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Original Article

Pharmacognostical, physicochemical and phytochemical evaluation of *Huberantha senjiana* (*Annonaceae*) leaf: An endemic tree of Gingee Hills Tamil Nadu India

[Evaluación farmacognóstica, fisicoquímica y fitoquímica de la hoja de *Huberantha senjiana* (*Annonaceae*): Un árbol endémico de Gingee Hills Tamil Nadu India]

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Abstract

Context: *Huberantha senjiana* (*Annonaceae*) is a small tree found in dry rocky soils. It is an endemic species native to the Gingee hills of the Villupuram district of Tamil Nadu, India. The literature survey provides no scientific evidence on its pharmacognostical as well as pharmacological aspect.

Aims: To evaluate the detailed pharmacognostical, physicochemical, and phytochemical characters of *H. senjiana* leaves.

Methods: The pharmacognostic evaluation, such as macroscopic and microscopic characters of fresh leaf and powder, was done. Physicochemical constants like moisture content, extractive values, ash values, foaming index, swelling index, and fluorescence analysis were carried out. The phytochemical screening test and elemental analysis of the powdered leaves were also studied.

Results: Microscopy of the leaf revealed a single layer of square-shaped epidermal cells, the adaxial epidermal layer is apostomatic, and the abaxial epidermal layer is stomatiferous with paracytic stomata, an arch-shaped vascular bundle, clusters of prismatic crystals, spongy parenchyma cells, and palisade cells. Prismatic calcium oxalate crystals, fibers, epidermal trichomes, palisade mesophyll, xylem vessels, and a fragment of the epidermal cell with paracytic stomata were also identified in the microscopy of powdered samples. The phytochemical analysis of sequential extracts showed the presence of alkaloids, flavonoids, cardiac glycosides, terpenoids, carbohydrates, tannins, and steroids. The elemental analysis revealed the presence of heavy metals within the standard limit and trace elements in different concentrations.

Conclusions: The pharmacognostical, physicochemical, and phytochemical data have been reported for the first time, which provides referential information for the right identification and standardization of this unexplored species.

Keywords: *Huberantha senjiana*; microscopy; pharmacognostical; physicochemical; phytochemistry.

Resumen

Contexto: *Huberantha senjiana* (*Annonaceae*) es un árbol pequeño que se encuentra en suelos rocosos secos. Es una especie endémica nativa de las colinas de Gingee del distrito de Villupuram de Tamil Nadu, India. La revisión de la literatura no proporciona evidencia científica sobre su aspecto tanto farmacognóstico como farmacológico.

Objetivos: Evaluar los caracteres farmacognósticos, fisicoquímicos y fitoquímicos detallados de las hojas de *H. senjiana*.

Métodos: Se realizó la evaluación farmacognóstica de caracteres macroscópicos y microscópicos de hoja fresca y polvo. Se llevaron a cabo constantes fisicoquímicas como contenido de humedad, valores extractivos, valores de cenizas, índice de formación de espuma, índice de hinchamiento y análisis de fluorescencia. También se estudió la prueba de cribado fitoquímico y el análisis elemental de las hojas en polvo.

Resultados: La microscopía de la hoja reveló una sola capa de células epidérmicas de forma cuadrada, la capa epidérmica adaxial es apostomática y la capa epidérmica abaxial es estomatífera con estomas paracíticos, un haz vascular en forma de arco, racimos de cristales prismáticos, células de parénquima esponjoso, y celdas en empalizada. En la microscopía de muestras en polvo también se identificaron cristales prismáticos de oxalato de calcio, fibras, tricomas epidérmicos, mesófilo en empalizada, vasos del xilema y un fragmento de la célula epidérmica con estomas paracíticos. El análisis fitoquímico de extractos secuenciales mostró la presencia de alcaloides, flavonoides, glucósidos cardíacos, terpenoides, carbohidratos, taninos y esteroides. El análisis elemental reveló la presencia de metales pesados dentro del límite estándar y oligoelementos en diferentes concentraciones.

Conclusiones: Se reportan por primera vez los datos farmacognósticos, fisicoquímicos y fitoquímicos, los cuales brindan información referencial para la correcta identificación y estandarización de esta especie inexplorada.

Palabras Clave: *Huberantha senjiana*; farmacognóstico; fisicoquímico; fitoquímica; microscopía.

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INTRODUCTION

The firstborn form of health care system known to humanity is an herbal medicine that has been used throughout human history (Barnes et al., 2007). The ancient people differentiated the toxic and nontoxic plants by a trial-and-error method and gained the knowledge of processing and combined the knowledge of medicinal plants to get consistent and promising results. The tribal people systematically composed the evidence from the herbal plants and passed it on to the succeeding generations to revise and update the previous properties and the new ones until today. Thus, the constant interest in medicinal plants projected the distinct herbal pharmacopeia and modified their processing and treatment (Petrovska, 2012; Yuan et al., 2016). The knowledge of the ethnic traditional system of medicine has played a most vital role in the invention of the novel products from the medicinal plants as therapeutic agents. Therefore, the pharmaceutical research and development sector focuses on the growth of plant-based drugs through an exploration of leads (Ghorpade et al., 2012). In recent days the renewed interest towards the drugs of natural origin is due to the simple reason, that it is a green medicine and has no harmful effect of synthetic drugs to human beings and the environment. The natural drug has the advantage of easy accessibility, more economical, and no side effects. Despite its advantage, it also suffers from the disadvantage of adulteration (Ekor, 2014). Plant ingredients are used as a raw material for the drug formulation in the conventional medical system. Therefore, it is essential to do the pharmacognostic and physicochemical evaluation to detect the adulteration and genuineness of the raw material used in the preparation. Usually, the traditional workers who engaged in different systems of complementary medicine collect the raw drugs based on the morphological and other organoleptic characters. Therefore, there is a chance of accidental collection of mistaken plant material. Consequently, it is essential to carry out a detailed anatomical, physicochemical, phytochemical study to avoid uncertainty (Mohan et al., 2010).

In 2008, floristic studies conducted in Gingee Hills identified an unusual group of plant species from the family *Annonaceae* (Muralidharan et al., 2015a). The features of this species were highlighted, differentiated, and compared to the newly defined similar genus *Hubera* (Chaowasku, 2013). "The species was named as Senji for the type locality, namely (Gingee)" (Muralidharan et al., 2015a). The name of the genus *Hubera* is similar to *Huberia* DC (1828: 167; Melastomataceae). Hence the nomenclature commission for vascular plants substituted the generic name *Hubera* with a fresh name *Huberantha* (Chaowasku et al., 2015; Muralidharan et al., 2015b). The newly identified species *H. senjiana* were chosen for the study based on the phylogenetic approach since there is no documented scientific evidence of ethnomedical use and biological activity of the plant. "*The phylogenetic approach for selecting the target species is a new method for drug discovery, which can be potentially used to select the candidate species for drug discovery*" (Bay-Smidt et al., 2011). "*Huberantha is distinguished from Polyalthia s. str. Mainly by several ovules and structure of pollen infratectum*" (Muralidharan et al., 2015a). The studies on the phylogenetic forecast of chemical diversity and potential biological activity of plants observed that there is a considerable correlation between phylogenetic and chemical diversity and bioactivity in the therapeutically important plant species (Rønsted et al., 2012). Consequently, there might be a correlation between chemical and biological activity with the closely related species like *Polyalthia longifolia,* which is used in the conventional system of medicine for the treatment of fever and possess antibacterial activity, cytotoxicity, antifungal activity, anti-inflammatory, and hepatoprotective activities (Chen et al., 2000; Marthanda Murthy et al., 2005; Chanda et al., 2011; Jothy et al., 2012). Even though many plants have been exhaustively explored for their phytochemical and pharmacological potency, its pharmacognostical study remains unexplored and considered to be the primary step in the standardization of raw drugs.

The macroscopic and microscopic study is the crucial phase to transform the right character and quality of the medicinal plants, which must be accomplished before conducting any other experiments according to WHO (WHO, 2011; Ghosh et al., 2017). Thus, the current research was devoted to examining the macroscopical, microscopical, physicochemical, and phytochemical characters exhaustively to provide valuable information to write a monograph. The identification will also provide valuable information for selecting the unexplored species to assess different biological activities in the drug discovery process.

MATERIAL AND METHODS

Solvents, reagents, and standards

Analytical grade chemicals and solvents like n-hexane 99%, chloroform 99%, ethyl acetate 99.9%, isopropyl alcohol 99% (Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India), methanol 99.9% (Merck Specialties' Pvt. Ltd., Mumbai, India) and ethanol 99.9% (Changshu Hong Sheng Fine Chemicals Pvt. Ltd., China) were used for the extraction. The reagents and chemicals used for the microscopic investi-

gation and phytochemical evaluation were acquired from Sigma Aldrich, India Pvt. Ltd. Milli-Q water and chemical reagents: Milli-Q water (Milli-Q10 TS) was used to prepare all reagents in the laboratory during phytochemical evaluation.

Apparatus

Rotary evaporator (Bucchi Labor Technik, Switzerland), rotary microtome (Weswox India), qualitative microscopy was performed using both Inverted Binocular microscope (Leica Microsystems Vertrieb, Germany) and compound microscope (Weswox India); eyepiece-10×; objective-10×,40×). For quantitative microscopic study, camera Lucida (Weswox India), and a black chart for drawing were used. The photograph was taken using a digital camera Nikon lab photos 2 microsystems, microwave oven (CEM MARS 5, CEM, Matthews, NC, USA), (AA 7000 AAS, Lab India Instruments Pvt Ltd) was used for elemental analysis.

Plant collection and authentication

The leaves of *Huberantha senjiana* were collected from Gingee Hills, Pakkamalai Reserve Forest, Devathanampettai, Tamil Nadu (12º10.176′ N, 79º19.300′E, 100 m), during May 2018, and the freshly collected twigs with intact leaves were identified using morphological features with reference specimen and authenticated by Dr. C. Murugan, Scientist 'E' and Head of Office, Botanical Survey of India Southern Regional Centre, T. N. A. U. Campus, Lawley Road, Coimbatore-641 003 (Reg. No of the certificate: BSI/SRC/5/23/2019/Tech. /3313) and a specimen (voucher no SK1677) was deposited for future reference in the Division of Molecular biology Herbarium, SRM IST, Tamil Nadu, India. A sufficient quantity of samples was collected for the study.

Preparation of powder and extracts

The leaves of *H. senjiana* were washed thoroughly under running tap water, dried in the shade at room temperature. One part was preserved in FAA (formalin 5 mL + acetic acid 5 mL+ 70% ethyl alcohol 90 mL) mixture for anatomical studies, and the remaining part was homogenized by mechanical grinder into a fine powder to a mesh size of 60 (sieve no. 60) and stored in an airtight container for macroscopical, microscopical, physicochemical and elemental analysis.

The shade-dried leaves were powdered using a mechanical grinder (sieve no. 40) and macerated sequentially in a bell jar with n-hexane, chloroform, ethyl acetate, isopropanol, and methanol at room temperature for phytochemical analysis. The excess solvents were removed with the help of a rotary evaporator (Bucchi Labor Technik, Switzerland) concentrated, dried and the extract was preserved at 4°C in an airtight container for future studies.

Pharmacobotanical characterization

Macroscopic study

Both the powder and the leaves of *H. senjiana* were subjected to morphological observation with the help of sensory organs to judge size, shape, color, venation, margin, odor, taste, base, texture, fracture, apex, petiole size, surface, point of attachment, dimensions, and composition of the lamina and the addition of fresh and dried leaves. Only color, odor, and taste were observed for powder as per the methods described (WHO, 2007; Evans et al., 2009).

Microscopic study

Qualitative analysis of H. senjiana leaf

To study the anatomy of the leaf, the specimen required for the study was collected from the healthful plant and secured in FAA (formalin 5 mL + acetic acid 5 mL + 70% ethyl alcohol 90 mL) for 24 h. Then the small fragments of leaves from the FAA were washed in Milli-Q water and dehydrated with a graded sequence of tertiary-butyl alcohol as per the specified method (Sass, 1940). The infiltration of the samples was carried out by slow addition of paraffin wax with 58-60°C melting point until the tertiary-butyl alcohol solution gets supersaturation. Then the specimens were cast into paraffin blocks.

Sectioning

The paraffin-embedded specimens were sectioned with Rotary Microtome (Weswox, India) to get the 10-12 µm thickness, and the dewaxing was done by the usual procedure (Johansen, 1940). The dewaxed sections were stained with toluidine blue as per the method (O'Brien et al., 1964). Since toluidine blue is a polychromatic stain that gives remarkably good results with some cytochemical reactions. The dye rendered pink color to the cellulose walls, blue to the lignified cells, dark green to the suberin, violet to the mucilage, and blue to the protein bodies, among others, wherever necessary sections were also stained with safranin, fast-green, and IKI for starch grains.

For studying the stomatal morphology, venation pattern, and trichome distribution, paradermal sections were carried out by peeling the epidermal layer corresponding to the surface of the leaf after partial maceration with Jeffrey's maceration fluid for clearing of the leaf (Sass, 1940). The macerated and cleared leaf was mounted temporarily with a drop of glycerine on a glass slide and observed under a microscope. Pow-

dered material was cleared with 5% sodium hydroxide and mounted in glycerin medium after staining to study and measure different cell components.

Photomicrographs

Photographs of diverse magnification were taken with Nikon Lab photo 2 microscopic unit. A bright field was used for normal observation, and polarized light was employed for the study of crystals, starch grains, and lignified cells. Since these structures have birefringent properties, they appear bright against a dark background when observed under polarized light. Microscopic description of tissues is added with micrographs where required. Scale bars were used to indicate the magnifications used, and the anatomical features were described (Easu, 1965; 1977).

Quantitative analysis *H. senjiana* leaf

The quantitative microscopic analysis was performed to define the stomatal index, stomatal number, vein islet number, vein termination number, and palisade ratio of leaves were studied by following the specified procedure given by the Ayurvedic Pharmacopoeia of India (Anonymous, 2008).

Powder analysis

A small amount of *H. senjiana* leaf powder was transferred into the drop of glycerol with the help of a moistened needle then stirred well to mix uniformly, a cover-glass was placed above, and the overflowing fluid was taken out by a piece of filter paper and observed under the microscope (WHO, 2011). The samples were stained with N/50 iodine to examine starches, 0.1% w/v phloroglucinol solution with a droplet of Con HCL to observe the lignified cells, and 5% $FeCl_3$ in alcohol was used for the observation of tannins (Evans, 2009). Individual constructions and cell substances like epidermal cells, fibers, prismatic crystals of calcium oxalate, and trichomes were detected at different magnifications and imaged using a camera (Nikon Lab photo 2).

Physicochemical analysis

The physicochemical parameters like moisture content (loss on drying), ash value (total ash, water-soluble ash, acid insoluble ash, and sulfated ash), swelling index, and foaming index were determined by following the standard procedures (Anonymous, 2008). The cold maceration technique was used to determine the extractive values according to the procedure described with slight modification (Ghosh et al., 2017). About 5 g of the coarse powder of *H. senjiana* was weighed accurately and placed in six conical flasks with a glass stopper separately in 100 mL of the following solvents (n-hexane, ethyl acetate, chloroform, isopropyl alcohol, methanol, ethanol, and water) macerated in a closed vessel for about 6 h with the help of a mechanical shaker and allowed to rest at normal room temperature (24 ± 2°C). The extracts were filtered with Whatman No.1 filter paper and reduced using a rotary evaporator, and dried in an oven at 105°C. The weight of the residue obtained was used to calculate the extract value in % w/w for all the solvents.

Determination of foreign matter

About 0.5 g of coarse powder was placed as a thin layer and visually examined for foreign matter using the magnifying lens (10× lens), and the percentage of foreign organic matter was calculated for a dry weight of the material taken in % w/w (WHO, 2011).

Fluorescence analysis

The fluorescence analysis of the leaf powder and the extracts were performed by following the standard methods (Chase and Pratt, 2010; Akbar et al., 2014). The *H. senjiana* leaf extracts in n-hexane, chloroform, ethyl acetate, isopropyl alcohol, and methanol were prepared by cold maceration and examined in visible light and UV light (short 254 nm and long 366 nm) and recorded.

Behavior of H. senjiana leaf powder with diverse reagents

The pale green-colored *H. senjiana* powder was treated with diverse chemical reagents such as sodium hydroxide, potassium hydroxide, glacial acetic acid, ammonia solution, sulphuric acid, ferric chloride, picric acid, and Iodine solution to determine the change in color and behavior of powders like floating or sinking according to the methods described (Dangar and Patel, 2018; Tang et al., 2018).

Determination of pH

The pH of the aqueous solution (1% and 10%) of the *H. senjiana* leaf powder was determined by following standard procedures (Dangar and Patel, 2018). The coarsely powdered leaf material was weighed accurately, and 1 g for the 1% solution and 10 g for the 10% solution was dissolved in 100 mL of Milli -Q water, and the calibrated pH meter was used to measure the pH of an aqueous soluble portion at room temperature (24 ± 2°C).

Phytochemical analysis

The phytochemical analysis to identify the plant's secondary metabolites was done according to the standard procedures (Evans, 2009).

Elemental analysis

The fresh leaves were dried at room temperature, pulverized in an electrical mill, sieved, and stored up in an airtight container for elemental analysis. All glassware and Teflon tube used for digestion were soaked in dilute nitric acid and rinsed with Milli-Q-water (Millipore, Merck). All the standard solutions were prepared using 1000 mg/L of stock solutions (Merck) and 5%v/v HCL diluent to dilute Hg, As and Se standards and the samples, while Milli-Q-water was used as a diluent to dilute Zn, Cr, Ni, Cu, Fe, Na, K, Ca, Co, Pb, and Cd standards and samples. The elemental analysis was carried out using Atomic Absorption Spectroscopy (AAS) system (AA 7000 AAS, Lab India Instruments Pvt Ltd).

The samples were digested in the microwave oven following the methods described with modification (Dghaim et al., 2015). About 0.5 g of the powdered plant material was transferred into a 100 mL Teflon tube and added with 5 mL of concentrated H_2SO_4 and 5 mL of HNO_3, mixed well and digested for 30 min and transferred to a container and diluted with small quantities of Milli-Q-water, filtered with Whatman No. 1 and transferred to a standard flask to make up to 50 mL. The sample was analyzed using an appropriate blank for the concentration determination of Zn, Cr, Ni, Cu, Fe, Na, K, Ca, Co, Pb, and Cd using Flame Atomic Absorption Spectroscopy (FAAS) using the carrier gas acetylene. The concentration of Hg, As, and Se was estimated by the Hydride Generation method (HG-AAS) equipped with AAA7000 using the carrier liquid HCL (5%v/v) and reducing agent 2% sodium borohydride ($NaBH_4$) in 0.5 % (w/v) sodium hydroxide (NaOH). The Milli-Q-water was used as a diluent for (FAAS) method and HCL (5%v/v) as a diluent in (HG-AAS) method.

The instrumental calibration was done using a blank solution to zero and standardized using various working standards of different concentrations. The standard samples were loaded to plot the linearity curve, followed by a test solution to determine the concentration of individual elements. The analysis was carried out in triplicate, and the absorbance was documented.

RESULTS AND DISCUSSION

The pharmacognostic study of medicinal plants deals with standardization, authentication, and superiority of each crude drug through macromorphological and micromorphological examination along with physicochemical and phytochemical investigation. The pharmacognostic study helped in recognizing the controversial class of plant species and preventing adulteration in dry powder. As the morphological identity of the plants was lost when they were dried and pulverized (Mohan et al., 2010), the identity and quality were determined by macroscopic and microscopic investigation before other analyses (WHO, 2011), and based on the pharmacognostic, physicochemical, and phytochemical properties we have standardized the leaves of *H. senjiana*.

Organoleptic and macroscopic evaluation

The morphological and organoleptic observation with the help of sensory organs leads to the identification of a particular species. For immediate documentation of the natural habitat of the plant and the elementary figures of the foliage like shape, apex, margin, base, and the nature of venation are routinely evaluated (Fig. 1A-B). The findings of sensory and macroscopic explanation of leaves are tabulated in (Table 1). The morphological features of the leaves are given as described earlier by (Muralidharan et al., 2015a) in their study.

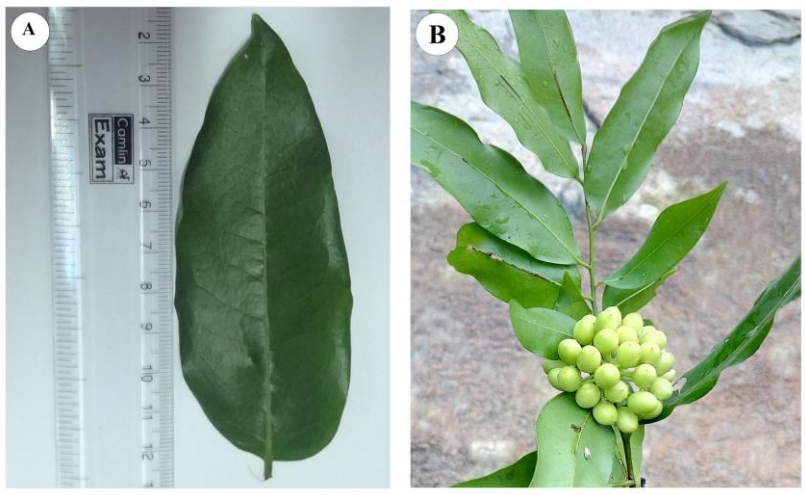

Figure 1. **(A)** Leaf; **(B)** *H. senjiana* leaf with fruit.

Table 1. Organoleptic and macro-morphological characterization of the leaf of *H. senjiana*.

Parameters	Observations
Leaf color on the upper side	Deep green
Leaf color on the lower side	Light green
Taste	Slightly bitter
Odor	Odorless
Surface of leaf	
Upper surface	Shiny
Lower surface	Smooth and shiny
Shape	Elliptic – Oblong
Margin	Pubescent
Apex	Acute to acuminate
Base	Oblique or rounded at base
Venation	Lateral
Length	6.5–13.5 in cm
Width	2.0–4.0 in cm
Petiole	
Length	5 mm
Powdered drug	
Color	Light green
Odor	Characteristic odor
Taste	Slightly bitter

Evaluation of microscopic characters

Qualitative analysis of H. senjiana leaf

The microscopic evaluation of the plant anatomy to distinguish the plant classes based on their internal structure and cellular composition are important as it is evident to differentiate among closely associated species of plants that might be morphologically and macroscopically similar (Baidoo et al., 2019).

Microscopic evaluation of transverse section (TS)

The TS of the leaf is dorsiventral and mesomorphic. It consists of a planoconvex midrib with smooth and even lamina (Fig. 2A). The midrib is bloated at the inferior surface with a small raise on the superior side, revealed adjacent laminar dorsiventral wings on both sides, the transverse section passing through the midrib showed as a single layer of square-shaped epidermal cells. The size of the midrib is found to be 450 µm in a vertical plane and 500 µm in a horizontal plane.

The midrib consists of a thick cuticle and epidermal cell on both the adaxial and abaxial sides. The epidermal cells are small and square. The cuticle above the epidermis is thin-layered with slightly elongated cells, which are thick and large on the adaxial surface when compared to the abaxial surface (Fig. 2A-B). There is a subepidermal, short layer of cells in the adaxial part of the midrib. The ground parenchyma tissue includes fairly wide angular, compact thick-walled parenchyma cells (Fig. 2A-B) located on the lower part of a midrib, which is less at the adaxial surface. Two small clusters of prismatic crystals form a circle (Fig. 3B). The vascular system consists of a planoconvex, a thick cylinder of fibers, which encloses several radii of vessels and thick darkly stained arc-shaped phloem strands found below the xylem vessels and the xylem vessel is lignified in nature. The vessels are wide, circular, thick-walled, and the protoxylem elements are directed upward (Fig. 3A). The vessels, fibers cylinder, ground parenchyma cells, and crystals appear bright under polarized light (Fig. 3B).

The lamina is uniformly smooth on surfaces. There is a thick cuticle on both the upper and lower epidermal cell layers. The adaxial and abaxial epidermal cells are wide and horizontally rectangular. The cells have rectangular thick calcium oxalate crystals. The lamina is found to be 149 µm thick (Fig. 3C). The mesophyll tissues are differentiated into adaxial, horizontal layers of long palisade cells and abaxial zone of spongy parenchyma cells (Fig. 3C). The palisade cells are narrow compact cells with dark dense cell

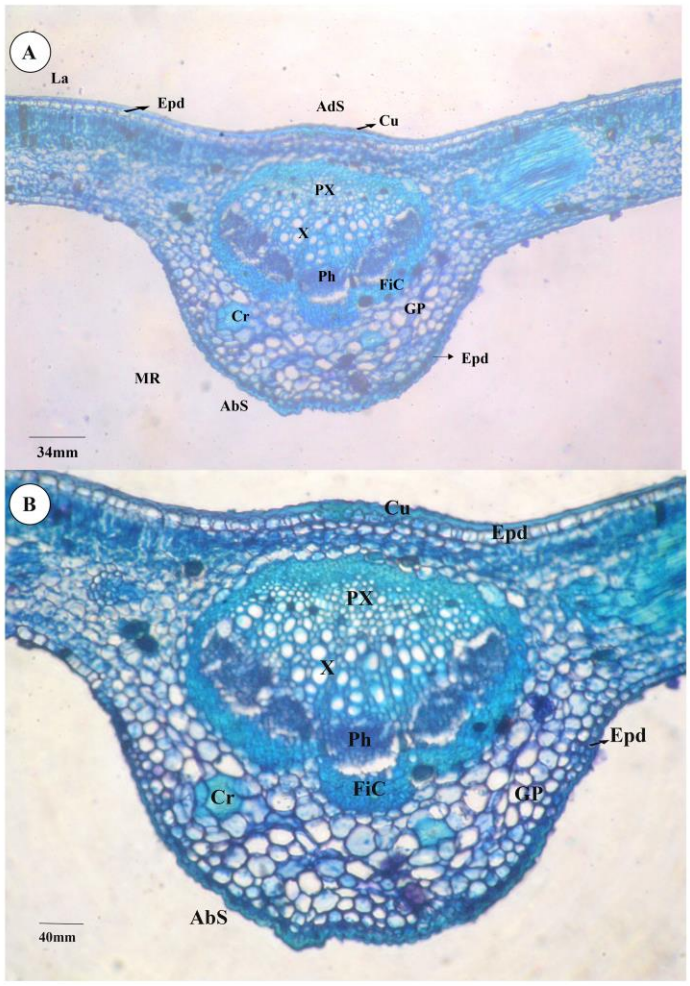

Figure 2. **(A)** Transverse section of *H. senjiana* leaf through midrib; **(B)** Transverse section of *H. senjiana* leaf midrib enlarged.

Abs: Abaxial side; **Ads**: Adaxial side; **Cr**: Crystal; **Cu**: Cuticle; **Epd**: Epidermis; **Fic**: Fiber cylinder; **GP**: Ground parenchyma; **La**: Lamina; **MIR**: Midrib; **Ph**: Phloem; **PX**: Protoxylem; **X**: Xylem.

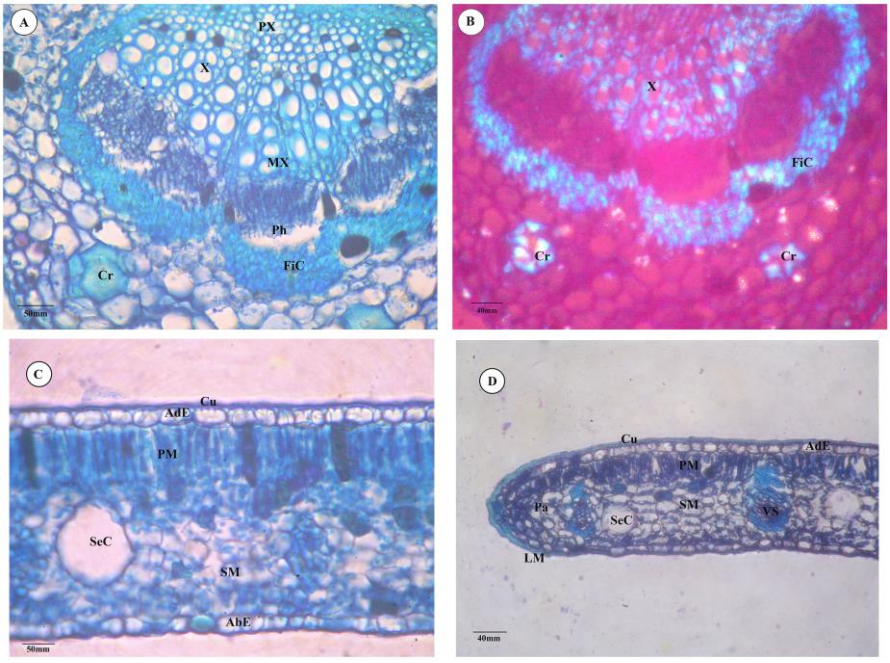

Figure 3. (**A)** TS of midrib-vascular system enlarge; **(B)** Vascular system of the midrib as seen under polarized light; **(C)** TS of lamina; **(D)** TS of leaf margin.

Cr: Crystal; **Fic**: Fiber cylinder; **MX**: Meta xylem; **Ph**: Phloem; **PX**: Proto xylem; **X**: xylem; **AbE**: Abaxial epidermis; **AdE**: Adaxial epidermis; **Cu**: Cuticle; **LM**: Leaf margin; **Pa**: Parenchyma; **PM**: Palisade mesophyll; **SM**: Spongy mesophyll; **SeC**: Secretory cavity; **VS**: Vascular strand.

Table 2. Leaf surface constants of *H. senjiana* leaf.

Parameters	Results
Stomatal number (upper)	9-(11.6)-15
Stomatal index (upper)	7.56- (8.68) - 10.13 (%)
Stomatal number (lower)	22-(33.8)-47
Stomatal index (lower)	17.88-(18.79)-23.5(%)
Vein islet number	17-(19)-21
Veinlet termination number	8-(10)-12
Palisade ratio	5-(6.6)-8

Estimates are reported as a scale from the lower limit to the upper limit acquired after 5 replicates. (lower limit- average – upper limit)

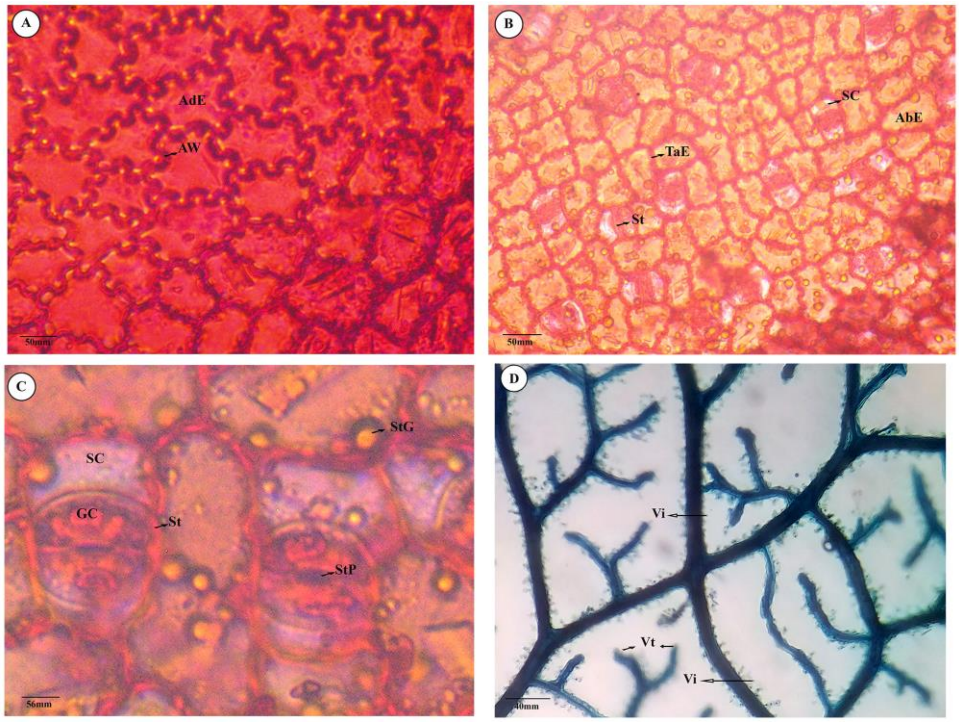

Figure 4. **(A)** Surface view of the adaxial epidermis; **(B)** Surface view of the abaxial epidermis showing stomata; **(C)** Two stomata showing paracytic subsidiary cells; **(D)** Surface view of the leaf showing vein islet and vein termination.

AdE: Adaxial epidermis; **AbE**: Abaxial epidermis; **AW**: Anticlinal wall; **GC**: Guard cell; **SC**: Subsidiary cell; **St**: Stomata; **Stp**: Stomatal pore; **StG**: Starch grains; **TaE**: Tangential epidermal cell wall; **Vi**: Vein islet; **Vt**: Vein termination.

contents. The spongy mesophyll cells are circular less compact, and have intercellular air cavities. Frequently there are wider circular thin-walled secretory cavities located in the mesophyll tissue (Fig. 3C)

The leaf margin (Fig. 3D) of the lamina is gradually narrow, ending with a thick blunt end, and the leaf margin is 120 µm thick. The lamina has a thick and smooth cuticle and fairly wide rectangular epidermal cells. The mesophyll tissue includes adaxial palisade cells and abaxial spongy parenchyma. There are small circular vascular strands enclosed with a fiber sheath. Wide circular cavities are also seen in the mesophyll tissue. The end of the lamina has a compact cluster of cells without mesophyll differentiation.

Quantitative analysis H. senjiana leaf

The analysis of leaf surface constants is important for the determination of adulteration in the leaf, which is used as a drug, and also it is useful to find the difference between closely associated species (Baidoo et al., 2019). The quantitative leaf constants analysis, such as stomatal number, stomatal index (adaxial and abaxial epidermis), vein islet number, veinlet termination, and palisade ratio, were done as per the standard procedure (Anonymous, 2008). The quantitative leaf microscopy results of triplicate samples are given in (Table 2). The surface of the leaf lamina was examined on upper and lower surfaces microscopically.

The adaxial layer of the epidermal cells is seen in the surface view (Fig. 4A). The epidermal cells are polygonal in outline with thick, highly wavy, and folded anticlinal walls. Sometimes the epidermal cells have dense beaded anticlinal walls. The adaxial epidermal layer is apostomatic. The epidermis of the abaxial surface is polygonal and angular in outline with thick wavy beaded walls (Fig. 4B). The epider-

mis is stomatiferous. The stomata are less frequent and random in distribution. The stoma has circular wide guard cells. The guard cells are 20 µm in a horizontal plane. The stomatal pore is small and elliptical. The stomata are exclusively paracytic type stomata. Each stoma has two rectangular subsidiary cells arranged parallel to the long axis of the guard cells, and hence the type of stomata is paracytic stomata (Fig. 4C). The photo documentation of the venation pattern, vein termination, and vein islet is shown in (Fig. 4D).

Powder microscopy

The plants are dried and powdered before being used or sold. The powdered crude drug loses its morphological identity and is easily prone to adulteration. Hence it is essential to regulate standards to find the adulteration in powdered drugs (Mohan et al., 2010). The leaf powder microscopy of *H. senjiana* showed fragments of the following elements: adaxial and abaxial epidermal cells, prismatic crystals, xylem vessels, epidermal trichomes, palisade mesophyll, the fragment of the epidermal cell with paracytic stomata and crystals. The adaxial layer of epidermal cells is seen in the surface view (Fig. 5B).

The cells are polygonal in outline with thick, highly wavy, and folded anticlinal walls. The abaxial epidermal cells are polygonal and angular in outline with thick wavy beaded walls with paracytic stomata (Fig. 5G). The epidermal trichome is occasionally seen in the powder. The trichome is long narrow, unbranched with uniseriate vertically elongated cells. The trichomes are up to 370 µm in length and 25 µm in thickness (Fig. 5C). The xylem vessel, which is lignified and broad pitted in nature, is observed (Fig. 5E). Fibers are common in the powder. They are long, thin, tapered at the end, and are non-septate. The fibers are either straight or twisted (Fig. 5A). The fiber is up to 600 µm long and 20 µm thick. Elongated rectangular calcium oxalate crystals that appeared bright under polarized light are seen in almost all epidermal cells (Fig. 5D). palisade parenchyma cells attached to upper epidermal cells (Fig. 5F) and crystals in between the ground parenchyma cells are observed, and the photo documentation was given in (Fig. 5H).

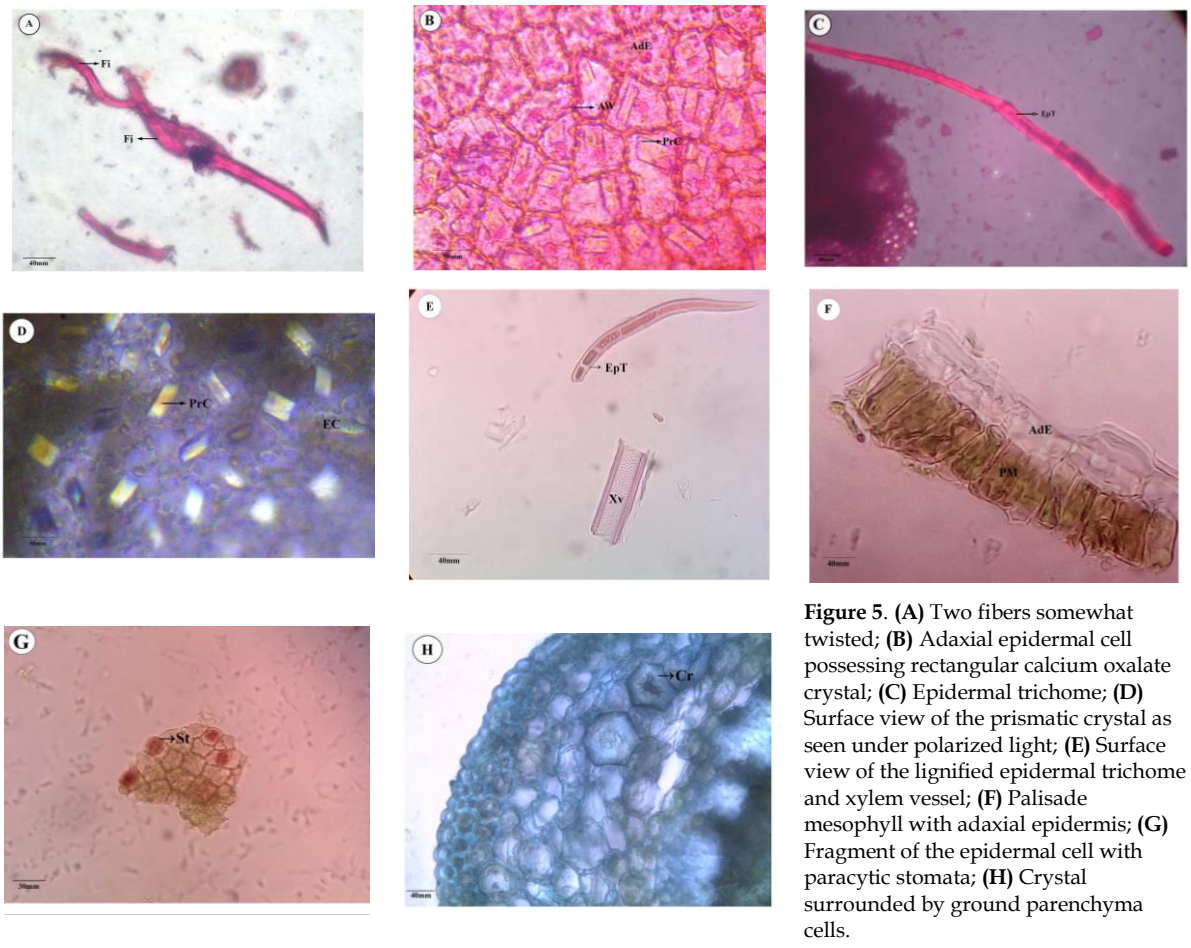

Figure 5. **(A)** Two fibers somewhat twisted; **(B)** Adaxial epidermal cell possessing rectangular calcium oxalate crystal; **(C)** Epidermal trichome; **(D)** Surface view of the prismatic crystal as seen under polarized light; **(E)** Surface view of the lignified epidermal trichome and xylem vessel; **(F)** Palisade mesophyll with adaxial epidermis; **(G)** Fragment of the epidermal cell with paracytic stomata; **(H)** Crystal surrounded by ground parenchyma cells.

AdE: Adaxial epidermis; **AW**: Anticlinal wall; **EC**: Epidermal cell; **PrC**: Prismatic crystal; **EpT**: Epidermal trichome; **Fi**: Fiber; **Xv**: Xylem vessel; **PM**: Palisade mesophyll; **St**: Stomata; **Cr**: Crystal.

Physicochemical properties

The assessment of physicochemical properties of the raw drugs is essential in identifying adulteration of crude drugs. The content of moisture in *H. senjiana* leaf powder was found to be 10.41 ± 0.13 %, which is not considered too high. Thus the growth of bacteria, fungi, or yeast will be inhibited.

Ash values

The ash value is the deposit leftover after heating the plant raw material at 400 ± 25°C in a muffle furnace, which denotes the presence of inorganic salts that occur naturally or adhering to the plant material from the environment or deliberately added as an adulterant by the humans. There are three different procedures to determine the ash value as total ash, acid-insoluble ash, and water-soluble ash. The total volume of ash left after ignition is the total ash. This comprises both 'physiological ash' obtained from the plant tissue itself and 'non-physiological ash', obtained from the rest of the extraneous matter sticking to the surface of the plant, and it is particularly important in assessing the purity of the drug based on the presence or absence of foreign inorganic matter such as metallic salts or silica. The acid-insoluble ash is a part of total ash, which measures the amount of silica as sand and siliceous earth. The water-soluble portion of the total ash sulfated ash is the presence of a residual substance that is not volatilized from the sample when it is ignited with sulphuric acid at 800 ± 25°C in a muffle furnace. The sulfated ash indicates the presence of inorganic impurities (Dave et al., 2010). The total ash of *H. senjiana* leaves as water-soluble ash, acid insoluble ash, and sulfated ash was evaluated, and the results were given in (Table 3). The low volume of ash values reveals that the non-physiological and inorganic matters like silica are present in less quantity in leaves of *H. senjiana* (Baidoo et al., 2019).

Foaming index

The saponins content in a plant material will produce a persistent foam as soon as when an aqueous solution of plant material is agitated. Thus, the capacity of foaming in an aqueous solution of extracts and plant materials was evaluated in terms of a foaming index. The results are given in (Table 3) showed that the foaming index of *H. senjiana* leaf powder was less than 100 (WHO, 2011).

Table 3. Physicochemical analysis of *H. senjiana* leaf powder and extracts

Parameter	Values
Foreign matter % (w/w)	0.77 ± 0.15
Loss on drying % (w/w)	10.53 ± 0.37
Total ash % (w/w)	7.44 ± 0.31
Water soluble ash % (w/w)	4.58 ± 0.30
Acid-insoluble ash % (w/w)	1.63 ± 0.33
Sulphated ash % (w/w)	4.5 ± 030
Foaming index (mL)	<100
Swelling index (mL)	Nil
pH (1% w/v solution)	6.51 ± 0.29
pH (10% w/v solution)	5.45 ± 0.28
Extractive values (mg/g)	
n-Hexane soluble extractive % (w/w)	6.49 ± 0.88
Chloroform soluble extractive % (w/w)	8.79 ± 1.01
Ethyl acetate soluble extract % (w/w)	7.64 ± 0.88
Isopropyl alcohol soluble extractive % (w/w)	11.81 ± 1.24
Methanol soluble extractive % (w/w)	14.39 ± 0.94
Ethanol soluble extractive % (w/w)	14.18 ± 0.78
Water soluble extractive % (w/w)	10.32 ± 0.40

Values are given as the mean ± standard deviation for three replicates in each experiment. Except for swelling index and foaming index.

Table 4. Fluorescence study of extracts and powder of *H. senjiana* leaf.

Plant extracts	Visible light	UV-254 nm	UV- 365 nm
n-Hexane extract	Dark brown	Dark green	Dark green
Chloroform extract	Dark brown	Dark brown	Dark green
Ethyl acetate extract	Dark green	Dark green	Dark green
Isopropyl alcohol extract	Dark green	Dark green	Greenish-yellow
Methanol extract	Dark greenish brown	Dark greenish brown	Dark greenish-yellow
Powder + reagent/solvent			
Powder as such	Dark green	Dark green	Yellowish green
Powder + water	Light green	Green	Fluorescence green
Powder +ethanol 95%	Dark green	Green	Yellowish green
Powder + conc. HNO_3	Yellowish green	Light green	Yellowish-brown
Powder + conc. H_2SO4	Black (charred)	Dark green	Brown in color
Powder + conc. HCl	Greenish-yellow	Dark green	Brown
Powder + $FeCl_3$	Greenish black	Dark green	Dark green
Powder + iodine solution (0.5 M)	Green-yellow	Green	Brownish green
Powder + NH_3 solution (25%)	Yellowish-brown	Light green	Fluorescence green

Swelling index

The different medicinal plant contains a definite medicinal value due to the presence of the variable constituents of pectin or hemicelluloses, mucilage and gum are among different herbal plants, hence leads to varying swelling properties of diverse herbal materials (WHO, 2011). The swelling index of *H. senjiana* leaf powder was zero, as given in (Table 3). The result indicates that the constituents responsible for the swelling properties are absent in the leaf part of *H. senjiana.*

Extractive value

The extractive value is an essential physicochemical parameter to establish the weight of crude extract to detect the exhausted and adulterated plant materials. It reveals the nature of the phytoconstituents of the crude drug and the solvent-soluble chemical constituent (Kabra et al., 2019). The experimental results revealed that the extractive yield of leaves of *H. senjiana* in methanol was highest, followed by ethanol, water, and IPA, and the lowest yield was obtained in hexane and chloroform (Table 3). The difference in extractive value in different solvents indicated that the development of bioactive standards of medicinal plants is guided by several internal and external factors. The excessive alcohol soluble extractive value revealed the existence of sugars, acids, and polar compounds like glycosides, phenols, and tannins (Kabra et al., 2019). The parameters given are valuable for the composition of an appropriate monograph for its correct identification.

Determination of pH

The assessment of pH is important to determine the dissolution, stability, and solubility of a drug as the acidic drug soluble in basic medium or basic drug soluble in acidic medium. The pH value also helps in developing the extraction procedure for the phytoconstituents from the medicinal plants (Dangar and Patel, 2018). The pH of aqueous extract (1% and 10%) of *H. senjiana* leaf powder is 6.51 ± 0.29 and 5.45 ± 0.28, respectively, which is weakly acidic, and the results are given in Table 3.

Fluorescence analysis

The powdered leaves and various solvent extracts of *H. senjiana* leaves were treated with diverse acidic and basic chemical reagents and observed under visible light, short (254 nm), and long (365 nm) UV light was detected, and the results are presented in (Table 4). The fluorescence assay is a valued, simple, and absolute technique for the determination of fluorescence compounds. Diverse compounds produce fluorescence once exposed to the UV light short and long wavelength (Akbar et al., 2014; Aslam and Afridi, 2018). The fluorescence analysis is considered to be the most important step in standardization with absolute scientific validation of crude drugs. It indicated the sign of chromophore in a crude drug. The different chemical reagents used may convert the chemical

constituents present into fluorescent derivatives, which show fluorescence in UV light, which is characteristic for a particular drug and may be useful in the detection of adulterants. Thus, the technique is extremely fast to distinguish from other species in the absence of other physiochemical evaluations (Prasanth et al., 2017).

Behavior of H. senjiana leaf powder with different chemical reagents

The powder analysis exhibited different color under ordinary daylight for different chemical reagents and the change in behavior of the leaf powder with different reagents were detected, and the results are given in (Table 5). The behavior of leaf powder with different chemical reagents is a unique method to detect the presence of adulterants in a crude drug is a fast technique to differentiate from suspicious species (Prasanth et al., 2017).

Preliminary phytochemical test of different fractions of *H. senjiana* leaves

The preliminary phytochemical screening of different fractions obtained by sequential extraction, such as n-hexane, ethyl acetate, chloroform, isopropyl alcohol, and methanol, showed the presence of various phytoconstituents like alkaloids, glycosides, flavonoids, terpenoids, steroids, and tannins. The phytochemical screening results of different fractions of *H. senjiana* leaves are presented in (Table 6). The presence of steroids, sterols, and triterpenoids was observed in n-hexane extract. The chloroform extract showed positive for steroids, alkaloids, and triterpenoids. The ethyl acetate extract showed the presence of flavonoids, and phenolic compounds, and the isopropyl alcohol extract responded positively for carbohydrates, tannins, phenolic compounds, saponins, alkaloids, and flavonoids. The methanolic extract yielded positive alkaloids, flavonoids, steroids, triterpenoids, saponins, carbohydrates, tannins, and phenols. The preliminary phytochemical screening was performed for the first time on this plant. Thus, the phytochemical screening benefits in identifying the different classes of the natural phytoconstituents leading to the quantification by different analytical methods and helps in identifying the pharmacologically active Phytocompounds (Evans, 2009; Kabra et al., 2019).

Elemental analysis

The toxicity due to heavy metals and other trace elements on human health and to the ecosystem has drawn significant interest in the current years. The plants play a significant role in transferring heavy metals and trace elements from the polluted land to humans. Thus, the heavy metals and trace elements have a circle in the food chain. Due to the low elimination rate of heavy metals via the kidney, even lower levels of heavy metals can cause damage to the human. The metals such as Na, K, Ca, Zn, Ni, Fe, Cu, Se, and Cr are the essential nutrients for the normal physiological and biological function of the human body. Despite its uses, a rise in the concentration above certain permitted limits become toxic to the human (Dghaim et al., 2015). The AAS analysis of fine powder revealed the occurrence of heavy metals like arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb) but observed to be within the permissible limit (WHO, 2007). The concentration of trace elements (Na, K, Ca, Co, Se, Zn, Ni, Fe, Cu, Cr) found to be variable, as given in (Table 7).

Table 5. The behavior of *H. senjiana* leaf powder with different chemical reagents.

Reagent	Color of solution	Behavior
Picric acid	Greenish-yellow	Sinking
Conc. H_2SO_4	Black (charred)	Floating
Aq. KOH	Yellowish brown	Floating
$FeCl_3$	Greenish black	Sinking
NaOH	Yellowish brown	Floating
Glacial acetic acid	Greenish-yellow	Partially floating
Iodine solution	Greenish-yellow	Partially floating
Ammonia	Yellowish-brown	Partially floating

Table 6. Preliminary phytochemical screening of *H. senjiana* leaf extracts.

Phytoconstituents	Name of Tests	Inference				
		n-Hexane	Chloroform	Ethyl acetate	Isopropyl alcohol	Methanol
Alkaloids	Dragondorff's test	-	+	-	+	+
	Mayer's test	-	+	-	+	+
	Wagner's test	-	+	-	+	+
Cardiac glycoside	Keller killiani test	-	-	-	+	+
Flavonoids	Ferric chloride test	-	-	+	+	+
	Shinoda test	-	-	+	+	+
Steroids and sterols	Liberman Burchard test	+	+	-	-	+
	Salkowski's test	+	+	-	-	+
Phenolic compounds	Ferric chloride test	-	-	+	+	+
Triterpenoids	Sulphuric acid test	+	+	-	+	+
Saponins	Foam test	-	-	-	+	+
Proteins and amino acids	Ninhydrin test	-	-	-	+	+
	Biuret test	-	-	-	-	+
Carbohydrates	Fehling's test	-	-	-	+	+
	Benedict's test	-	-	-	+	+
	Molisch's test	-	-	-	-	+

(−) Absent; (+) Present.

Table 7. Elemental analysis of leaf powder of *H. senjiana*.

Heavy metals	Standard limit* (ppm)	Concentration (ppm)
Lead (Pb)	10	1.141 ± 0.06
Cadmium (Cd)	0.3	0.292 ± 0.05
Arsenic (As)	3	0.448 ± 0.05
Mercury (Hg)	1	0.739 ± 0.07
Essential trace elements		
Sodium (Na)	NA	1.615 ± 0.10
Selenium (Se)	NA	0.256 ± 0.03
Iron (Fe)	NA	0.909 ± 0.08
Copper (Cu)	NA	0.368 ± 0.06
Nickel (Ni)	NA	1.568 ± 0.10
Zinc (Zn)	NA	0.491 ± 0.07
Chromium (Cr)	NA	ND
Cobalt (Co)	NA	0.325 ± 0.04
Calcium (Ca)	NA	1.700 ± 0.09
Potassium(K)	NA	2.180 ± 0.09

ND: Not detectable; NA: Not applicable; Results are expressed as mean ± SEM

CONCLUSION

The results from the current investigation of leaves of *H. senjiana* provide critical information on pharmacognostic standardization, physicochemical evaluation, phytochemical screening, and elemental analysis for the first time. The macroscopic and microscopic study revealed significant features, which are helpful in the discovery and authentication of this plant. The important studies on physicochemical parameters the phytochemical screening to determine the phytoconstituent profile and the toxic elements were studied. The data obtained will help in the preparation of the monograph and act as a root of knowledge for investigators, manufacturers, and customers to regulate quality and as a reference to explore the related species, which is not being explored scientifically. Despite the above study, further studies need to be carried out in phytochemical isolation to explore the phytochemistry and *in vitro* screening in different cell lines to explore the pharmacology of the rare growing endemic species.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Contribution	Pandiyan R	Ilango K
Concepts or ideas	x	x
Design	x	x
Definition of intellectual content	x	x
Literature search	x	
Experimental studies	x	
Data acquisition	x	
Data analysis	x	x
Statistical analysis	x	
Manuscript preparation	x	
Manuscript editing	x	x
Manuscript review	x	x

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