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Comparative Chemoprofile Variation Study of Bhumyamalaki (*Phyllanthus fraternus Webst*) Collected from Different Bhoomi Desha of Karnataka by Using RP-UPLC Method

Dr. Ramesh Gaddi[1], Dr. Rajashekhar.S.Ganiger[2], Dr. Lambani Shobha[3], Dr. Abhishek[4]
[1, 3, 4]*PG scholar, Department of Dravyaguna, TGAMC Ballari, RGUHS Karnataka*
[2]*Professor and HOD, Department of Dravyaguna, TGAMC, Ballari, RGUHS Karnataka*

Abstract: ***As per the survey of WHO, 88% of all countries are estimated to use traditional medicines. Ayurveda is bestowed science of life which is mainly based on herbs. It is a treasure of amazing herbal medicine but still many important medicinal plants which are used in treatment are need to be standardized. The drug Tamalaki mentioned in Swasahara and Kasahara dashemani of Charaka Samhita which is popularly known as Bhumyamalaki (Phyllanthus fraternus webst). Phyllanthus fraternus Webst (syn. P.niruri auct. Pl.non Linn) is one of the species belongs to Euphorbiaceae family is considered as Bhumyamalaki, which is commonly found as a weed in Central and southern India. At present, many species of Bhumyamalaki are found in natural habitat. In Ayurveda, seers have given importance to Bhoomi Desha i.e the natural habitat of dravyas is important in Dravya collection wherein they have emphasized to collect medicinal plants from suitable desha. Many literatures survey revealed the presence of various phyto-constituents in Phyllanthus fraternus such as Phyllanthin, Hypophyllanthin, Nirphyllin, Phillnirurin, Niranthin, Niruriside, Securinine, Limonene, 4-Methoxy- securinine, 4-Methoxy-norsecurinine, Niruretin, Phyllanthol, Phyllanthenol, Phyllanthenone, Lintetralin, Astragalin, Cymene, Niruodine and Phyllanthamide. The present work aims to see the possible changes in Phyto constituents seen in a same species of Bhumyamalaki i.e. Phyllanthus fraternus Webst which is grown in different Desha like Jangaladesha, Sadharanadesha and Anoopadesha, Quantification of Phytochemicals are done by using TLC and RP-UPLC.***
Result : The Percentage of Phytoconstituents Phyllanthin and Hypophyllanthin was found to be more in anupa desha sample when compared to other two deshas sample.
Keywords: Bhumyamalaki, Desha, TLC, RP-UPLC, Phyllanthin, Hypophyllanthin.

I. INTRODUCTION

Aushadha is one among Trisutra. The concept of “Jagatyevam Aushadham” is explained in Samhita. Aushadha, alleviates the diseases by bringing back the tridosha to normalcy. The dravya to act as an effective medicine, the proper processing plays a pivotal role. As Acharya Charaka opines, even a proper processed poison or tikshna dravya can be an effective medicine.

As per the servey of WHO 88% of all countries are estimated to use traditional medicines.[1] The drug Tamalaki mentioned in Swasahara and kasahara dashemani of Charaka samhita which is popularly known as Bhumyamalaki.[2] According to Bhavaprakasha synonym of Bhumyamalaki is Tamalaki.[3] The plant is stomachic and fresh juice of the plant is used for the treatment of jaundice.[4] At present, many species of Bhumyamalaki are found in natural habitat. Depending on habitat the quality and quantity of dravyas varies.[5] Charaka acharya has explained about Bheshaja pariksha in which he has mentioned about importance of Guna of dravyas and the Desha i.e Natural habitat.[6] Phyllanthus fraternus Webst (syn. P.niruri auct. Pl. non Linn) is one of the species belongs to Euphorbiaceae family is considered as Bhumyamalaki, which is commonly found as a weed in Central and southern India.[7] Many literature survey revealed the presence of various phyto-constituents in Phyllanthus fraternus such as Phyllanthin, Hypo-phyllanthin, Nirphyllin, Phillnirurin, Niranthin, Nirtetralin, Niruriside, Securinine, Limonene, 4-Methoxy-securinine, 4-Methoxy-norsecurinine, Niruretin, Phyllanthol, Phyllanthenol, Phyllanthenone, Lintetralin, Astragalin, Cymene, Niruodine and Phyllanthamide.[8] This plant is also known to be highly effective in treating in different diseases such as Hepatitis, Cold, Flu, Tuberculosis, Viral infection, Anemia, Biliary and Urinary disorders and other bacterial and fungal infections. This plant has the ability to act as antioxidant, antinociceptive, hepato protective and antifibromyalgic and diabetic effects etc.[9]

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Therefore, it becomes an important study to know the particular phytoconstituent and its concentration in all the 3 samples by ethanolic extract analytically. Hence the study entitled "Comparative chemoprofile variation study of bhumyamalaki (phyllanthus fraternus webst) collected from different bhoomi desha of karnataka by using rp-uplc method"

II. OBJECTIVES OF THE STUDY

1) Collection of Phyllanthus fraternus Webst from three different bhoomi deshas of Karnataka.
2) To carry out the comparative study of collected Phyllanthus fraternus Webst on Pharmacognostic, Physicochemical and phytochemical analysis.
3) To compare Quantitative Phyllanthin and hypophyllanthin phytoconstituents of Phyllanthus fraternus Webst collected from different bhoomi deshas by using RP-UPLC method.

III. MATERIALS AND METHODS

A. Collection, Authentication And Preparation Of Trial Drug Bhumyamalaki

1) Collection Of Trial Drug

Trial drug collected from 3 different deshsas of Karnataka.
a) Jangala Desha - Ballari-Sanduru surroundings of Karnataka
b) Sadharana Desha - Hubballi-Yallapura surroundings of Karnataka
c) Anupa Desha - Mangaluru -Udupi surroundings of Karnataka.
* The genuinity of the trial drug was confirmed by Professor Dr. Manjunath Ajanal, Rajiv Gandhi Education Society Ayurveda Medical College, Rona.
* The trial drug was authenticated as Bhumyamalaki (Phyllanthus fraternus webst)

2) Preparation Of Trial Drug

5 kgs of Bhumyamalaki drug (Panchanga) was collected from the different bhumi deshas (Jangala, Sadharana and Anupa) of Karnataka, whole panchanga was dried for 15 days till it completely dried. After drying 2.0 kg of drug was obtained, which was later made into coarse powder by using pulverizer and fine powder using mixer grinder.Finally, 1kg of fine powder and 1 kg of coarse powder was obtained.PREPARATION OF ETHANOLIC EXTRACT OF BHUMYAMALAKI The ethanolic extract was carried out by General method, The Standard Operating procedure was followed.

3) Materials required

Coarsely powdered drug, solvent, glass beaker, cotton plug, Stirrer.
Procedure: Firstly, the Solvent ethanol 100ml was taken in the graduated Conical flask and noted. Coarsely powdered drug is taken in a filter paper and weighed for 25gms on a digital weighing scale and noted. A tight cotton plug is kept on the opening of the conical flask to avoid evaporation of the solvent Gently shake the solution and kept for overnight. Next day the solution is filtered using funnel, filter paper, stand and filtrate is taken in a China dish and measured then kept over the steam bath/water bath till the liquid content evaporates. At last, the extract was in the form of flakes/layer was obtained.The physical characters of the extract were noted and preserved.

IV. MORPHOLOGICAL AND ORGANOLEPTIC STUDIES OF THE TRIAL DRUG

The morphology of the sample (panchanga) was examined by the naked eye and also by using a simple microscope to note the characteristic features. The organoleptic characters were determined as per the API guidelines. Drug Phyllanthus fraternus webst. was also observed for the following sensory features:- Shabda, Sparsha, Rupa, Rasa,Gandha.

A. Microscopic studies

1) Powder Microscopy

Powder microscopy was carried out as per standard operating procedure.
For powder microscopy a finely powdered sample was treated with phloroglucinol:Hcl (1:1) to stain lignified tissues. The treated powder was also mounted in glycerine and observed under the trinocular microscope at 10x. Photographs at different cellular structures were taken with a camera (Magcam make; model no: DC14) attached with a microscope.

2) Transverse Section Studies

The transverse section was performed by the free-hand section technique. The sections were mounted in glycerine and observed under a microscope. The thinnest section with the highest clarity was stained with phloroglucinol reagent, and histological analysis was carried out to observe different cellular structures and inclusions under a trinocular microscope (Olympus make; model no: CX21i) at 10× and 40× magnification. Photomicrographs were taken with a camera (Magcam make; model no: DC14) attached with a microscope.

3) Physicochemical Evaluation

The Physicochemical parameters like ash values, extractive values, loss on drying of samples were determined by using coarse powder as per the standard guidelines of The Ayurvedic Pharmacopoeia of India (API).

4) Preparation of Extract

A coarsely powdered panchanga sample (5 g) of *P.fraternus* was soaked in a conical flask containing 50 ml ethanol and fitted with a rubber cork and kept for about 24 hrs and then it is filtered after that kept on a waterbath until the water content evaporate and only extract is remains, The dried extracts were redissolved in ethanol and used for phytochemical screening, chromatographic and spectroscopic studies.

5) Phytochemical Screening

A part of the redissolved extract was treated with different reagents to detect the presence of phytochemical groups like alkaloids, flavonoids, phenols, etc.[15,16]

B. Fingerprint Analysis

TLC analysis

Carry out thin-layer chromatography on a TLC plate using phyllanthin and hypophyllanthin as reference standards.
Extract: Ethanol, Mobile Phase (Solvent system) -- Toluene: ethyl acetate: formic acid: water (20:60:15:5)
Stationary phase – marked TLC plate.
Mobile phase was measured and taken by using pipette in a beaker and kept closed.
Filtrate which has already been prepared should be kept for heating on a water bath till liquid part evaporates. Then dried part is collected and diluted by adding 5-10 ml of methanol and mixed well. This solution is ready to use. Like these 3 solutions of 3 samples has to be prepared (Jangala, sadharana, anupa) in separate china dishes.Procedure: Mark the TLC sheet/Plate by using markers as Jangala Sadharana and Anupa side by side (Parallelly). Apply 10 µl each of the test solution by using micro capillary tube (using sharp end) on a TLC plate as bands of 10 mm on the respective markings, Heat the plate at 110° for about 5 minutes or till the bands are clearly visible. The chromatogram obtained with test solution shows a band at R -0.50 corresponding to that of Hypophyllanthin and at R, -0.35 corresponding to that of Phyllanthin and the profile should be similar to the one given in the TLC Then it has to be dried completely and kept in the mobile phase beaker. Here TLC plate is known as Stationary Phase. Stationary phase is kept in the mobile Phase.Till the bands are appear partially or fully on the TLC plate. Removed the Plate and observed by keeping in the visible light/white light, the bands which are visible can be noted and measured the lengths and widths, then finding R_f Value same is done for Short UV and Long UV rays by keeping in the
UV Chamber, The chromatogram obtained with test solution shows a band at R -0.50 corresponding to that of hypophyllanthin and at R, -0.35 corresponding to that of phyllanthin and the profile should be similar to the one given in the TLC by comparing the R_f values obtained with the Standard values mentioned in the API books or Qualitative Standards of Indian Medicinal Plants. Confirming which particular group of phytochemicals is present in the samples. After, that wrap the TLC Plate in the aluminium foil for safety storage.

V. ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY (RP-UPLC) ANALYSIS:

Estimation of Phyllanthin.
Ethanolic Extract of Bhumyamalaki.
Reversed Phase Ultra Performance Liquid Chromatography conditions:
Column: RP-UPLC C-18 column
Size: 4mm×250nm

Mobile phase: orthophosphoric acid (OPA) and acetonitrile: methanol (1:1)
Stationary phase: Silica
Injection volume: 20µl Flow rate: 1ml/min
Wave length: 254nm
Run time: 20 minutes.
Instrument used: Shimadzu SPD-10A.
Chromatographic Conditions
High-performance liquid chromatography was performed with Waters 2695 Alliance system with a 2998 photodiode array detector (PDA). Two compounds were separated on a reverse-phase 250 mm × 4.6 mm, 5 µ, symmetry C8 column (Waters). The mobile phase was prepared from 0.1% OPA (solvent A) and acetonitrile:methanol (1:1) (solvent B). The mobile phase was degassed and filtered through 0.45-µm filter before use. The gradient program used was as follows: initial 0–12 min, from A–B (60:40 v/v); 12–30 min, linear change from A–B (60:40 v/v) to A–B (20:80 v/v); 30–31 min, linear change from A–B (20–80 v/v) to A–B (05–95 v/v); 31–40 min, constant change from A–B (5–95 v/v); 40–41 min linear changes from A–B (5–95 v/v) to A–B (60–40 v/v); 41–45 min, constant change from A–B (60–40 v/v). The mobile phase flow rate was 1 mL min−1 Before the first injection, the column was saturated for 30 min with the initial mobile phase. The column temperature was maintained at 40 °C.The injection volume was kept 20 µL. The PDA was set at 230 nm to acquire the chromatogram. A UV spectrum was acquired in the range 200–400 nm. All three compounds were identified by comparing the retention times and spectra obtained from sample and standard solutions. The present work was performed in an airconditioned room maintained at 25 °C Mobile phase preparation: The mobile phase used here is Ortho-phosphoric acid OPA and Acetonitrile and methanol in the ratio of 1:1 which means 350ml of Acetonitrile and make up to 500ml by adding 150ml of UPLC grade water. This mobile phase solution is filtered by Membrane filtration method and Degas is done by sonicator for 15minutes. Standard preparation: The standard Phyllanthin with the concentration 1mg/ml was dissolved in mobile phase. Sample preparation: Bhumyamalaki solution 10µl/ml and ethanolic extract 10mg/ml were dissolved in mobile phase.
Procedure:

- ❖ The mobile phase was forced through the packed column with flow rate of 1ml/min and under pressure gradient elution 254nm.
- ❖ The chromatograph with the concentration 1mg/ml is done by injecting 20µl of

standard Phyllanthin and Hypophyllanthin solution.

- ❖ The chromatograph of Bhumyamalaki choorna solution with concentration 10µl/ml was done.
- ❖ The computer recorded the peaks of Absorbance of the substance.
- ❖ The report contains the retention time (RT).
- ❖ The amount of Phyllanthin and Hypophyllanthin present in all the sample was

Estimated using formula.

VI. RESULTS

All the tests were done as per the rules and regulations of the drugs and cosmetic act 1940. All the tests were done under the guidance of the experts and responsible authority of the lab. The Analytical study carried out for Bhumyamalaki choorna, the following results are documented.

TABLE1
PHYSICOCHEMICAL STUDY – JANGALA SAMPLE

Sl. No	Test	Result
1	Ash value	7.5 %
2	Acid insoluble ash	3.5 %
3	Water soluble extract	12.2%
4	Alcohol soluble extract	5.2 %
5	Loss on drying	7.16 %

TABLE 2
PHYTOCHEMICAL STUDY OF JANGALA SAMPLE

Sl.No	Test	Results
1	Carbohydrate	Present
2	Reducing sugar	Present
3	Non- reducing sugar	Absent
4	Monosaccharide	Present
5	Protein	Present
6	Amino acid	Present
7	Tannin	Present
8	Alkaloid	Present
9	Steroid	Absent
10	Saponin	Absent
11	Oils and fats	Absent
12	Flavonoids	Absent

TABLE 3
TLC ANALYSIS OF JANGALA SAMPLE *Extract:* Methanol
Mobile phase – (Toluene, ethyl acetate, formic acid : water :: 20:60:15:5)

Sl. No	Wavelength	Rf Value Result
1	White light/ visible light	0.166, 0.28
2	Short UV wave (254nm)	0.25, 0.464
3	Long UV wave (365nm)	0.559

TABLE 4
PHYSICOCHEMICAL STUDY – SADHARANA SAMPLE

Sl. No	Test	Result
1	Ash value	23 %
2	Acid insoluble ash	15.5 %
3	Water soluble extract	15.6%
4	Alcohol soluble extract	3 %
5	Loss on drying	6.3 %

TABLE 5
PHYTOCHEMICAL STUDY OF SADHARANA SAMPLE

Sl.No	Test	Results
1	Carbohydrate	Present
2	Reducing sugar	Present
3	Non- reducing sugar	Absent
4	Monosaccharide	Present
5	Protein	Absent
6	Amino acid	Present
7	Tannin	Present
8	Alkaloid	Present
9	Steroid	Absent
10	Saponin	Absent
11	Oils and fats	Absent
12	Flavonoids	Present

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TABLE 6
TLC ANALYSIS OF SADHARANA SAMPLE
Extract: Methanol
Mobile phase – (Toluene, ethyl acetate, formic acid : water :: 20:60:15:5)

Sl. No	Wavelength	Rf Value Result
1	White light/ visible light	0.107, 0.166, 0.25
2	Short UV wave (254nm)	0.107, 0.25
3	Long UV wave (365nm)	0.107, 0.25, 0.63, 0.892

TABLE 7
PHYSICOCHEMICAL STUDY – ANUPA SAMPLE

Sl. No	Test	Result
1	Ash value	13.5 %
2	Acid insoluble ash	8.0 %
3	Water soluble extract	15.6%
4	Alcohol soluble extract	7.0 %
5	Loss on drying	8.08%

TABLE 8
PHYTOCHEMICAL STUDY OF ANUPA SAMPLE

Sl.No	Test	Results
1	Carbohydrate	Present
2	Reducing sugar	Present
3	Non- reducing sugar	Absent
4	Monosaccharide	Absent
5	Protein	Absent
6	Amino acid	Absent
7	Tannin	Present
8	Alkaloid	Absent
9	Steroid	Present
10	Saponin	Present
11	Oils and fats	Absent
12	Flavonoids	Absent

TABLE 9
TLC ANALYSIS OF ANUPA SAMPLE
Extract: Methanol
Mobile phase – (Toluene, ethyl acetate, formic acid : water :: 20:60:15:5)

Sl. No	Wavelength	Rf Value Result
1	White light/ visible light	0.166, 0.273
2	Short UV wave (254nm)	0.261
3	Long UV wave (365nm)	0.273, 0.63, 0.892

A. The Transverse section of Stem of Jangala Sample

The transverse section of matured stem has of 3-5 layers of cork, composed of thinwalled, tubular, tangentially elongated and radially arranged cells; epidermis consists of a single layer barrel-shaped cells with a thick cutinized outer wall. The epidermis is followed by cortex which consists of a continuous ring of collenchymatous tissue, followed by a chlorenchymatous layer and a zone of oval, tangentially elongated, thin walled, parenchymatous cells which has patches of phloem. The cells of the outer most layer of the cortex are comparatively larger and compactly arranged, the cells of the innermost layer of the cortex are tangentially

elongated; secondary phloem is narrow, composed of a sieve tube, companion cells and phloem parenchyma; secondary xylem consists of vessels, tracheids, fibres, and xylem parenchyma; pith is situated at the centre, composed of thin-walled, circular to oval parenchymatous cells which contain starch grains and calcium oxalate crystals

B. Powder Microscopy

The powder microscopy revealed polygonal epidermal cells in surface view, fragments of rectangular to pentagonal cork cells were observed. The fragments of fibres, pitted and lignified spiral vessels are visible. Scattered rosette and prismatic crystals of calcium oxalate are seen.

C. The transverse section of Stem of Sadharana Sample

The transverse section of matured stem has of 3-5 layers of cork, composed of thin-walled, tubular, tangentially elongated and radially arranged cells; epidermis consists of a single layer barrel-shaped cells with a thick cutinized outer wall. The epidermis is followed by cortex which consists of a continuous ring of collenchymatous tissue, followed by a chlorenchymatous layer and a zone of oval, tangentially elongated, thin-walled, parenchymatous cells which has patches of phloem. The cells of the outer most layer of the cortex are comparatively larger and compactly arranged, the cells of the innermost layer of the cortex are tangentially elongated; secondary phloem is narrow, composed of a sieve tube, companion cells and phloem parenchyma; secondary xylem consists of vessels, tracheids, fibres, and xylem parenchyma; pith is situated at the centre, composed of thin-walled, circular to oval parenchymatous cells which contain starch grains and calcium oxalate crystals

D. Powder Microscopy

The powder microscopy revealed polygonal epidermal cells in surface view, fragments of rectangular to pentagonal cork cells were observed. The fragments of fibres, pitted and lignified spiral vessels are visible. Scattered rosette and prismatic crystals of calcium oxalate are seen.

E. The transverse section of Stem of Anupa Sample

The transverse section of matured stem has of 3-5 layers of cork, composed of thin-walled, tubular, tangentially elongated and radially arranged cells; epidermis consists of a single layer barrel-shaped cells with a thick cutinized outer wall. The epidermis is followed by cortex which consists of a continuous ring of collenchymatous tissue, followed by a chlorenchymatous layer and a zone of oval, tangentially elongated, thin-walled, parenchymatous cells which has patches of phloem. The cells of the outer most layer of the cortex are comparatively larger and compactly arranged, the cells of the innermost layer of the cortex are tangentially elongated; secondary phloem is narrow, composed of a sieve tube, companion cells and phloem parenchyma; secondary xylem consists of vessels, tracheids, fibres, and xylem parenchyma; pith is situated at the centre, composed of thin-walled, circular to oval parenchymatous cells which contain starch grains and calcium oxalate crystals

F. Powder Microscopy

The powder microscopy revealed polygonal epidermal cells in surface view, fragments of rectangular to pentagonal cork cells were observed. The fragments of fibres, pitted and lignified spiral vessels are visible. Scattered rosette and prismatic crystals of calcium oxalate are seen.

Morphology of bhumyamalaki of trividha desha.



Fig 1. Jangala



Fig 2.Sadharana



Fig 3. Anupa

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Transverse Section Of Fruit And Root Of Bhumyamalaki

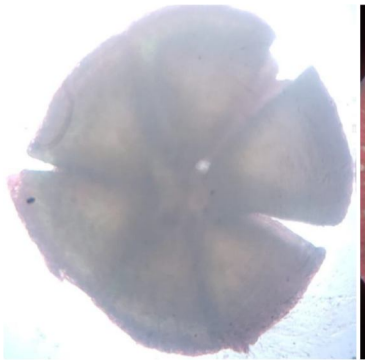


Fig 4

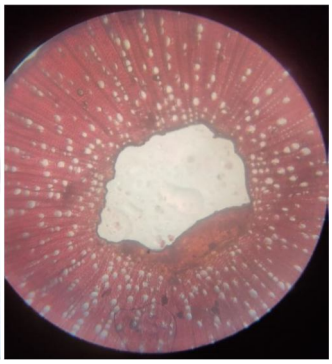


Fig 5

POWDER MICROSCOPY OF BHUMYAMALAKI



Fig 6

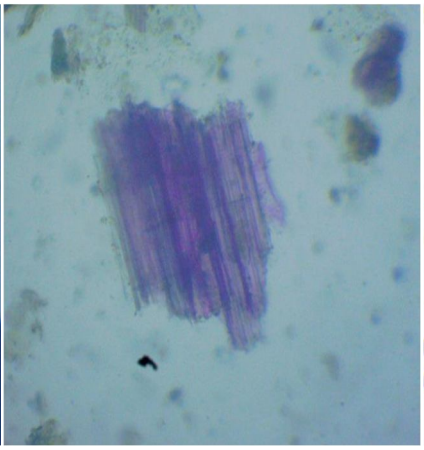


Fig 7

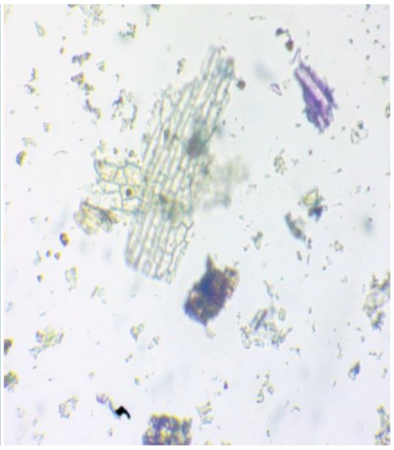


Fig 8

TLC OF METHANOLIC EXTRACT OF PANCHANGA OF BHUMYAMLAKI CHURNA

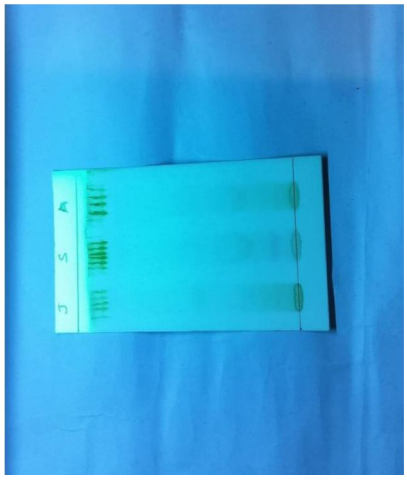


Fig 9. Observation in Short-UV

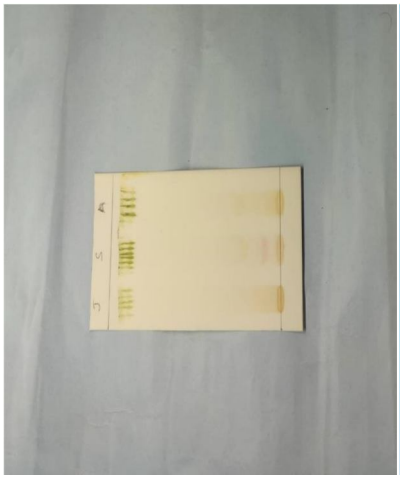


Fig 10. Observation in Visible light

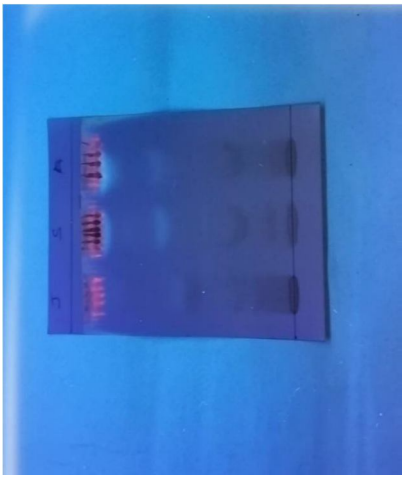


Fig 11. Observation in long-UV

VII. DISCUSSION

Bhumyamalaki is an important plant in the Indian system of medicine. The exhaustive literature survey revealed the pharmacological importance of the panchanga of Bhumyamalaki, Quality control by morphological and microscopical techniques plays an important role in the establishment of the appropriate identity of medicinal plants. Hence, the present study was planned to develop the botanical and phytochemical quality standards of the plant Phyllanthus fraternus.

The morphological study revealed a tap root system with a thin and tapered end. Roots were terminally branched with a length of 2.5–11 cm and thickness of 0.7–1.2 cm. The color of the roots was light brown, with no odour and taste. To study the anatomical features, powder microscopy and transverse section were done. The microscopic evaluation of powdered panchanga showed the presence of prismatic and rosette crystals. cellular structures like xylem vessels, spiral xylem vessel, pitted xylem vessels, cork cells and phyllanthus fibre cells with tannin content were also found. The transverse section was carried out to have a complete picture of the anatomical structure of the stem, root and the cell arrangement, cell positions, cell content etc. The transverse section showed cork cells followed by the cortex, phloem region, xylem region, medullary rays and pith from the periphery to the center. As per "The Ayurvedic Pharmacopoeia of India, standardization of plant drugs. The results of different parameters including loss on drying,ash values,extractive values were determined, The preliminary phytochemical analysis revealed the presence of alkaloids, triterpenoids, flavonoids, steroids and phenolics and these phytochemical groups are also reported.The chemical fingerprint analysis has more potential to reveal the chemical nature of plant constituents in totality than the marker analysis; therefore, in the present study, two analytical techniques, namely TLC and RP-UPLC were used to develop the chemical profile of Phyllanthus fraternus.

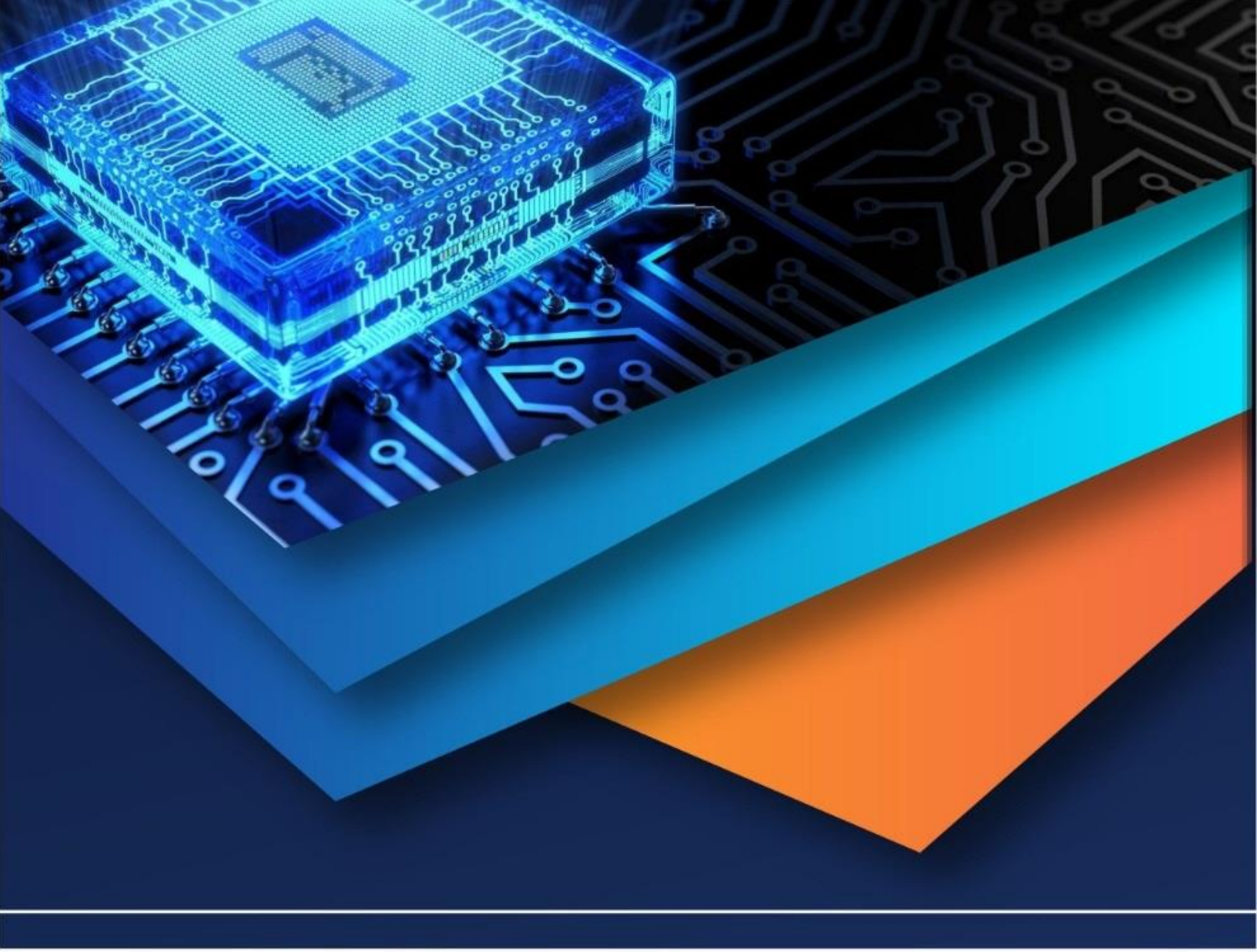
RP-UPLC was used to develop the fingerprint profile to have more details of the plant.All technical information generated from different analytical techniques supported the presence of lignan components as a major class of chemical constituents of Phyllanthus fraternus. These results can be further used to chemically explore the Phyllanthus fraternus for the isolation and characterization of chemical components. Further, important isolated components can be evaluated for their ethnopharmacological properties using in vitro and in vivo models. Moreover, the developed quality parameters can be used as a discriminative tool to differentiate the morphologically similar species.

VIII. CONCLUSION

The present study has generated a set of phyto-pharmacognostical quality parameters of Phyllanthus fraternus following macroscopic, anatomical, microscopical, physicochemical and chemical fingerprint (TLC, RP-UPLC) analysis. The generated data can serve as an investigative tool for the quality assessment, authentication and distinguishing medicinally significant plant species of the Phyllanthus.

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