Authentication of herbs by physiological & morphological studies through HPTLC, ICP-MS, UV Spectroscopy, Genetic Engineering & PCR

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Abstract- The increasing global market for herbal medicinal products (HMPs) require stringent quality control measures to combat adulteration, misidentification, and contamination. Traditional reliance on organoleptic and morphological studies alone is inadequate for complex, processed, or powdered materials as it contains more than 50 species. This article focus on research on *Eclipta alba* (Bhringaraj) by combined application of Physiological & Morphological studies, High-Performance Thin-Layer Chromatography (HPTLC), Inductively Coupled Plasma Mass Spectrometry (ICP-MS), UV Spectroscopy (UV), and Genetic Engineering (DNA Barcoding/PCR) as an integrated, multi-disciplinary strategy for the definitive authentication and quality assessment of herbal drugs. This synergistic approach provides a three-tiered confirmation—botanical origin, chemical profile, and safety (heavy metal) profile—essential for the standardization and regulation of herbal medicine.

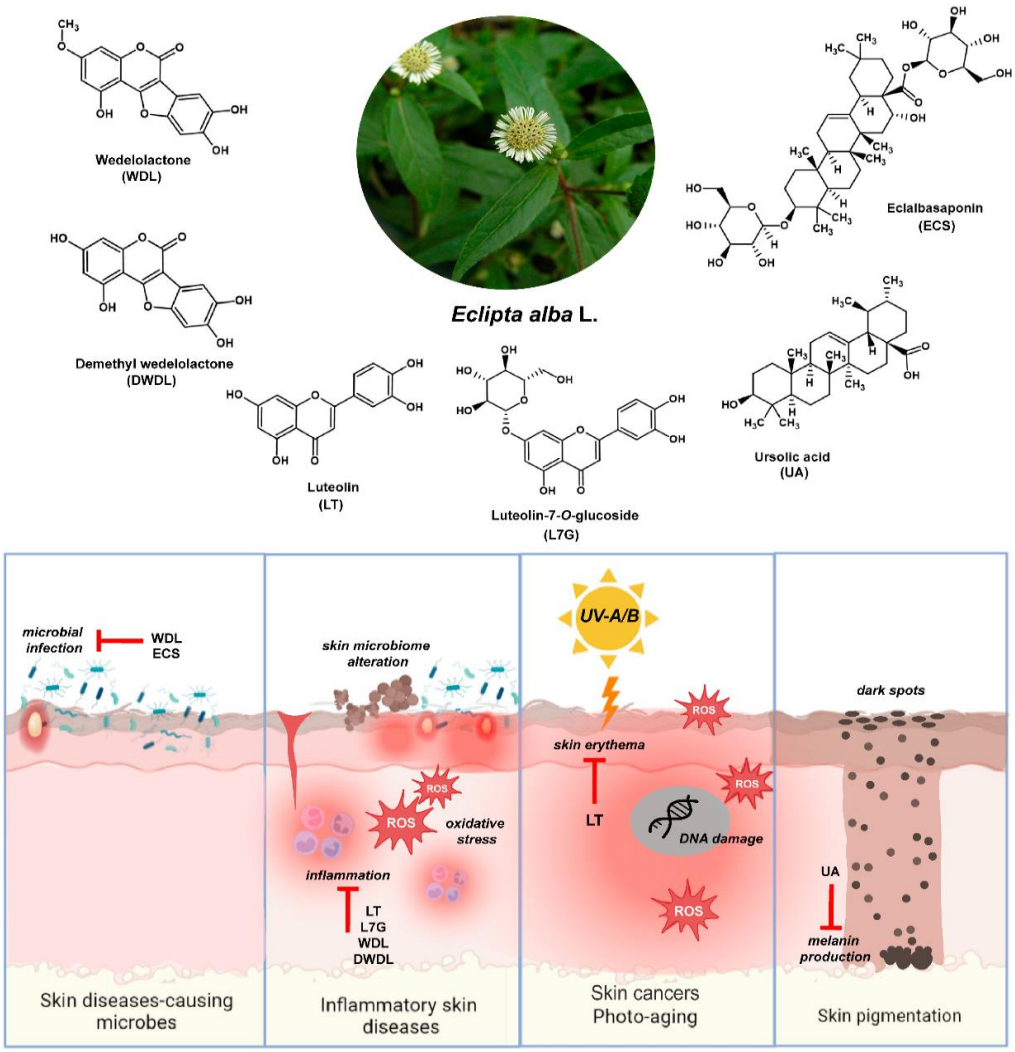
Introduction

For making an herb to globalize it is required to stringent quality control measures to combat adulteration, misidentification, and contamination. Authentication is the fundamental first step in the quality control of herbal materials, ensuring the correct plant species and part are used for therapeutic purposes. The World Health Organization (WHO) and international pharmacopoeias emphasize the need for robust methods to guarantee the safety, efficacy, and consistency of herbal products. Adulteration, where a genuine herb is intentionally substituted with an inferior or toxic alternative, poses a major threat to public health and erodes consumer trust. A comprehensive authentic-cation protocol must integrate classical and modern analytical techniques to provide an irrefutable identity profile. Bhringaraj is a small branched perennial herb. It is commonly found in India, China, Taiwan, Philippines, Japan and Indonesia. This herb has traditionally been used in ayurvedic medicine for being a liver tonic and having beneficial effects on diabetes, eye health, and hair growth.

Bhringaraj (*Eclipta alba*) oil is a well-

known for hair tonic to keep hair dark and stop hair loss. Sometime it is also termed as 'king of the hair'. *E. alba* decocted in coconut oil, is referred as 'cooling' oil. It is used externally for 'hot' and inflammatory like headaches, sinusitis and ear infections. The herb is helpful in heat problems. The whole plant of *Eclipta alba* works as a best medicine for hair growth. The presence of β-sitosterol in *Eclipta alba* help to rebuild hair in androgenic alopecia. β-sitosterol is usually used for heart disease, modulating the immune system, prevention of cancer, as well as for rheumatoid arthritis, tuberculosis, hair loss and benign prostatic hyperplasia. Other activities recorded include trypanocidal and mosquito larvicidal. Standardization, a tool in the quality control process thus, simply provides a quality assurance program for manufacturing of any herbal formulation. For quality control of herbal materials or their extracts one needs to proceed by selecting one of the different phytoconstituents of the product, preferably the one showing maximum desired bioactivity and subsequent method of quantification of that specific constituent is required to be developed.

HPTLC as well as HPLC are modern adaptations of chromatography with better and advanced separation efficiency and detection limits. HPTLC can be utilized to identify as well as quantify
the phytoconstituents present in a medicinal plant. These identification is required for regulatory perspective to ensure the efficacy, quality as well safety of the herbal drugs present in a plant. Presence of trace elements can be seen in medicinal plants. Low quantities of heavy metals like lead (Pb), cadmium (Cd), arsenic (As) and mercury (Hg) are toxic for human health when exposed for a long time. According to World Health organization the maximum perm-issible levels in food and drug materials of heavy metals as given for cadmium (Cd) and lead (Pb) which are 0.3 and 10 mg/kg respectively[1-11].



Material and Method

Equipments and reagents

All chemical used for HPTLC and UV Spectroscopy as well as physiochemical method are Analytical Grade and acid and standard used for ICP-MS analysis use ICP Grade.

HPTLC from CAMAG, ICP-MS from Perkin Elmer with model 1100, UV Spectroscopy from Shimadzu with model UV 1800.

Physiological and Morphological Studies

Physiological (organoleptic) and morphological (macroscopic and microscopic) evaluations remain the conventional starting point, especially for crude, whole, or fragmented herbal drugs.

- **Macroscopic Examination (Morphology):** Involves assessing external features such as size, shape, color, texture, fracture, and specific features like hairs or venation patterns.
- **Microscopic Examination (Physiological/Anatomy):** Involves the study of cellular and tissue elements, which are often unique to a species. Diagnostic features include the shape of trichomes, stomatal index, arrangement of vascular bundles, and the presence

of crystals (e.g., calcium oxalate) or starch grains.

High-Performance Thin-Layer Chromtography (Hptlc)

HPTLC is a powerful, economical, and high-through put chromatographic tech-nique widely used for herbal quality control and authentication.

- **Principle:** HPTLC separates the complex mixture of compounds in an herbal extract based on their differential partitioning between a stationary phase (e.g., silica gel plate) and a mobile phase (solvent system).
- **Sample Preparation:** weighed about 2 g of powder add 25 ml methanol reflux on water bath for 15 minutes, cool and filter. Standard of authentic E. alba is also prepared in the same manner. Apply 10 micro liter each of the standard and test solution on the TLC plate as a band of 10 mm. Develop the plate about 8 cm from the line of application.
- **Solvent system:** Toluene: acetone: formic acid (11: 6: 1).

Loss on Drying

Dry the evaporating dish for 30 min under the same conditions to be employed in the determination. Place about 5 to 10 g of powder/drug accurately weighed in a tared evaporating dish. Place the loaded bottle in the hot air oven. Dry the test specimen at 105 °C for 3 hours and weigh. Continue the drying and weighing at half an hour interval until difference between two successive weighing corresponds to, not more than 0.25 per cent.

Total Ash Value

Incinerate about 2 to 3 g, accurately weighed, of the ground drug in a tared platinum or silica dish at a temperature not exceeding 600 °C until free from carbon, cool in a desiccator for 30 min and weigh without delay. If carbon free ash cannot be obtained in this way, exhaust the charred mass with hot *water*, collect the residue on an ash less filter paper, incinerate the residue and filter paper, add the filtrate, evaporate to dryness, and ignite at a temperature not exceeding 600 °C. Calculate the percentage of ash with reference to the air-dried drug.

Alcohol Soluble Extractive

Macerate 5 g of the air dried drug, coarsely powdered, with 100 ml of *alcohol* of specified strength in a closed flask for 24 hours, shaking frequently during 6 hours and allowing to stand for 18 hours. Filter rapidly, taking precautions against loss of solvent, evaporate 25 ml of the filtrate to dryness in a tared flat bottomed shallow dish and dry at 105 °C, to constant weight and weigh. Calculate the percentage of alcohol-soluble extractive with reference to the air-dried drug.

Water Soluble Extractive

Macerate 5 g of the air dried drug, coarsely powdered, with 100 ml of chloroform water in a closed flask for 24 hours, shaking frequently during 6 hours and allowing to stand for 18 hours. Filter rapidly, taking precautions against loss of solvent, evaporate 25 ml of the filtrate to dryness in a tared flat bottomed

shallow dish and dry at 105 °C, to constant weight and weigh. Calculate the percentage of alcohol-soluble extractive with reference to the air-dried drug.

UV- Visible Spectroscopy (UV)

UV-Visible spectroscopy is a rapid, non-destructive technique used to characterize and quantify compounds containing chromo-spheres. In this research we use UV for determination of Total tannin and total flavonoids.

Estimation of Total Tannin Content

The tannins were determined by A.M. Diaz,

Analytical method. Absorbance for test and standard solutions were measured against the blank at 720 nm with an UV/ Visible spectrophotometer. The tannin content was expressed in terms of mg of tannic acid equivalents/ g of dried sample.

Sample preparation

Weight accurately about 0.1 g finely powered sample in 100 ml of purified water extract it with the help of condenser at 100°C using water bath for 1 hr, cool and decant the dissolved extract in to 500 ml volumetric flask. Washed the residue with purified water and make the volume up mark with same solvent. Filter the extract through whatman filter paper no.1. Discard first 50 ml of filtrate use next filtrate for analysis.

Standard preparation

Weight accurately about 100 mg of standard tannic acid in 100 ml standard flask and make up to volume with water (standard stock solution). Pipette out 1 ml from the above solution and makeup to 100 ml with water (standard solution).

Reagent preparation: 1. Prepare 1 % potassium ferri cyanide in water.
2. Prepare 1 % ferric chloride in water

Procedure: Take 1 ml of standard solution in 10 ml volumetric flask. Add 1 ml potassium ferri cyanide and 1 ml of ferric chloride. Mix well and make the volume up to 10 ml with purified water. Exactly 30 min after addition of the reagent read the optical density at 720 nm against reagent blank. Reagent blank is prepared by a diluting 1 ml potassium ferri cyanide and 1 ml of ferric chloride to 10ml with purified water.

Test solution: Take 0.2 ml of test solution and follow the same procedure as that as standard and measure the test absorbance (T) against reagent blank.

Test blank: Take 0.2 ml of test solution and make up to 10 ml with purified water and measure the absorbance (TB) against water.

Note-All the optical density readings should be taken exactly 30 min after additions of the reagents.

Determination of Flavonoids

The flavonoid content was determined by aluminum chloride method using Quercetin as standard. Extracts and Quercetin were

prepared in (10 mg/ mL). 0.1 mL of extract was mixed with 0.9 mL of distilled water in test tubes, followed by addition of 75 µL of 5% sodium nitrite solution. After 6 minutes, 150 µL of 10%aluminium chloride solution was added and the mixture was allowed to stand for further 5minutes after which 0.5 mL of 1M Sodium hydroxide was added to the reaction mixture. Then add 2.5 ml of distilled water and mixed well. The absorbance was measured immediately at 510 nm using a spectrophotometer. A calibration curve was generate during various concentrations of Quercetin (20-100µg). Blank consist of all the reagents, except for the extract or Quercetin is substituted with 0.1ml of Results were expressed as the Quercetin equivalence (QE) of the sample was expressed in mg/g of the extract.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

ICP-MS is an ultra-sensitive technique for the determination of trace and ultra-trace elements, critical for safety and geographical authentication.

Sample Preparation: sample is prepared with the help of Anton Paar microwave digester model no 3001. Weigh accurately 0.250 g sample in vessel and add 8 ml of ICP grade nitric acid swirl the content and seal the vessel after completion of acid reaction. Now put sealed vessel into the assembly and put the lid of assembly after that put it into the turnable plate of digester and run the program as prepared care should be taken that the sample after digestion should be clear and no residue should be left. The prepared sample will go for ICP-MS Analysis.

Prepare the method and sample file in the ICP-MS instrument by using Syngistix software and run the sample after getting daily performance check passed. Result of heavy metal will present in PPM.

Molecular Authentication: Genetic Engineering and Pcr-Based Tech-niques

DNA-based methods provide the most definitive species identification, unaffected by the plant's age, physiological state, or processing steps.

DNA Barcoding and Polymerase Chain Reaction (PCR)

DNA barcoding, a technique derived from genetic engineering principles, uses short, standardized gene regions to identify species.

Process: 1. DNA Isolation: Extracting DNA from the herbal material.

2. **PCR Amplification:** Using the Polymerase Chain Reaction (PCR) with universal primers (e.g., *ITS2*, *matK*, or *rbcL*) to amplify the target barcode region[10].
3. **Sequencing and Analysis:** Sequencing the amplified fragment and comparing the resulting sequence against validated reference databases (e.g., GenBank or BOLD).

4. **Authentication Value:** PCR-based DNA barcoding provides a **molecular fingerprint** that can reliably distinguish between closely related species (substitutes) and detect low-level adulteration in powdered or multi-component formulations. Species-specific PCR assays are also developed for rapid screening of known adulterants [12-23].

Result and Discussion

Macroscopic Study

The plant is herbaceous; the leaves are opposite and dark green. The stem and leaves bear characteristic white hairs. The inflorescence is found at the axis of the leaves.

Microscopic Study

A transverse section of the root shows the following characters:

1. An outer cork layer which consists of reddish brown cells (Fig.1a).
2. The cortical zone inner to the epidermis consists of Parenchymatous cells, which are thin walled, and loosely arranged (Fig.4b). They are 38-50-75µ X 30-45-50µ. They contain starch grains upto 2.5µ in diameter. The cortical zone shows the presence of resin canals with brownish black contents in them.
3. The stele shows an outermost endodermis, which is not very distinct. Inner to the endodermis, pericyclic fibres are seen (Fig.1c), arranged in patches as a ring. These fibres are 18-50µ in diameter. The phloem lies inner to these fibres (Fig.1d).
4. The centre of the stele is filled with the xylem elements (Fig.1e). The xylem vessels are arranged in close radial rows surrounded by the xylem fibres. 1-2 seriate medullary rays are also seen. The vessels have a diameter ranging from 15-50µ.

A transverse section of the stem shows the following characters:

1. An epidermis consisting of rectangular cells which are 20-25µ X 10-13µ (Fig.3a). Some of the cells bear multicellular glands (Fig.5a).
2. The hypodermis lies inner to the epidermis and consists of collenchyma (Fig. 2a, 3b) which are thick walled cells having a diameter of 15-30µ.
3. The cortex is made up of loosely arranged, thin walled parenchymatous cells filled with starch grains measuring 45-75µ in diameter (Fig.2b, 3c). The inner cortex shows a few resin canals (Fig.4e).
4. The stele consists of the outermost pericyclic fibres (Fig3d, 4b). The cambium is in-between the phloem and is found outer to the xylem (Fig.4c), which occurs towards the pith (Fig.4d). The secondary xylem just beneath the cambium is formed as a continuous ring unlike the primary xylem, which is formed in bundles.
5. The central pith is large and consists of loosely arranged parenchyma (Fig.2d).

A transverse section of the lamina of the leaf shows the following characteristics:

1. An outermost epidermis which has characteristic hairs and multicellular glands (Fig.7a, 8).
2. The palisade parenchyma that

consists of elongated cells (Fig.6a) and the spongy parenchyma which has circular cells (Fig.6b). Both these constitute the mesophyll tissue.

3. The lower epidermis bears characteristic hairs.

Diagnostic features:

1. All parts of the plant except the roots bear characteristic white hairs.
2. The leaves are opposite and bear the inflorescence in the axis.
3. The root and the stem show resin canals in the cortical region.
4. The stem and leaves bear multicellular glands.

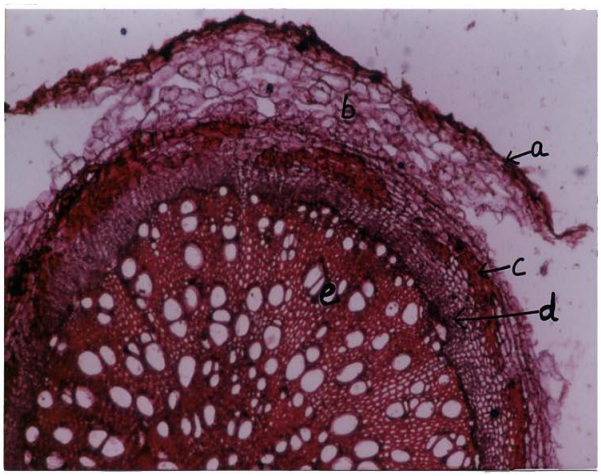


Figure- 1 TS of root (4 x)
a. Cork
b. Cortex
c. Pericyclic fibres
d. Phloem
e. Xylem

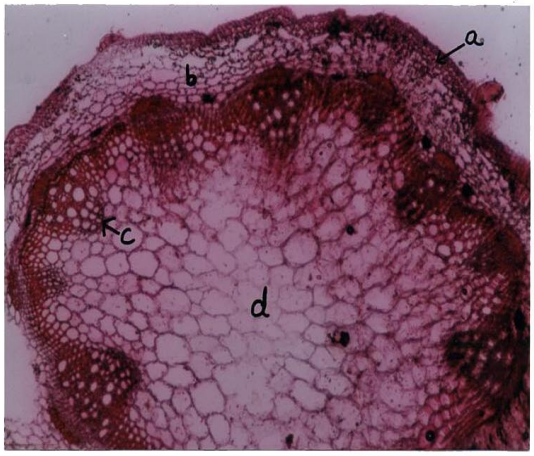


Figure-2 TS of stem (4 x)
a. Hypodermis
b.Cortex
c. Vascular bundles
d. Pith

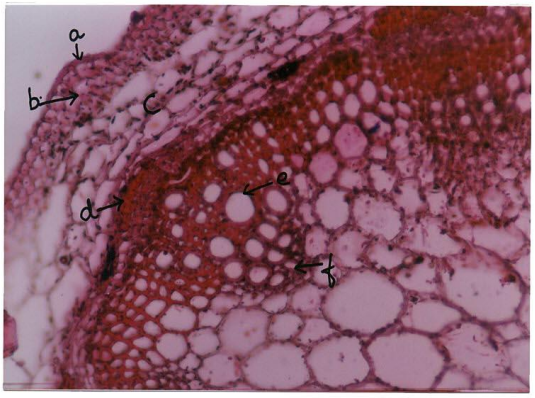


Figure- 3 TS of stem (10 x)
a. Epidermis
b. Hypodermis
c. Cortex
d. Pericyclic fibres
e. Metaxylem
f. Protoxylem

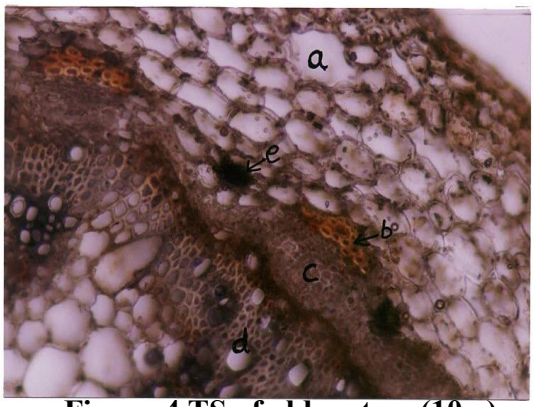


Figure-4 TS of older stem (10 x)
a. Cortex
b. Pericycle
c. Phloem
d. Xylem
e. Resin canal

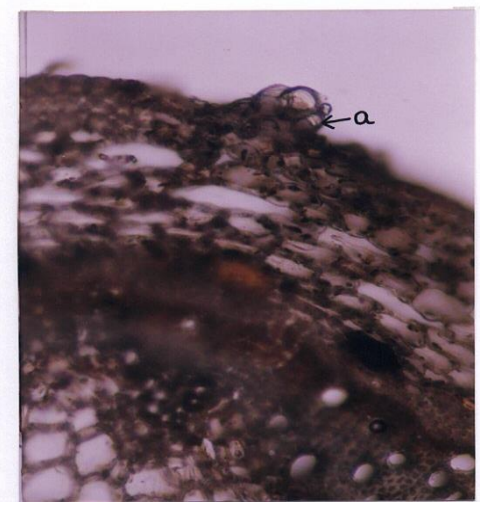


Figure- 5 TS of stem (40 x)
a. Multicellular gland

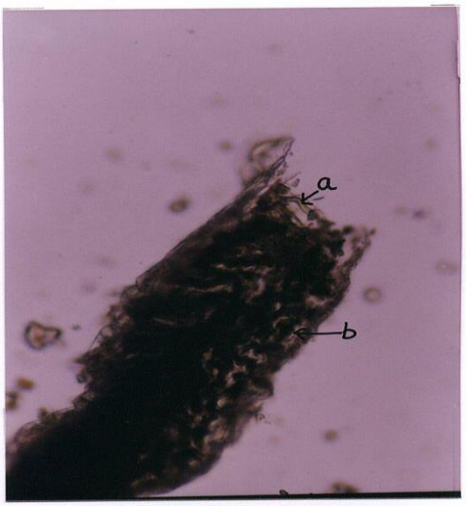


Figure-6 TS of lamina of leaf (10 x)
a. Pallsade parenchyma
b. Spongy parenchyma

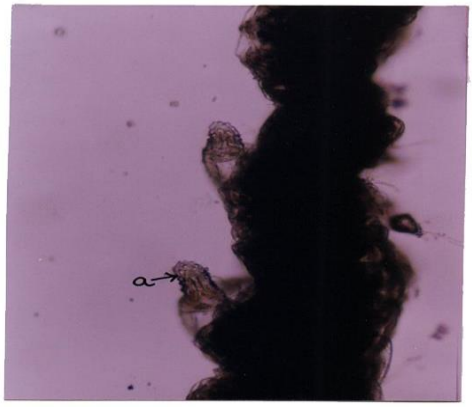


Figure- 7 TS of lamina of leaf (10 x)
a. Glandular hairs

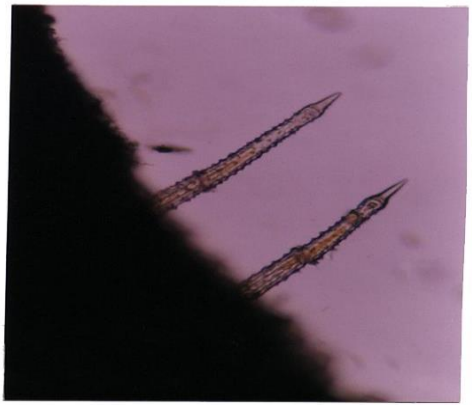
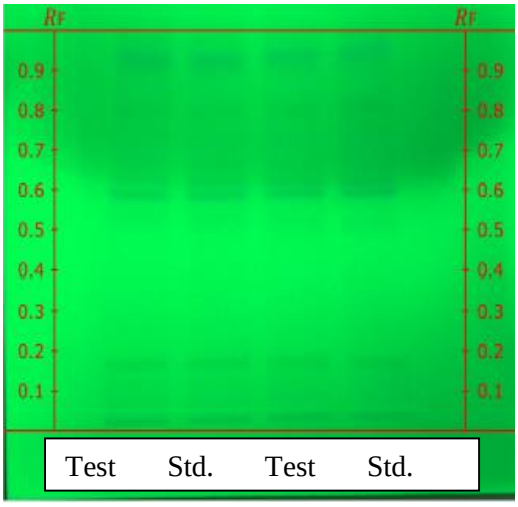
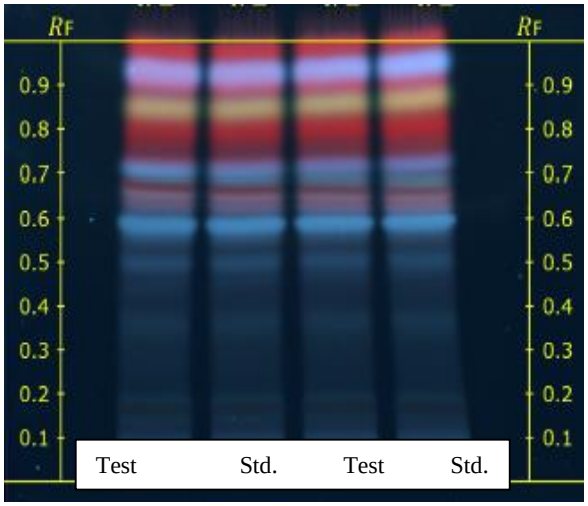


Figure- 8 Glandular hairs of the leaf (10 x)

HPTLC Chromatogram and analysis:



Chromatogram at 254 nm



Chromatogram at 366 nm

Table-01 Physicochemical characters of *Eclipta alba* powder

Sr. No.	Parameters	Observation
1	Foreign matter	0.78 % w/w
2	Acid-insoluble ash	8.57 % w/w
3	Water soluble extract	25.02 % w/v
4	Alcohol-soluble extractive	14.43 % w/v
5	Loss on drying at 105 °C	4.2 % w/w
6	Total Ash value	17.43 % w/w

Table-02 Total Tannin Content determine by using UV Spectroscopy technique

Sr. No.	Absorbance	Total Tannin content mg/g equivalent to Tannic acid.
01	0.345	320
02	0.351	322
03	0.339	318
Mean ± SD		320 ± 2

Table-03 Total Flavonoid Content determine by using UV Spectroscopy technique

Sr. No.	Absorbance	Total Flavonoids content mg/g equivalent to Quercetin.
01	0.198	130
02	0.197	128
03	0.199	132
Mean ± SD		130 ± 2

Table-04 Heavy Metal determination by Perkin Elmer ICP-MS

Sr. No.	Heavy Metals			
	Lead (Pb) (PPM)	Cadmium (Cd) (PPM)	Arsenic (As) (PPM)	Mercury (Hg) (PPM)
01	0.95	0.35	0.05	0.004
02	3.15	0.62	0.07	0.009
03	1.45	0.54	0.10	0.007

Conclusion

Authentication of herbs by physiological and morphological studies through HPTLC, ICP-MS, UV Spectroscopy & PCR evaluation of *Bhringaraja* (*Eclipta alba* Hassk.) for whole plant provided specific parameters that will be useful in scientific evaluation, identification and authentication of the particular species and its medicinal products. This study could serve as a

constructive reference to allow further in-vivo analysis which can be conducted to evaluate the extent of protective effects of *Eclipta alba* against chemically induced cellular damage. The present investigations revealed that *Eclipta alba* contain significant amount of phenols and flavonoids while heavy metals in a regulatory limit and hence can be globally used for manufacturing of herbal preparation.

Disclaimer Statement

Authors declare that no competing interest exists. The products used for this research are commonly used products in research. There is no conflict of interest between authors and producers of the product.

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