pharmacognostical characters and HPTLC finger print profile of *P. amarus* as distinctive features for authentication, identification and standardization purposes. The study includes highlighting the pharmacognostical characters and HPTLC finger print profile of *P. amarus* as distinctive features for authentication, identification and standardization purposes.

MATERIAL AND METHODS

A. Collection and authentication of plant material: Whole plant of *Phyllanthus amarus* Schum and Thonn family- Euphorbiaceae collected from different parts of Karad region western Maharashtra. The collected species submitted to Dept. of Botany Yashwantrao Chavan College of Science, Karad for initial authentification [Reference- Authentification certificate-22-072011]. The Dept. of Botany asked us to re verify the species taking official help from Botanical survey of India [BSI], Pune. The plant species deposited as herbarium authenticated by BSI, Pune [Reference No. BSI/WC/Tech./2012/644,].

B. Standardized extracts of *Phyllanthus amarus*:

a. Standardized aqueous extract of *Phyllanthus amarus* whole plant

(PAAE)-The standardized extract of *P. amarus* whole plant (water extract) [Reference No: SR/KN/CL/1/2012-L12030241 dated 10/03/2012], was procured as a gift sample from Chemiloids Ltd., Vijaywada.

b. Standardized methanolic extract of *Phyllanthus amarus* leaf (PAME)- The standardized methanolic extract of *P. amarus* leaf (Methanol extract contains >2.5% of Phyllanthin and Hypophyllanthin) Report No: FP1112042-PA/11 LOT 05 was procured as a gift sample from Natural Remedies Pvt. Ltd., Bangalore.

C. Standardized hydro-methanolic extract of *Phyllanthus amarus* leaf (PAHME)- The standardized hydro methanolic extract of *P. amarus* leaf (60% Methanol Hydroalcoholic extract contains >5% of Corilagin) - Report No: FP1102034- PA/ 11LOT /02 were procured as a gift sample from Natural Remedies Pvt. Ltd., Bangalore.

C. Pharmacognostic studies [11-14]

1. Morphologic study of Phyllanthus species [11-14] Morphological study carried out as per the reported methods. Organoleptic evaluation wan done by means of sense organs, which provide the simplest as well as quickest means to establish the identity and purity to ensure quality of a particular drug. Organoleptic characters such as shape, size, colour, leaf structure like margin, apex, base surface, venation and inflorescence, etc evaluated.

2. Microscopic study of Phyllanthus species [12-14]- For the microscopic studies, the dried plant materials [root and stem] boiled for 10-15 minutes in distilled water to become soft and then the transverse section obtained. The leaves were soaked in normal water for two hours. For microscopic studies, freehand transverse sections of root, stem and leaf taken. The staining and mounting of the sections performed following usual procedures of plant micro techniques. The sections of the plant materials stained mainly by phloroglucinol and HCL and mounted with help of few drops of glycerine. The advanced Motic microscope was used for taking microphotographs of the sections under 10 x and 45 x power of microscope.

3. Physico-chemical analysis [12-14]- Physico-chemical values such as the percentage of total ash, acid-insoluble ash, water-soluble ash as well as water soluble and alcohol soluble extractives were calculated as per the standard procedures and were in accordance with procedures mentioned in Indian Pharmacopoeia.

4. Preliminary Phytochemical screening [12-14]-The extracts of *P. amarus* species

subjected to preliminary phytochemical evaluation using qualitative chemical tests for detecting the presence of the phytoconstituents such as lignans, tannins, flavanoids, alkaloids, glycosides, phenolic compounds, phytosterols, carbohydrates, proteins and amino acids.

5. HPTLC analysis [11-14]- Advanced phytochemical investigations performed on HPTLC for identification and characterization of bioactive extracts, with the help and supervision of experts from Anchrom lab Mumbai. The details of the instrumentation parameters and procedure for finger print analysis is outlined as follows-

Instrumentation and chromatographic conditions utilized for HPTLC analysis

Instrument-CAMAG Linomat 5 "Linomat5_080222" S/N 080222 (1.00.12)

- **(a)** Spotting device.—Linomat V Automatic Sample Spotter (Camag, Muttenz, Switzerland).
- **(b)** Syringe.—100 µl (Hamilton, Bonaduz, Switzerland).
- **(c)** TLC chamber.—Glass twin-trough chamber (20 x10 x 4 cm; Camag).
- **(d)** Densitometer.—TLC Scanner 3 linked to winCATS software (Camag).
- **(e)** HPTLC plates.—20 x 10 cm, 0.2 mm layer thickness, precoated with silica gel 60 F254, Cat. No. 1.05548, E. Merck KgaA, Darmstadt, Germany.

Linomat 5 application parameters- Spray gas: Inert gas; Sample solvent type: Methanol; Dosage speed: 150 nl/s; Predosage volume: 0.2 ul; Syringe size: 100 µl; Number of tracks: 4- 8; Application position: 8.0 mm; Band length: 8.0 mm; Solvent front position: 80.0 mm.

Preparation of Phyllanthus Sample Solutions- Dried powdered extracts of *P. amarus (*200 mg) re-extracted exhaustively with methanol using a sonicator for 1 h on a water bath. The methanol soluble portion filtered used for the further HPTLC analysis. The stock solution of the sample, having concentration of 0.4 mg/ml (0.4 µg/µl) was prepared.

Mobile Phases for General Finger Print Analysis- Various mobile phases were tried such as, Toluene: Ethyl acetate (80:25); toluene: ethyl acetate: formic acid (60:20:20) for development of common chromatograms. Out of the various mobile phases tried, Toluene: Chloroform: Ethanol (4:4:1, v/v) gave the best resolution for development of common chromatogram for the analysis of the components of the extracts under study from each other.

General Finger Print Analysis- HPTLC aluminum plates pre-coated with silica gel were as the stationary phase. The plates not pre-washed with any solvent prior to

chromatography. The samples spotted in the form of bands, with the help of a Camag 100 micro liter syringe using a Camag Linomat V (Switzerland) sample applicator. A constant application rate 150 nL/s employed. The slit dimension was kept at 6 mm × 0.45 mm, with a scanning speed of 20 mm/second, and a data resolution of 100 µm/step was employed.

The composition of the mobile phase was toluene: chloroform: ethanol (4:4:1). The linear ascending development carried out in twin trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 30 minutes at room temperature (25 ± 2°C). The length of the chromatogram run was 80 mm. Subsequently, the plate allowed dry at room temperature. The separated bands on the HPTLC plates scanned over the wavelength of 200 – 540 nm. The source of radiation utilized was the deuterium illumination (D2 lamp) for 254 nm, Mercury (Hg) for 366 nm and for 540 nm. The images captured on Camagreprostar 3 with win-CATS software 4.05.

RESULTS AND DISCUSSION

1. Morphologic characters- *P. amarus* is an herb that grows up to 10-75 cm high and is erect. It is commonly found as a weed in fallow lands clearings river beds and in wastelands. The dried aerial part of the plant is used as drug and consists of interwoven mass of crumpled leaves, stem and secondary branches bearing leaves fruits and flowers. The photograph of whole plant of *P. amarus* is depicted in **Figure 1**. The description is as follows:

Stem and secondary branches: Stem is cylindrical with 2 to 3 mm in diameter. Internodes are 5 to 15 mm in length, branching from the base. Each branch is 4 to 5 cm in length. Longitudinally ridged at places; fracture is short, fractured surface is hollow in the centre; main stem branches from the base bears 4 to 5 cm long deciduous branches which are about 1 mm in diameter having scarious lanceolate small stipules at the base and 8 to 12 more pairs of leaves on either side. Fruits, flowers and leaves get detached on drying and then branches appear like rachis of unipinnate compound leaf.

Leaves: They are simple, alternate, distichous, arranged very closely, obovate to oblong. They are lanceolate 4- to 9 mm in length and 3 to 4 mm in width. Leafs are shortly petiolate or subsessile, obtuse, entire, glabrous, reticulate, pinnate, lateral veins less conspicuous on the upper surface.

Flowers: They are small, unisexual axillary, solitary or in clusters. There 3 united stamens with 5 sepals.

Fruits: They are tricarpellary globose capsule, 3 to 5 mm in diameter, pedicel is short, fruiting perianth is acute. 6 seeds are present in a fruit and are trigonous.

2. Microscopic characters-The observations of the studied microscopic characters compiled in a comparative manner with respect to three important parts of the plant viz. root, stem and leaf.

a) **Root-** The TS of root shows epidermis a single layer of thin walled cells. The cortex region with 6-8 layers of parenchymatous cells without intercellular spaces. The inner cortex consists of patches of macrosclereids. The vascular cylinder consisting of 5-8 layers secondary phloem cambium and 25-40 layers of secondary xylem along with fibers (pits rare, bordered; ends tapering; wall tetra-to hexagonal), vessel members (long with tails at both ends, pits circular, bordered; perforation plate simple). The xylem parenchyma is thin-walled with uniseriate rays, 3-8 cells high, usually heterogenous type while the pith is parenchymatous. The microscopic features of root **Figure 2**.

b) **Stem-** The TS of the stem is circular in outline and shows central pith occupying the major area of the section, encircled by continuous band of xylem and a ring of discontinuous periclyclic fibers, narrow parenchymatous cortex, a layer of epidermis and collenchymatous narrow hypodermis. The detailed TS at 40 x shows a layer of epidermis, embedded with stomata, at places bearing papilla and covered with thick cuticle, a narrow band of chlorenchymatous hypodermis lies underneath this followed by 2 to 3 rows of chlorenchymatous cortex, pericycle is characterized by discontinuous ring of groups of thin walled fibers. Phloem is narrow, parenchymatous, cambium is distinct, xylem consists of radial rows of vessels tracheids, thin walled fibres, parenchyma and uniserate to biseriate medullary rays; pith is wide and parenchymatous; cells getting disintegrated on drying developing cavity in the centre, cluster and rosette crystals of calcium oxalate throughout the parenchymatous cells of the cortex and the pith. The microscopic features of stem see **Figure 3**.

c) **Leaf-** The transverse section of the leaf passing through midrib is

slightly elevated on the lower side and flat on the upper side. It shows layer of upper epidermis, its cells being bigger in size than the lower one and cover with thin cuticle. At places it is papillose and embedded with stomata, underneath the upper epidermis lies a layer of palisade in continuation with the midrib. Meristele of the midrib consists of radiate xylem and an arc of phloem; underneath the palisade layer of lamina lie 2 to 4 rows of spongy parenchyma traversed with obliquely cut vascular bundles and prismatic and rosette crystals of calcium oxalate. The microscopic features of leaf **Figure 4**. The important cellular characters of the leaf like type of stomata cell wall, margin of lamina, nature of crystals and nature palisade tissue in midrib reported in comparative manner in **Table 1**. While the other important cellular characters of the leaf like number of stomata, indices of stomata, palisade ratio, vein islet number and vein termination number tabulated in **Table 2**.

3. Powder characteristics- Powder characteristics of *P. amarus* has been outlined as follows-

a) **Root:** Powder of the root represented abundant fibers, vessel members, fragments of cork cells, macrosclereids, simple starch grains (which were either solitary or clustered).

b) **Stem:** Powder of the stem represented abundant fibers, tracheids vessels, and fragment of cork cells, crystals and very few starch grains.

c) **Leaf:** Powder of the leaves represented fragments of lamina with sinuous epidermal cell walls, pallisade cells, spongy cells vascular tissues, trachieds, crystals and pieces of stomata.

4. Ash values and specific solvent soluble values- The percentage values of total ash, acid-insoluble ash and water-soluble ash **Table 3**. For details of alcohol-soluble and water-soluble extractives see **Table 3**.

5. Extracts of the Phyllanthus species for chronic pain modulating potential- As per the search from the herbal market it was evident to us that most of the herbal drug manufacturers are preparing standardized extracts. Standardized extracts individually possessing medicinal potential are incorporated formulations as herbal medicines. Taking the current market trend into consideration we decided to procure extracts of *Phyllanthus amarus* from two

most renowned herbal drug and extracts manufacturers. The standardized extract of *P. amarus* whole plant (water extract) was procured as a gift sample from Chemiloids. While the standardized methanolic extract of *Phyllanthus amarus* leaf and the standardized hydro methanolic extract of *Phyllanthus amarus* leaf procured as a gift sample from Natural Remedies. The various physical characteristics of the extracts used in the present study outlined in **Table 4**.

6. Preliminary Phytochemical screening of standardized *P. amarus* extracts- Phytochemical screening results have shown the presence of many valuable compounds such as lignans, flavonoids, tannins, polyphenols, triterpenes, sterols and alkaloids in the extracts of two Phyllanthus species. The details of the test utilized and their results summarized in **Table 5**.

7. HPTLC Finger Print analysis of Phyllanthus extracts- Diverse compositions of the mobile phase for HPTLC analysis tested in order to obtain high resolution and reproducible peaks. The TLC procedure used to develop the mobile phase for the four phyllanthus extracts. The extracts spotted on the TLC plate and different individual solvents as well as a combination of solvents tried, to get a good separation. For HPTLC analysis through HPTLC techniques, optimization of solvent system was done using combination of solvent system of varying polarity.

Initially the solvent system used was toluene: ethyl acetate in varying ratios (2: 1, 85:15 v/v) tried but the plate was not well resolved. Of the various mobile phases tried, toluene: chloroform: ethanol (4:4:1, v/v) gave the best resolution for development of common chromatogram for the analysis of the components of the extracts under study. Well-defined spots obtained when the chamber saturated with the mobile phase for more than 20 minutes, at room temperature. HPTLC fingerprint study demonstrates unique finger print pattern for the similar solvent system. The HPTLC plate pictures are depict under specific heading of wavelength with the selected solvent system.

Chromatographic finger print analysis of phyllanthus extracts taken at 254 nm wavelength shows Rf unique loci at 0.26. PAAE, PAHME, PAME and PFHEE showed presences of 6, 4, 4 and 7 compounds respectively with maximum % of area covered by PAHME extract. HPTLC finger print profile and 3d spectra of phyllanthus extracts taken at 254 nm wavelength recorded in the **Figure 5**. The details of Rf range, No of Peaks, No of Auto Generated tracks and Maximum height of peaks is summarized in **Table 6**.

The maximum height peak of 290.5 observed with PAAE extract. The number of auto generated peaks for PAAE, PAHME, and PAME extracts were 6, 7 and 4 respectively.

Chromatographic finger print analysis of phyllanthus extracts taken at 366 nm wavelength shows Rf unique loci at 0.68. PAAE, PAHME and PAME showed presences of 2, 8, and 11 compounds respectively with maximum % of area covered by PAAE extract. The maximum height peak of 518.6 observed with PAHME extract. The number of auto generated peaks for PAAE, PAHME, PAME extracts were 2, 4, 3 respectively. HPTLC finger print profile and 3d spectra of phyllanthus extracts taken at 366 nm are recorded in the **Figure 6**. The details of Rf range, No of Peaks, No of Auto Generated tracks and Maximum height of peaks is summarized in **Table 7**.

Chromatographic finger print analysis of the derivatised phyllanthus extracts taken at 366 nm wavelength shows Rf unique loci at 0.41. PAAE, PAHME, PAME showed presences of 7, 8, 12 compounds respectively with maximum % of area covered by PAHME extract. HPTLC finger print profile and 3d spectra of subsequent derivatization of phyllanthus extracts taken at 366 nm wavelength is depicted in **Figure 7**. The maximum height peak of 407.6 observed with PAME extract. The number of auto generated peaks for PAAE, PAHME, PAME extracts were 7, 8, 12 respectively. The details of Rf range, No of Peaks, No of Auto Generated tracks and Maximum height of peaks is summarized in **Table 8**.

Chromatographic finger print analysis of the phyllanthus extracts taken at 540 nm wavelength shows Rf unique loci at 0.68. PAAE, PAHME, PAME showed presences of 10, 9, 9 compounds respectively with maximum % of area covered by PFHEE extract. HPTLC finger print profile and 3d spectra of phyllanthus extracts taken at 540 nm are recorded in **Figure 8**. While the other details of Rf range, No of Peaks, No of Auto Generated tracks and Maximum height of peaks is summarized in **Table 9**. The maximum height peak of 472.7 observed with PAHME extract. The number of auto generated peaks for PAAE, PAHME, PAME extracts were 6, 6, 4 and 4 respectively.

Figure 1: *P. amarus* whole plant

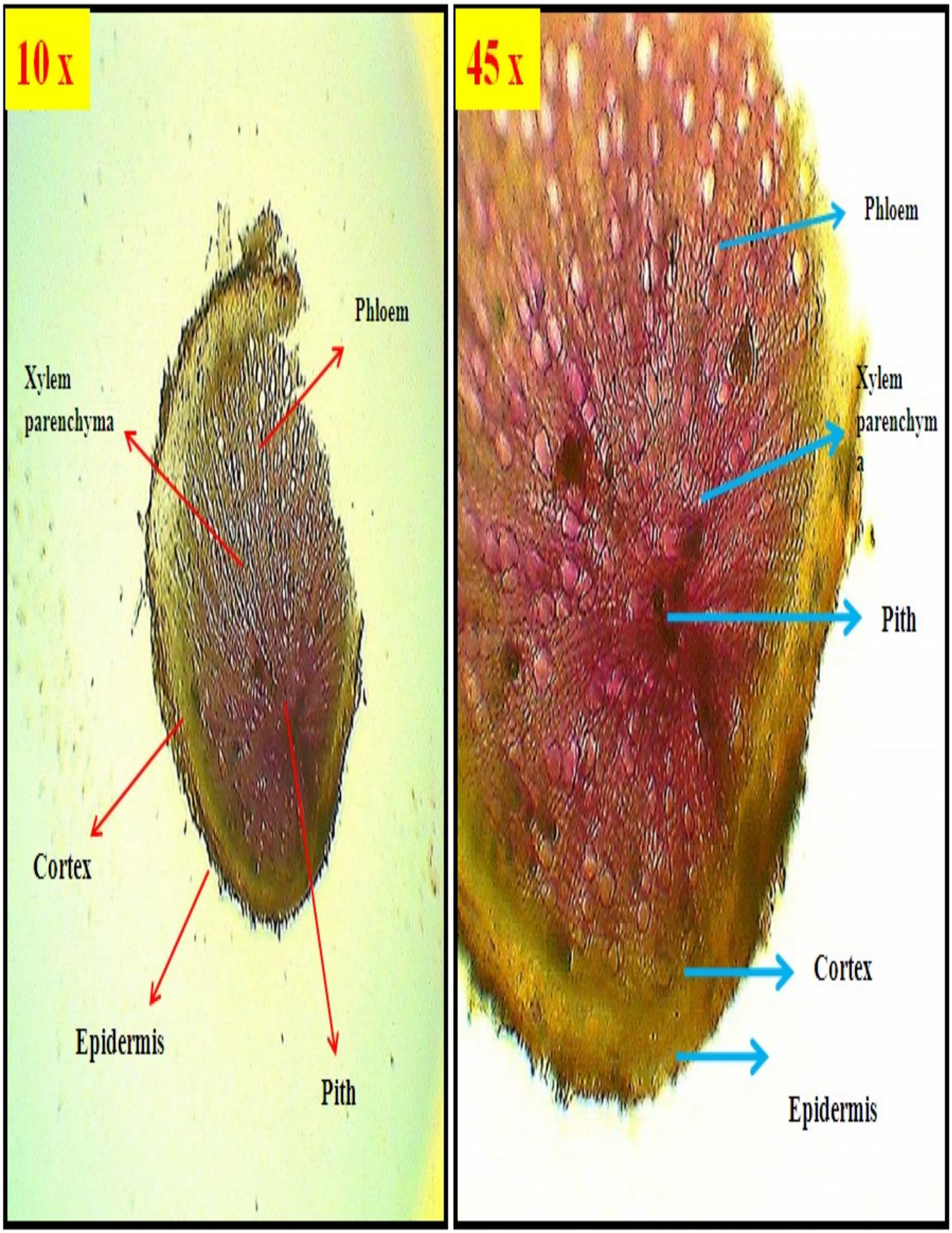

Figure 2: Observed microscopical features of Root of *Phyllanthus amarus*

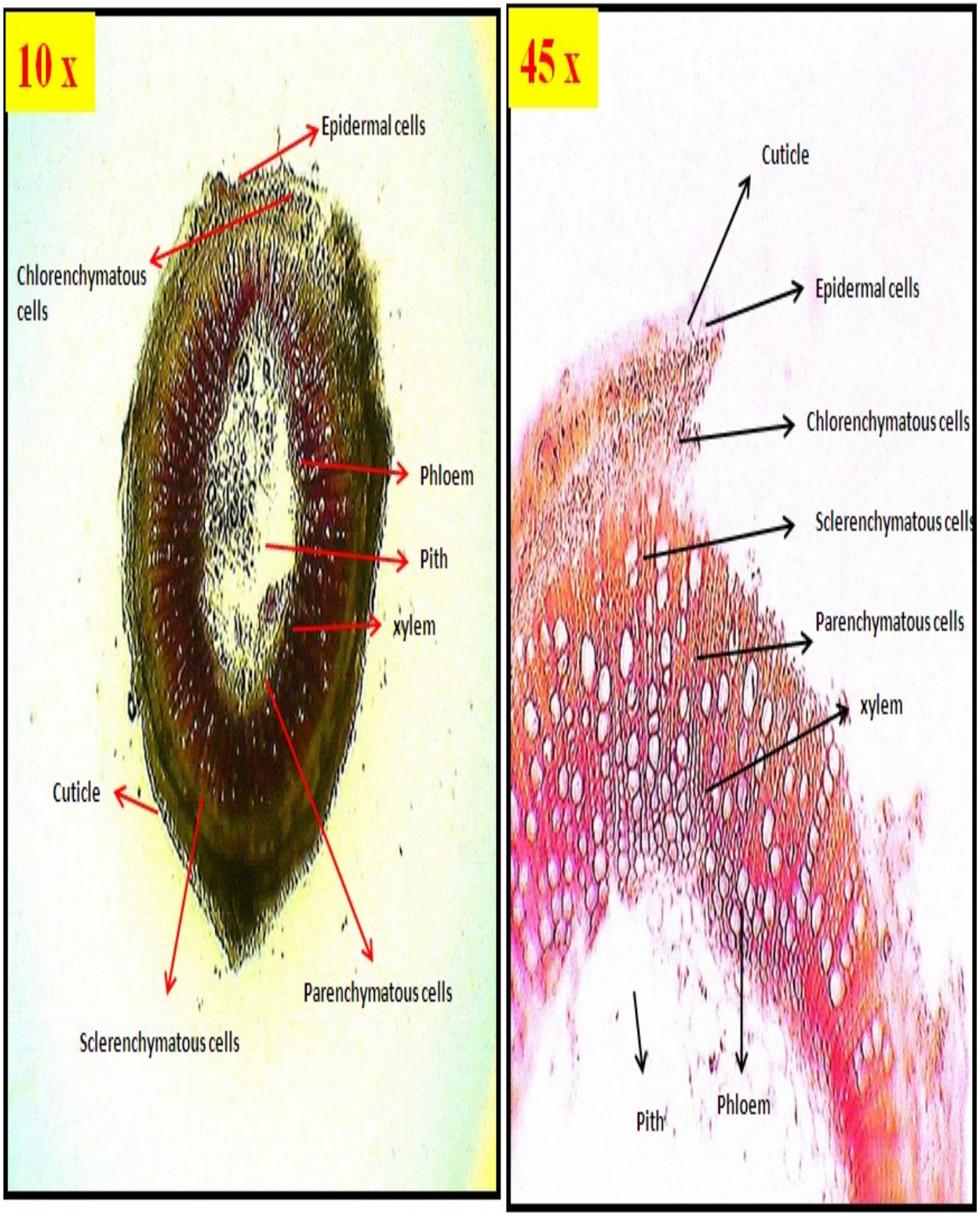

Figure 3: Observed microscopical features of Stem of *P. amarus*

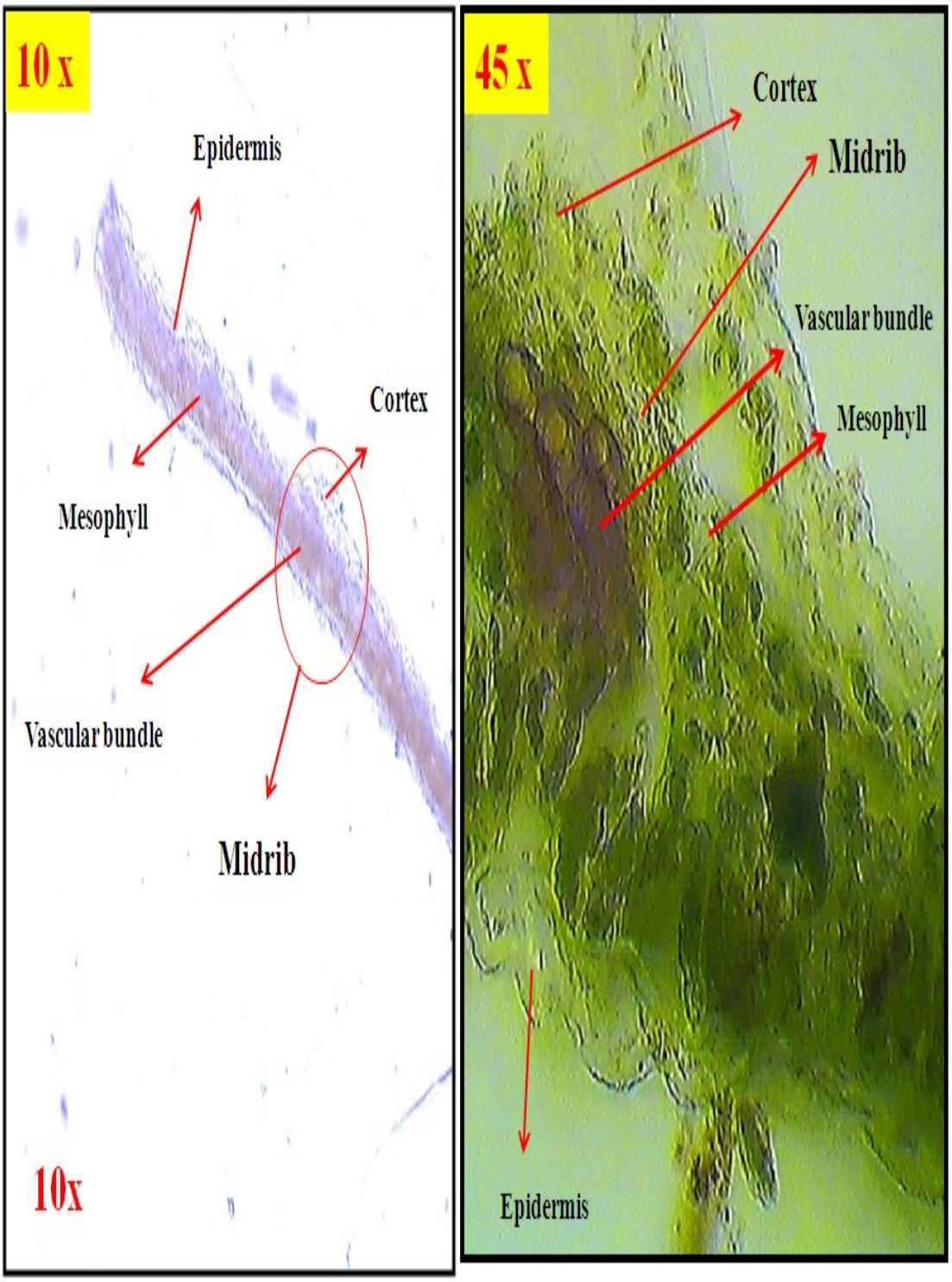

Figure 4: Observed microscopical features of Leaf of *P. amarus*

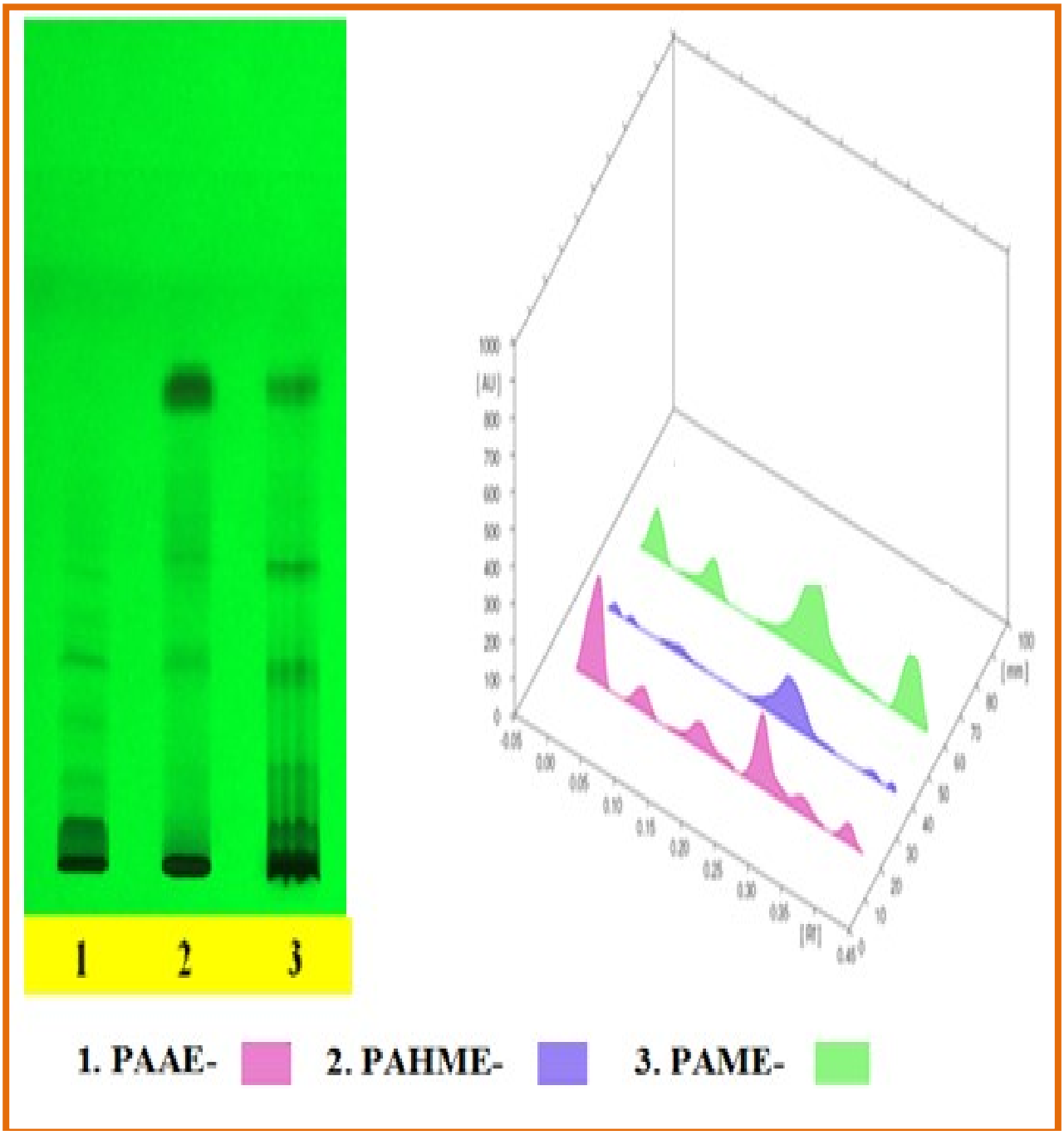

Figure 5: Phytochemical finger print and 3d spectra of phyllanthus extracts taken at 254 nm wavelength

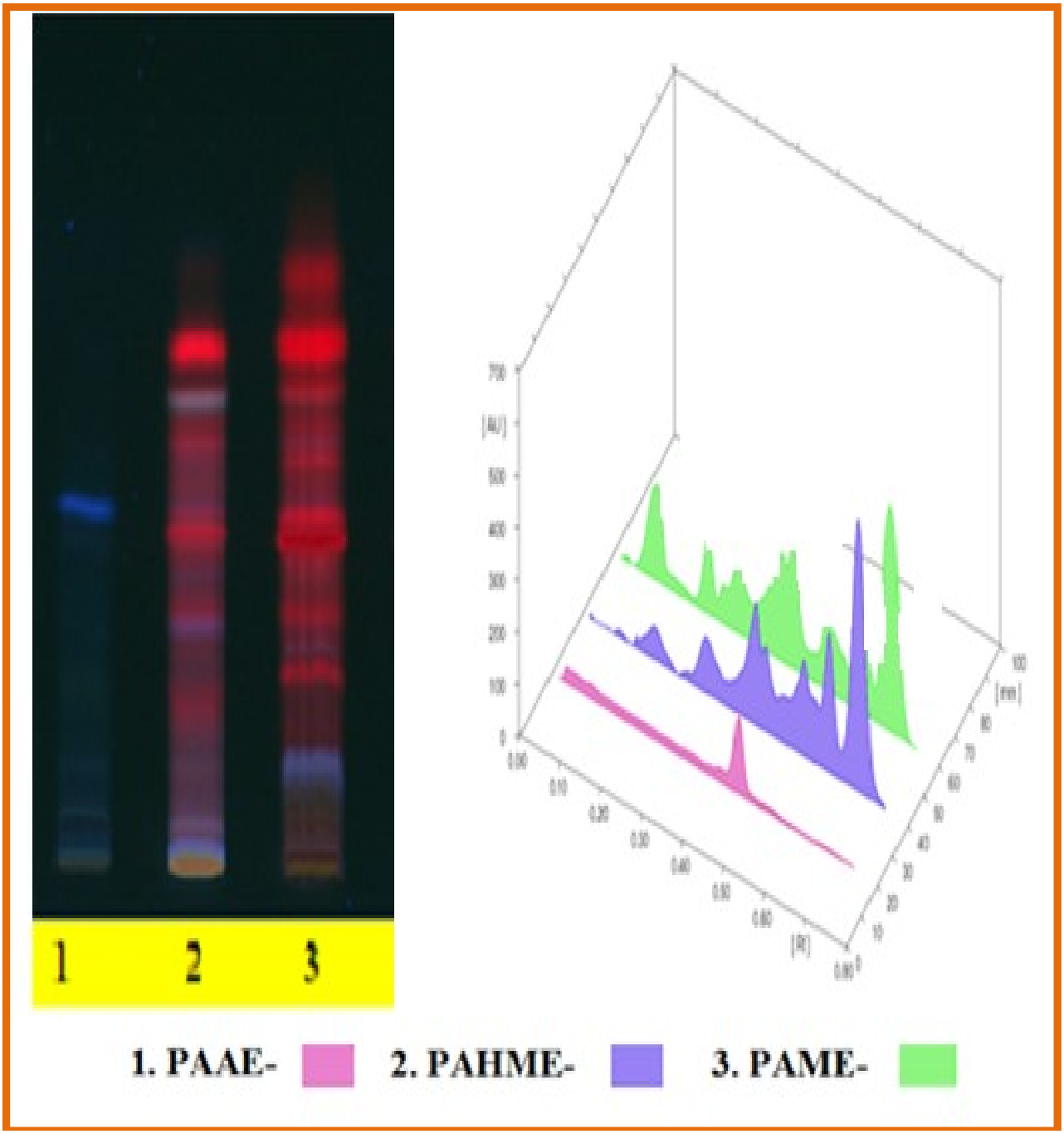

Figure 6: Phytochemical finger print and 3d spectra of phyllanthus extracts taken at 366 nm wavelength

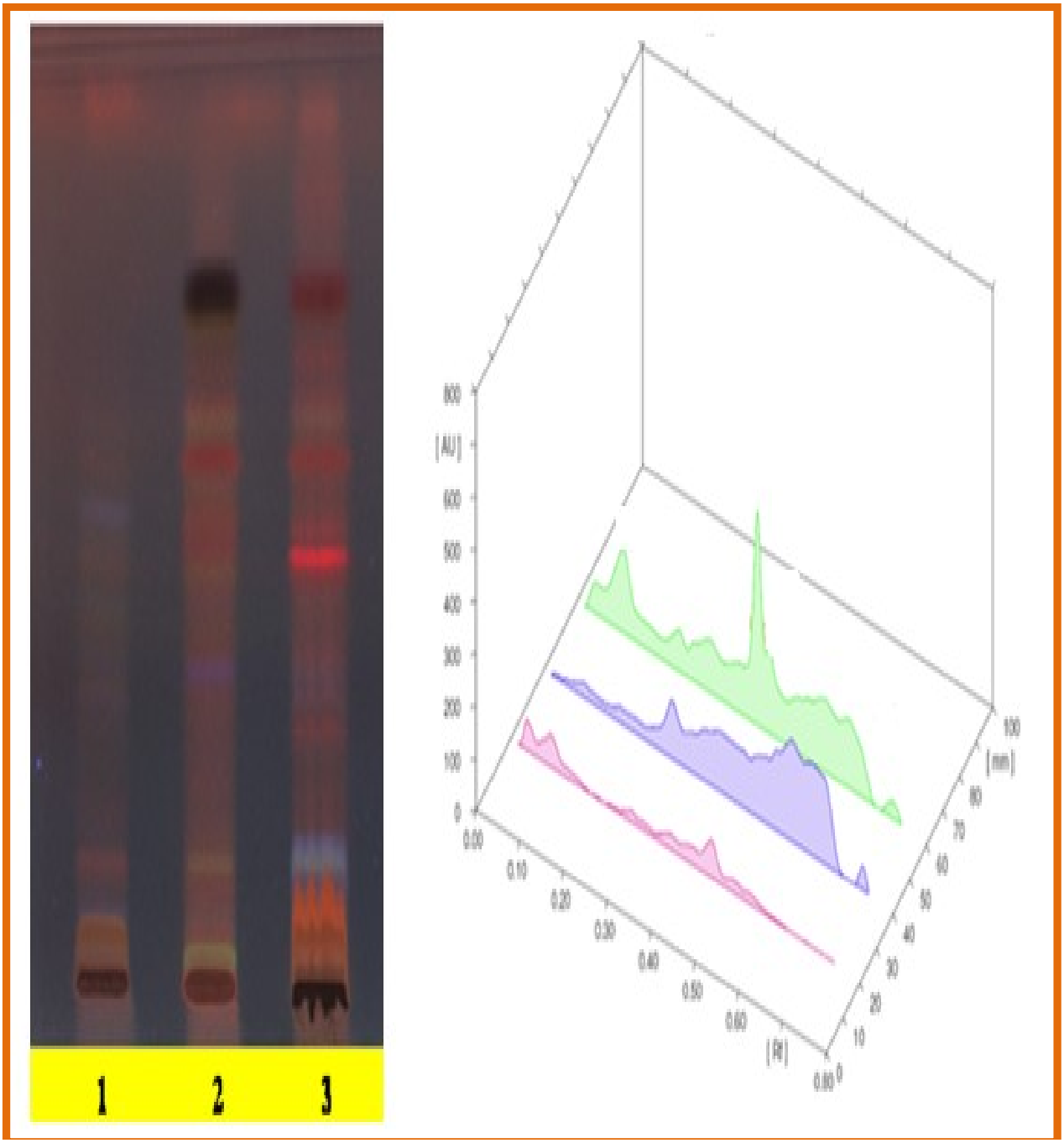

Figure 7: Phytochemical finger print and 3d spectra of derivatised Phyllanthus extracts taken at 366 nm wavelength

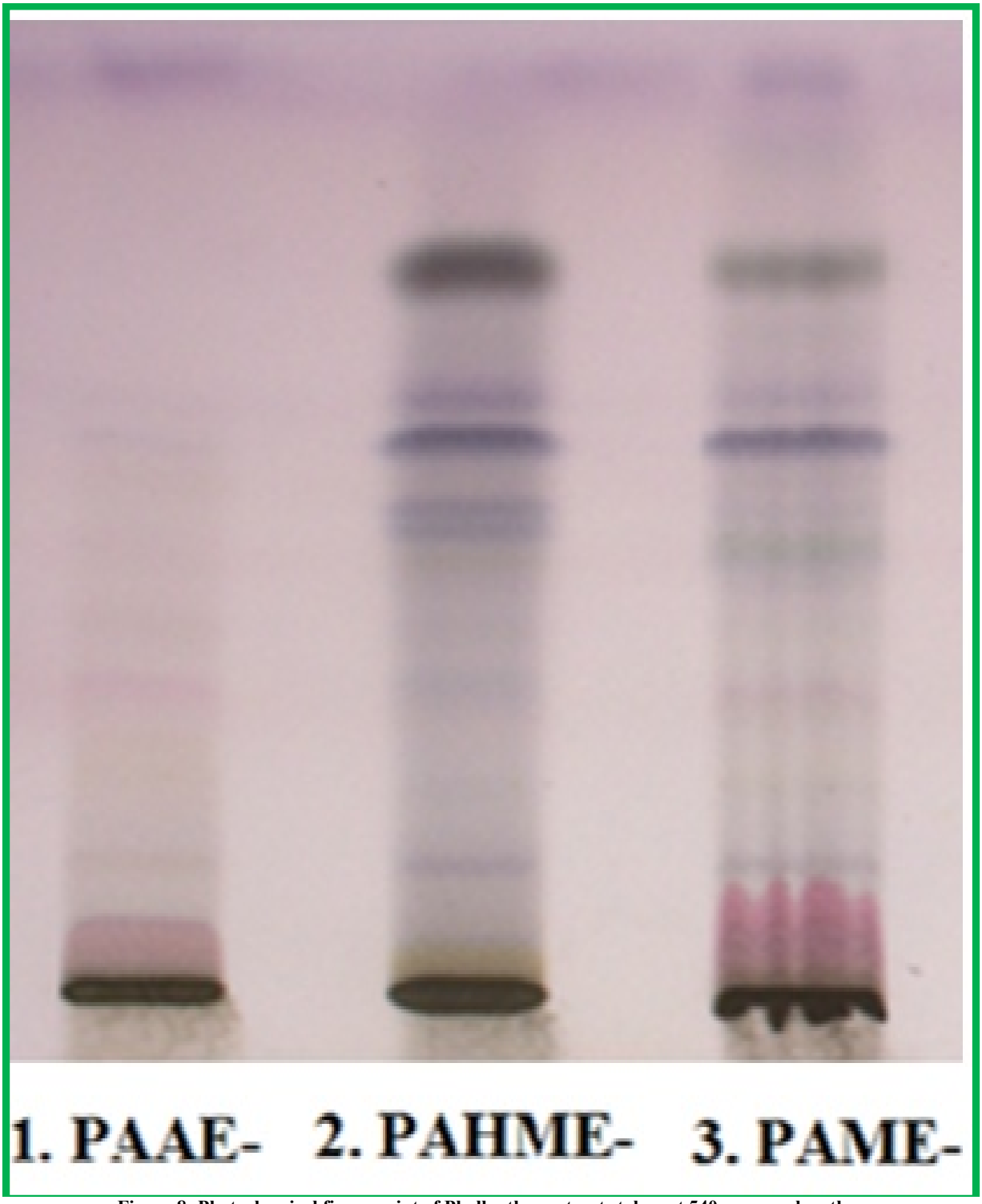

Figure 8: Phytochemical finger print of Phyllanthus extracts taken at 540 nm wavelength

Table 1: Important reported comparative cellular characters of the leafs

Stomatal type	Cell wall	Margin of lamina	Crystals	Palisade tissue in midrib
Paracytic and anisocyctic	Wavy	Papillae like out growths at some places	Clusters rarely prismatic	Discontinuous

Table 2: Other important reported comparative cellular characters of the leafs

Stomatal numbers	Stomatal indices	Palisade ratio	Vein islet number	Vein termination number
218 - 230	28–30	13–14	16–18	42–49

The values are represented as the lower and upper limits of three observations; n = 3.

Table 3: Percentage of ash and extractive values of *Phyllanthus amarus*

Parameters	Values in %
Total ash	6.00-6.50
Acid-insoluble ash	0.30 - 0.50
Water-soluble ash	0.10 - 0.30
Alcohol-soluble extract	18.00 -20.00
Water-soluble extract	22.00 –24.00

The values are represented as the lower and upper limits of three observations; n = 3.

Table 4: Characterization of extracts by Physical methods

SR. NO.	TEST	*Phyllanthus amarus*		
		PAAE	PAME	PAHME
1	Colour	Brown	Brown to dark brown powder	Green to Light green powder
2	Odour	Characteristic	Characteristic	Characteristic
3	Taste	Characteristic and agreeable	Characteristic and acrid	Characteristic and bitter
4	Solubility			
	Distilled Water	Good	Good	Good
	DMSO	Excellent	Excellent	Excellent

Table 5: Characterization of extracts for various chemical constituents by chemical methods

CHEMICAL TEST	*Phyllanthus amarus*		
	PAAE	PAME	PAHME
As per the COA of the manufacturing laboratory	Extract contains 81.74% of water soluble extractives	Extract contains > 2.5% of Phyllanthin and Hypophyllanthin	Extract contains > 5% of Corilagen
Test for Flavonoids Shinoda test Lead acetate	+	++	+
Test for Tannins Ferric chloride Lead acetate	++	++	++
Test for Alkaloids Dragendroff's Test Hager's Test	-	++	++
Test for Triterpenoids	+	++	+
Test for Sterols Salkowaski Test	-	+	+
Test for Reducing sugars (Fehlings test)	+	+	+
Test for Saponins Foam Test	+	-	+
Test for Glycosides (Molisch test)	+	+	+
Test for LIGNANS	++	++	++

Interpretation of results: (−) absent; (+) low; (++) good.

Table 6: Chromatographic finger print analysis of phyllanthus extracts taken at 254 nm wavelength

Name of extract	Parameters					
	Rf range	Rf of Unique loci	No of Peaks	No of Auto Generated tracks	Max Height	Area % range
PAAE	0.03 -0.40	0.27	6	6	48.5 - 290.5	5.98 - 36.36
PAHME	0.03 -0.39	0.26	4	7	16.6- 128.0	2.37- 81.76
PAME	0.02 -0.40	0.25	4	4	92.6 - 225.0	11.52 - 50.89

Table 7: Chromatographic finger print analysis of phyllanthus extracts taken at 366 nm wavelength

Name of extract	Parameters					
	Rf range	Rf of Unique loci	No of Peaks	No of Auto Generated tracks	Max Height	Area % range
PAAE	0.04 - 0.46	0.46	2	2	30.1 - 146.3	18.79 - 81.21
PAHME	0.09 - 0.68	0.43 , 0.68	8	4	12.5 - 518.6	0.44 - 34.75
PAME	0.03 - 0.68	0.68	11	3	10.6- 431.3	0.15 - 26.72

Table 8: Chromatographic finger print analysis of derivatised Phyllanthus extracts taken at 366 nm wavelength

Name of extract	Parameters					
	Rf range	Rf of Unique loci	No of Peaks	No of Auto Generated tracks	Max Height	Area % range
PAAE	0.04 - 0.04	0.09, 0.46	7	4	19.7 - 71.7	6.37 - 28.54
PAHME	0.09 - 0.73	0.57	8	4	26.7 - 192.1	2.28 - 29.20
PAME	0.04 - 0.72	0.41	12	8	32.5 - 407.6	1.40 - 18.31

Table 9: Chromatographic finger print analysis of Phyllanthus extracts taken at 540 nm wavelength

Name of extract	Parameters					
	Rf range	Rf of Unique loci	No of Peaks	No of Auto Generated tracks	Max Height	Area % range
PAAE	0.03 - 0.55	0.27	10	6	11.5 - 72.0	1.76 - 25.08
PAHME	0.09 - 0.68	0.68	9	6	14.0 - 472.7	0.36 - 29.65
PAME	0.03 - 0.68	0.51, 0.68	9	4	27.0 - 387.8	0.98 - 29.70

CONCLUSION

The results presented in this study could serve as diagnostic parameters for proper identification as well as preparation of a monograph on *Phyllanthus amarus*.

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Conflicts of interest- We all the authors declare no conflict of interest.

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