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Morpho-anatomical and histochemical comparison of *Centella asiatica L.* and hydrocotyle verticillata Thunb

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Abstract
The present study focuses on the morpho-anatomical studies and histochemical studies for identifying the similarities and differences between the two plants. As both the plants are often confused as the same plant that is used for brain development and have been mistakenly consumed by the peoples, we carried out the study to distinguish the unique features of both the study plants. The morphological and the anatomical studies showed that both the plants possess both similar and different characters. Plants possess different phytochemical constituents which is responsible for the defence mechanism in plants. The histochemical studies revealed that both the study plants possess almost same kind of phytoconstituents but the localization of such phytoconstituents differ among the two plants. Thus this study helps for the proper identification and authentication of the study plants.

Keywords: Morpho-anatomical, *Centella asiatica*, *Hydrocotyle verticillata* and riparian

Introduction
The plants are the sources of producing many conventional drugs. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Ahmad *et al*., 2015) [1]. The bioactive components of these plants are great sources for new therapeutic agents. These therapeutic agents are of great importance nowadays because these pose new hope for disease prevention (Daminar and Bajo, 2013) [4]. It has been estimated that 14 - 28% of higher plant species are used medicinally and that 74% of pharmacologically active plant derived components were discovered after following up an ethnomedicinal use of the plants.

The different vegetation distribution in India and Tamil Nadu are Forest vegetation, Grassland vegetation, Riparian vegetation, Aquatic and semi aquatic vegetation. Riparian vegetation comprises plant communities that grow laterally to rivers and streams. Riparian vegetation is affected by both flood process and the characteristics of landforms that are shaped by floods (Bendix *et al*., 2000) [2]. The study plants *Centella asiatica* and *Hydrocotyle verticillata* belongs to the family Apiaceae are collected from the riparian vegetation which are rich in phytoconstituents and possess more medicinal value.

The plant *Centella asiatica* is commonly seen in the borders of rice fields. The leaves resemble the structure of human brain. It has been used by Ayurvedic medical practitioners for almost 3000 years to treat wounds, mental and neurological disorders, artherosclerosis, microbial infections and cancer (Pokhrel and Neupane, 2021) [10]. *Centella asiatica* is a commonly used herb in the Varmam treatment and is mentioned as 'Vetri pachilai' and 'Vallarai' in Siddha Varmam literatures. It is administrated both as internal and external medicine (Vanmathi Palraj and Harihara Mahadevan, 2018) [16].

The plant *Hydrocotyle verticillata* Thunb. is commonly known as water pennywort. It usually grows in freshwater swamps, lagoons and along streams and rivers, sometimes partially submerged. Its juice is used for the treatment of fevers. The poultice is used for the treatment of wounds and boils. The decoction of the plant is used for the treatment of abscesses, colds, coughs, hepatitis, influenza, pruritus and sore throat (Umate and Deogade, 2020) [15].

As the pharmacognostical study of *Centella asiatica* L. and *Hydrocotyle verticillata* Thunb.has not yet been studied fully. The present study aimed to reveal the differences between the plants *Centella asiatica* and *Hydrocotyle verticillata* through morphological, anatomical and histochemical analysis.

Materials and methods
Collection and identification of plants
The plant *Centella asiatica* L. and *Hydrocotyle verticillata* Thunb. were collected from the bank of Bhavani river in Erode district, Tamilnadu. The authenticity of the plants was confirmed by referring the research articles.

Macroscopical analysis
Macroscopic characters of simple determination technique which includes

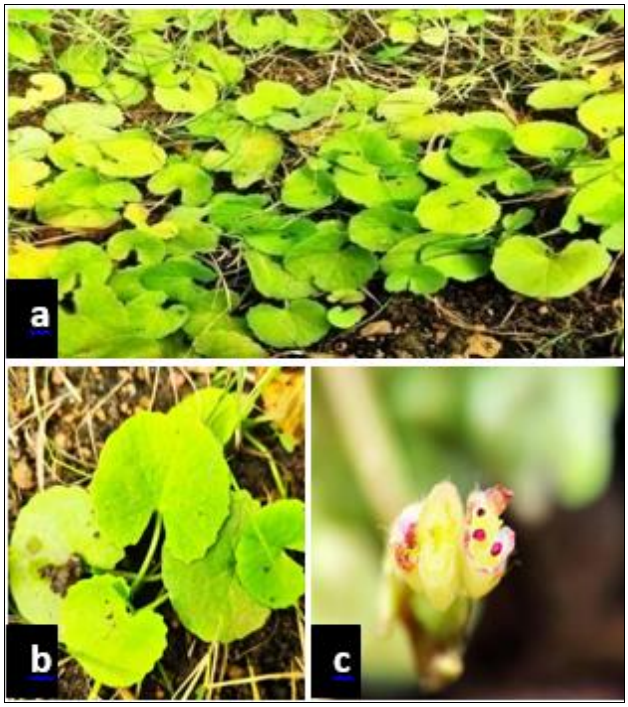


Fig 1: Images of *Centella asiatica*, a- habit, b- leaves, c- inflorescence

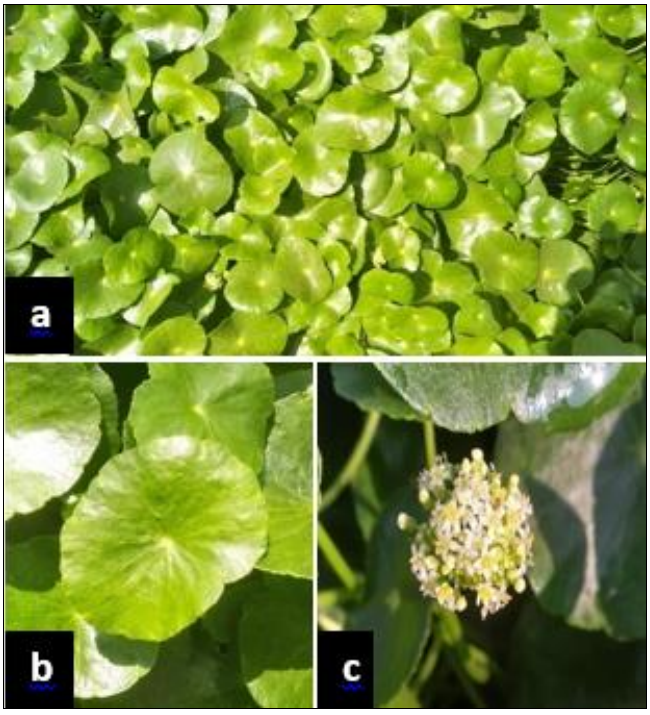


Fig 2: Images of *Hydrocotyle verticillata*, a- Habit, b- Leaves, c- Inflorescence

organoleptic studies like shape, size, colour, odour of plant and its extra features. The entire plant of *Centella asiatica* and *Hydrocotyle verticillata* were analysed (Gamble and Fischer 1915 - 1936) [6] in the field and photographed under original environment and evaluated botanically.

Histochemical analysis

Sectioning

The free hand sections were taken in the petiole. The thickness of the sections was according to Johansen (1940) [8]. The sections were stained with the respective reagents used for histochemical analysis as per the method of O'Brien *et al.*, (1964) [9]. Glycerine mounted temporary preparations were made for macerated cleared materials. Powdered materials of different parts were cleared with NaOH and mounted in glycerine medium after staining. Different cell components were studied and measured.

Pictomicrographs

Photographs were taken with different magnification in Nikon lab photo 2 microscopic unit. Magnifications were indicated by the scale bars in the micrographs. The colour identification indicates the presence of the certain compounds.

Microscopical characterization was made by employing standard sectioning and staining methods as per standard procedure described by O'Brien *et al.*, (1964) [9]. The cell arrangements, size and shape of cell, cell inclusions, synthesis and distribution were localized histochemically in the petiole of study plants using respective reagents and were recorded on a photonic microscope (Model Ax70 TRF, Olympus optical). Fresh free hand sections of petioles of *Centella asiatica* and *Hydrocotyle verticillata* were taken. The fresh free hand sections of the petiole were used for the histochemical study and were treated with the respective reagents to localize the presence or absence of metabolites synthesis and their storage area.

Test for Alkaloids

Dragendorff's reagent consists of two solutions: Solution A – 1.7g basic bismuth subnitrate in 100ml water/acetic acid (4:1 v/v) and Solution B – 40.0g Potassium iodide in 100ml of water. Dragendorff's reagent was prepared by mixing 5ml of Solution A + 5ml of Solution B + 20ml acetic acid + 70ml water. Sections are placed in Dragendorff's reagent. The development of orange brown colour indicates the presence of alkaloids. (Yoder and Mahelberg, 1976) [17]

Test for Starch

Freehand sections were placed in iodine- potassium iodide solution (0.2g of iodine dissolved in 2% of potassium iodide solution). Starch appears blue or black in few seconds (Johansen, 1940) [8].

Test for Carbohydrates

Freehand sections were placed in the Fehling's solution (Equal amount of Fehling's solution A and Fehling's solution B) and then mounted to observe the carbohydrates that are stained Pink.

Test for Proteins

Freehand sections were immersed in the Biuret reagent for few seconds. The development of light blue to dark blue colour indicates the presence of proteins.

Test for Flavonoids

Freehand sections were placed in the 25% Lead acetate solution. Flavonoids are stained yellow in colour.

Test for Lipids

Free hand sections were placed in the Sudan I solution. The development of Yellow colour indicates the presence of lipids.

Test for Phenols

Freehand sections were placed in the alcoholic ferric chloride solution and then mounted for microscopic observation. The Green colour indicates the presence of phenols.

Test for Tannin

Freehand sections were dipped in the solution containing neutral ferric chloride and few drops of sodium carbonate. The dark black colour indicates the presence of tannin.

Results

Morphological studies

Centella asiatica

The plant *Centella asiatica* is a quite aromatic, prostrate, perennial, stoloniferous, creeper herb, which height up to 15 cm. Stem is striated, glabrous and rooting at the nodes. Leaves are simple, alternate, exstipulate, petiolate, all leaves arising from rhizomes, 7 – 8 in number. Lamina reniform, marginal shallow crenate to dentate, green in colour, 1.2 to 5.0 cm in diameter. Inflorescence axillary, umbel, each umbel consists of 3 or 4 flowers. Flowers incomplete, bisexual, irregular, bracteate, pedicellate and pedicel is short. Stamens 5, epiphyllous, anthers bilobed and filaments are short. Ovary superior, bicarpellary, syncarpous, locules 2, styles 2, terminal, stigma simple. Fruit cremocarp (Fig – 1).

Hydrocotyle verticillata

The plant *Hydrocotyle verticillata* is a herbaceous groundcover perennial, glabrous, succulent prostate herb. Stem creeping, rooting at the nodes, with long stolon. Leaf simple, alternate, leaflets orbicular and palmately lobed, margin serrate, shiny, blackish green above and pale beneath, prominently veined with long petiole. Inflorescence long arising from the axil of the leaves. Each flower subtended by a minute bract at the base. Stamens usually 5. Ovary orbicular, glabrous; style filiform, divaricate. Fruits sub-orbicular, broader than long, laterally compressed with prominent ribs (Fig – 2).

Anatomical studies

Petiole of *Centella asiatica*

The transverse section of the petiole is angular in outline. The epidermis is compactly arranged and covered with thick cuticle. The epidermis is followed by 3- 4 layers of collenchymatous hypodermis is present. It is followed by 2-3 layers of chlorenchymatous cells. The vascular bundles are present in the parenchymatous zone. The bundles are embedded in the parenchymatous region. Each vascular bundle consists of phloem on adaxial side and xylem on abaxial side with sclerenchymatous bundle cap (Fig – 3A).

Petiole of *Hydrocotyle verticillata*

The transverse section of the petiole shows wavy in outline. The epidermis is compact and made up of barrel shaped rectangular cells which is covered with thick cuticle. The cortex is distinguished into collenchymatous hypodermis, chlorenchymatous region and parenchymatous ground tissue. The vascular bundles are embedded in the ground tissue with 'U' shaped xylem which is surrounded by phloem (Fig – 3B).

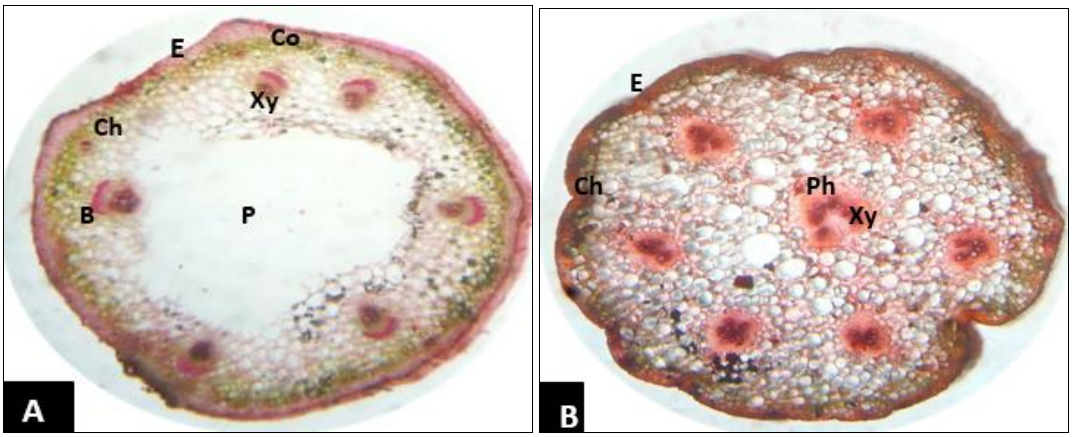
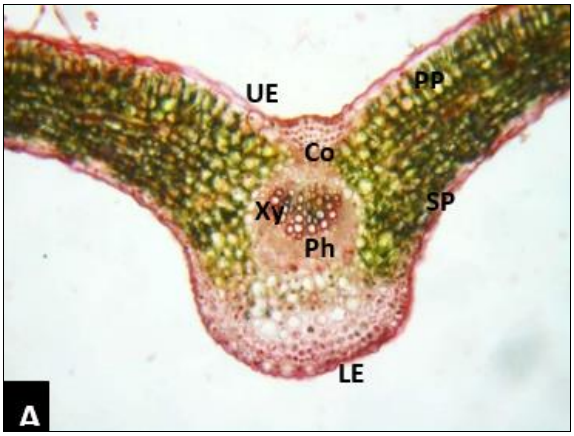


Fig 3: Images showing Transverse section of petiole of A- *Centella asiatic*a and B- *Hydrocotyle verticillata,* E – Epidermis, Co – Collenchymatous cortex, Ch – Chlorenchymatous cortex, B – Bundle cap, Xy – Xylem, Ph – Phloem, P – Pith.

Leaf of *Centella asiatica*

The Transverse section of the leaf shows the mesophyll region and lamina region. The epidermis is compactly arranged and covered with thick cuticle. Beneath the cuticle, epidermis is present. In the mesophyll region, following the upper epidermis and above the lower epidermis, there is a presence of collenchymatous region. The collenchymatous region, followed by parenchymtous region is present. The vascular bundle is present in the midrib region with xylem in exarch condition. In the lamina region, the epidermis is followed by 3 – 4 layered radially elongated palisade parenchyma cells. It is then followed by spongy parenchyma. Stomata is present (Fig – 4A).



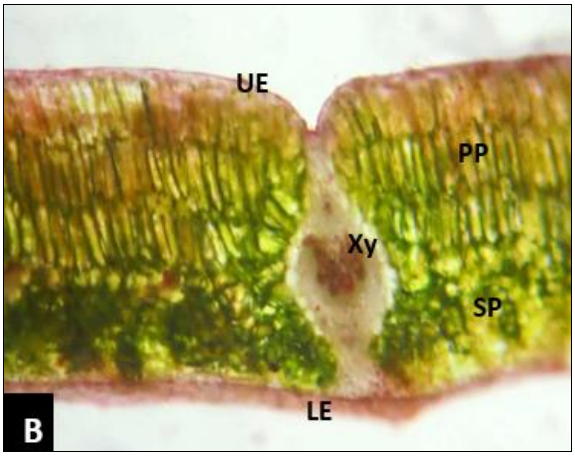


Fig 4: Images showing transverse secction of leaves of A- *Centella asiatica* and B- *Hydrocotyle verticillata*, UE – Upper Epidermis, Co – Collenchymatous hypodermis, PP – Palisade Parenchyma, SP – Spongy Parenchyma, Xy – Xylem, Ph – Phloem, LE – Lower Epidermis.

Leaf of *Hydrocotyle verticillata*

In the transverse section of the leaf, the epidermal layer is present in the upper and lower side of the leaf. It is the single layer of parenchymatous cells without intercellular spaces. The epidermis is followed by cortex. The cortex is distinguished into palisade parenchyma and spongy parenchyma. In the midrib region, the epidermis is followed by the collenchymatous region. The vascular bundle consists of xylem and phloem. Stomata is present (Fig – 4B).

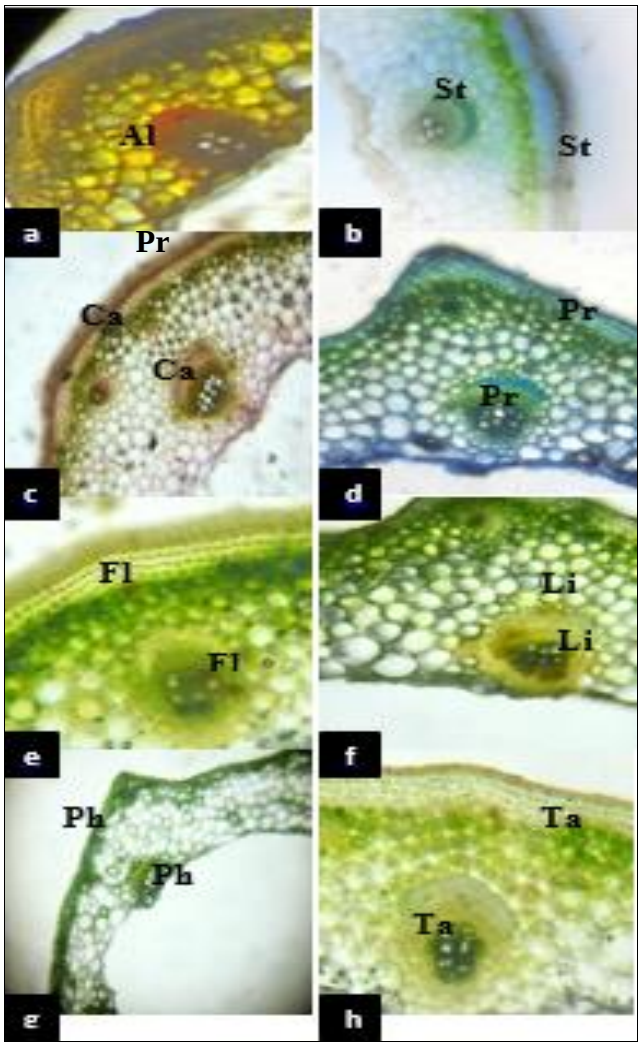
Histochemical studies

Histochemistry is the study of distribution of chemical compounds within biological cells using histological techniques such as histology stains, indicators and microscopy. It has been carried out to detect various phytochemical groups localized in different tissue zones of the plant parts. The results revealed the presence of proteins, lipids, alkaloids, flavonoids, carbohydrates, tannins, phenols and starch in various tissue zones of the petioles of *Centella asiatica* and *Hydrocotyle verticillata* which is represented in the Figure 5 and 6. This test highlights that vascular bundles and cortical zone are the main sites for the synthesis or storage of different phytochemical groups.

Centella asiatica

Alkaloids

Alkaloids are degradation products of protein. They were investigated by using Dragendorff's reagent.



Al – Alkaloids Fl - Flavonoids Ca – Carbohydrate Ph - Phenols
St – Starch Li – Lipids Pr – Proteins Ta –Tannin

Fig 5: Images showing Histochemical analysis of plant *Centella asiatica;* a- Alkaloids, b- Starch, c- Carbohydrates, d- Proteins, e- Flavonoids, f- Lipids, g- Phenols, h- Tannin.

The Dragendorff's reagent was observed in the sclerenchymatous bundle cap region which surrounds the vascular bundle. The alkaloids containing cells become reddish orange in colour (Fig – 5a).

Flavonoids
The localization of flavonoids by 25 % lead acetate solution is indicated by the yellow colour. The yellow colour was observed in the epidermis, few regions of cortex and in the phloem regions (Fig – 5e).

Carbohydrates
Carbohydrates were observed in the epidermis, few regions of cortex and bundle cap when the sections were placed in the Fehling's solution (Fig – 5c).

Lipids
Lipids are widely distributed in the plant body and they probably occur in small amounts in every plant. In taxa under study, lipids were localized in the cells of epidermis, few regions of cortex region and in the phloem by using Sudan I solution (Fig – 5f).

Phenols
Phenols were distributed mostly in the epidermis, cortex, xylem and in the phloem regionswhen the sections were placed in the alcoholic ferric chloride solution (Fig – 5g).

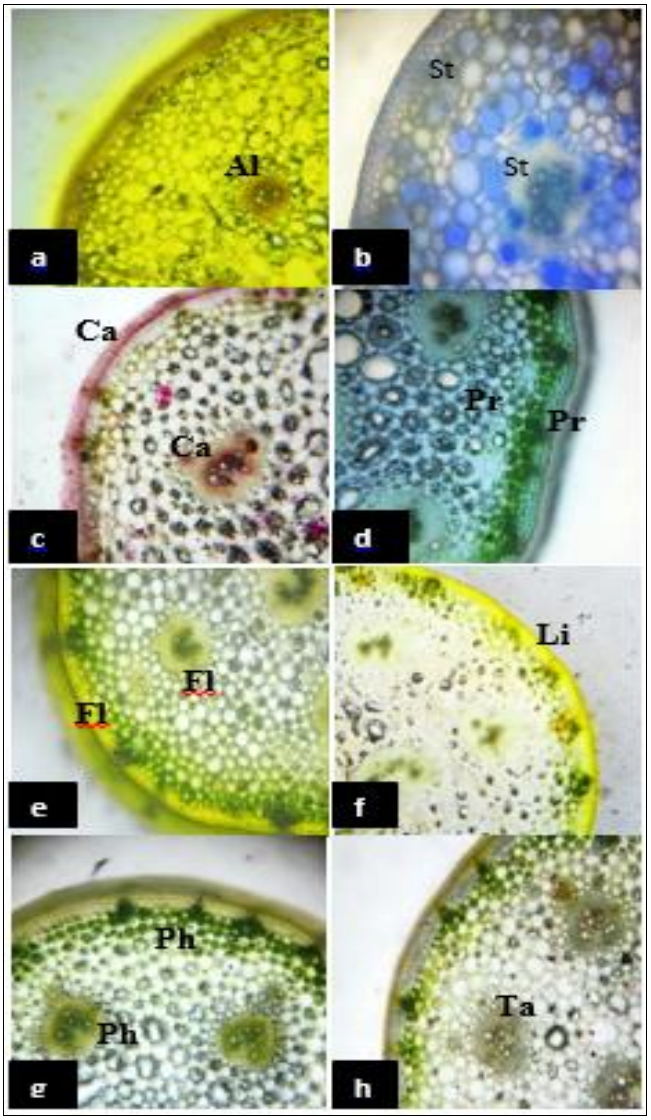
Protein
Protein was the major constituent of the living protoplast but they also occur temporarily as inactive ergastic substance. Ergastic protein is known as storage material and found deposited in amorphous and or crystalline forms. This Biuret reagent indicates that the proteins are found in the epidermis, cortical region and in the bundle cap in the taxa under study (Fig – 5d).

Starch
Starch is the principal ergastic substance of the protoplast. The iodine in potassium iodide solution helps to localize starch in the petiole especially in the epidermis and in few regions of cortex of *Centella asiatica* (Fig – 5b).

Tannin
Tannins were observed in some regions of cortex, xylem and some intercellular space of petiole in the test plant when it is placed in the solution containing neutral ferric chloride and sodium carbonate (Fig – 5h).

***Hydrocotyle verticillata* Thunb.**
Alkaloids
Alkaloids are degradation products of protein. They were investigated by using Dragendorff's reagent. The Dragendorff's reagent was observed in the epidermis and in the xylem region. The alkaloids containing cells become reddish orange (Fig – 6a).

Flavonoids
The localization of flavonoids by 25% lead acetate solution is indicated by the yellow colour. The yellow colour was



Al – Alkaloids Fl - Flavonoids
St – Starch Li - Lipids
Ca – Carbohydrate Ph - Phenols
Pr – Proteins Ta – Tannin

Fig 6: Images showing Histochemical analysis of plant *Hydrocotyle verticillata;* a- Alkaloids, b- Starch, c- Carbohydrates, d- Proteins, e- Flavonoids, f- Lipids, g- Phenols, h- Tannin.

Observed in the epidermis, few regions of cortex and in the phloem region (Fig – 6e).

Carbohydrates
The Fehling solution shows the presence of carbohydrates in the epidermis, few regions of cortex and in the xylem tissues (Fig – 6c).

Lipids
Lipids are widely distributed in the plant body and they probably occur in small amounts in every plant. In taxa under study, the Sudan I solution is used to localize lipids and it is found in the cells of epidermis, cuticle region, few cells of cortex and phloem (Fig – 6f).

Phenols
Phenols were observed in the few regions of cortex and xylem when placed in alcoholic ferric chloride solution (Fig – 6g).

Protein
Protein was the major constituent of the living protoplast, but they also occur temporarily as inactive ergastic substance. Ergastic protein is known as storage material and found deposited in amorphous and or crystalline forms. Protein was observed in the epidermis, cortical region and in the phloem of *Hydrocotyle verticillata* when the sections are placed in Biuret reagent (Fig – 6d).

Starch
Starch is the principal ergastic substance of the protoplast. In the present work, the iodine in potassium iodine solution helps to localize starch in few regions of the cortex (Fig – 6b).

Tannin
Tannin is a heterogeneous group of phenol derivatives, usually related to glycosides. Tannins were observed in the regions of cortex in the taxa under study when it is placed in a solution containing neutral ferric chloride and sodium bicarbonate (Fig – 6h).

Discussion and Summary
The comparative analysis of *Centella asiatica* and *Hydrocotyle verticillata* through anatomical and histochemical approach is limited. The medicinal property of the plants are due to the presence of various phytoconstituents. These are responsible for the antimicrobial activity of the plant extracts. The present study focused on the comparative analysis of *Centella asiatica* and *Hydrocotyle verticillata* on the basis of morphology, anatomy, histochemical studies.

Morphological studies
The present study revealed that the leaves of the *Centella asiatica* are reniform but the plant *Hydrocotyle verticillata* possess orbicular leaves. Chua *et al*., (2022) [3] reported that *Centella asiatica* has kidney shaped leaves with second-order veins branched off at the intervals of several first-order veins but *Hydrocotyle verticillata* posses rounded leaves with multiple first-order veins by using in-house leaf image recognition system.

Table 1: Histochemical analysis of *Centella asiatica* L. and *Hydrocotyle verticillata* Thunb.

S. No.	Test	Reagents used	Localization		Colour
			Centella asiatica	***Hydrocotyle verticillata***	
1.	Alkaloids	Dragendorff's reagent	Bundle cap	Epidermis, xylem	Reddish orange
2.	Starch	Iodine – potassium iodide solution	Epidermis, cortex	Cortex	Blue
3.	Carbohydrate	Fehling's A & B	Epidermis, cortex, bundle cap	Epidermis, cortex, xylem	Pink
4.	Protein	Biuret reagent	Epidermis, cortex, bundle cap	Epidermis, cortex, phloem	Blue
5.	Flavonoids	25% Lead acetate	Epidermis, phloem, cortex	Epidermis, phloem, cortex	Yellow
6.	Lipids	Sudan I	Epidermis, cortex, phloem	Cuticle, Epidermis, Cortex, phloem	Yellow
7.	Phenols	Alcoholic ferric chloride	Epidermis, cortex, xylem, phloem	Cortex, xylem	Green
8.	Tannins	Ferric chloride and sodium carbonate	Cortex, xylem, intercellular space	Cortex	Dark blue to black

Anatomical studies
The anatomical structure of *Centella asiatica* and *Hydrocotyle verticillata* are used to discriminate both of the study plants. There is a presence of hollow pith in the petiole of *Centella asiatica* but it is not seen in *Hydrocotyle verticillata*. The outline is angular in *Centella asiatica* but in *Hydrocotyle verticillata*, it is wavy. In the petiole of *Hydrocotyle verticillata*, the study made by Umate and Deogade (2020) [15] revealed that the epidermis is followed by hypodermis and central ground tissue and the vascular bundles are abruptly distributed all over the ground tissue. Sudhakaran (2017) [12] identified the hollow pith in the petiole of *Centella asiatica.*
The transverse section of the leaf of *Centella asiatica* have a slight depression on the midrib region whereas the transverse section of the leaf of *Hydrocotyle verticillata* is more or less linear. Sudhakaran (2017) [12] reported that the lamina was flat and much reduced in dimension compared to the midrib in *Centella asiatica.*

Histochemical studies
Histochemical analysis is one of the valuable standardization processes of quality control of crude drugs to locate the presence of ergastic cell contents in the histological zones of the plant (Prabhu *et al.,* 2011) [11]. Chemical analysis and biological assays are very important aspects in pharmacognostic evaluation of medicinal plants (Trease and Evans, 1983) [14]. The study was designed to notice whether the histochemicals can deliver some unique features which are helpful to plant taxonomy or not. Information concerning the histochemical study of *Centella asiatica* and *Hydrocotyle verticillata* are limited. The presence of some ergastic cell contents such as carbohydrate, protein, lipids, alkaloids, flavonoids, tannins, phenol and starch in various tissue zones of the petioles are micro-photographed.
In the present study the results revealed the localization of alkaloids by Dragendroff's reagent and the same has been indicated by the reddish orange colour which was observed in the sclerenchymatous bundle cap regions of plant *Centella asiatica* and in the xylem and epidermal regions of *Hydrocotyle verticillata*. This results are in accordance with the study made by Dhale *et al*., (2011) [5] in the stem of *Adhatoda zeylanica* in that study, the alkaloids are present in the regions of epidermis, cortical parenchyma, vascular bundle and pith parenchyma.
In the present study the flavonoids were localized by the 25% lead acetate solution in the epidermis, few regions of cortex and phloem region in both the plants of *Centella asiatica* and *Hydrocotyle verticillata* which was indicated by the yellow colour. In the same way, the flavonoids are identified in the regions of epidemis, parenchymatous region of mesophyll, phloem and epithelial cells of the leaves of *Hedera helix* (Sulborska *et al.,* 2022) [13].
In the present study the carbohydrates were observed in the epidermis, few regions of cortex and bundle cap in *Centella*

asiatica and in *Hydrocotyle verticillata*, it was observed in the epidermis, few regions of cortex and xylem regions. Correspondingly, the glucoside (the degradation product of carbohydrate) was localized in the regions of epidermis, pith parenchyma and vascular bundles of stem of *Adhatoda zeylanica* (Dhale *et al*., 2011) [5].

Lipids are widely distributed in the plant body and they probably occur in small amounts in every plant. In *Centella asiatica*, the lipids were observed in the cells of epidermis, few regions of cortex and in the phloem regions whereas in *Hydrocotyle verticillata* it was observed in the cells of epidermis, cuticle region, few cells of cortex and phloem regions. The histochemical analysis of leaves of *Hedera helix* revealed the presence of lipids in the regions of collenchymatous hypodermis, palisade and spongy parenchyma, phloem and epithelial cells (Sulborska *et al.*, 2022) [13].

In the present study the phenols were noticed in epidermis, cortex, xylem and in the phloem in *Centella asiatica*. In *Hydrocotyle verticillata*, it was noticed in the regions of cortex and xylem tissues. In the leaf of *Hedera helix*, the phenols were localized in the regions of epidermis with cuticle, collenchymatous hypodermis, palisade and spongy parenchyma (Sulborska *et al.*, 2022) [13].

In the present study the proteins were observed in the phloem, cortical region and epidermal regions of *Centella asiatica*. Similarily, proteins were present in epidermal cells, cortical cells and bundle cap regions in *Hydrocotyle verticillata*. The proteins are localized in the regions of epidermis and scattered cells of cortex in the stem of *Adhatoda zeylanica* (Dhale *et al*., 2011) [5].

In the current study the presence of starch (dark blue patches) was observed in the epidermal and cortical regions of *Centella asiatica* and in *Hydrocotyle verticillata*, it was found only in few cortical regions. Likewise, in *Alpinia calcarata*, few starch grains are present in the few regions of cortex and stele (Girija *et al*., 2014) [7].

In the present study tannin (black coloration) was displayed in some regions of cortex, xylem and in some intercellular spaces of petiole of *Centella asiatica* and in the *Hydrocotyle verticillata*, it was identified in the cortical regions. Sulborska *et al.*, (2022) [13] localized the tannin content in the regions of epidermis, phloem and epithelial cells of the leaf of *Hedera helix*.

In the present study, the anatomical analysis of the plant *Centella asiatica* and *Hydrocotyle verticillata* revealed that both the plants shows more or less similar anatomical cell organisation. The histochemical studies showed that both the study plants consists of similar phytoconstituents in different regions of petiole.

Thus the results obtained by the present study is used to differentiate the plants efficiently. This method can be used for the authentication of the drugs used in Siddha, Ayurveda and other herbal systems of medicine. This is an easy and effective method for the correct identification of genuine herbs from adulterants.

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