International Journal for Pharmaceutical Research Scholars (IJPRS)

V-4, I-3, 2015 ISSN No: 2277 - 7873

RESEARCH ARTICLE

Pharmacognostical Evaluation of Leaves of Two Medicinally Important Species of *Clerodendrum*

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Manuscript No: IJPRS/V4/I3/00139, Received On: 05/07/2015, Accepted On: 08/07/2015

ABSTRACT

The present study aimed to evaluate various pharmacognostical standards which include macroscopy, microscopy, determination of leaf constants and Physicochemical parameters, starch grain detection, preliminary phytochemical screening and trace metal analysis of the leaves of *Clerodendrum serratum* (L.) Moon and *Clerodendrum viscosum* Vent. *C. serratum* and *C. viscosum* leaves were successively extracted with petroleum ether (60 –80°C), chloroform and methanol by soxhlation. Microscopy was carried out by taking the thin transverse sections of the leaves. Leaf constants viz., stomatal number, vein-islet number, veinlet termination number and palisade ratio were determined. Detection of starch grains was carried out by treating the powder leaf with dilute Iodine solution. Ash and extractive values were also determined. The preliminary phytochemical screening of the extracts was carried out to detect various phytocompounds. Trace metal analysis was performed to determine the elemental concentration using PIXE technique. The microscopy of the leaves of *C. serratum* and *C. viscosum* shows the presence of cuticle, epidermis, trichomes, stomata, palisade layer, spongy parenchyma, collenchyma, vascular bundles and parenchymatous cells. Simple starch grains are observed in leaves of both the species. The leaf constants and physicochemical parameters of *C. serratum* and *C. viscosum* leaves were also determined. The trace metal analysis indicated the presence of higher percentage of K and Ca in leaves of both the species. The pharmacognostical evaluation of the leaves of *C. serratum* and *C. viscosum* serves as a basis for proper identification, physical evaluation and monograph of this plant.

KEYWORDS

Clerodendrum Serratum (L.) Moon, *Clerodendrum Viscosum* Vent., Pharmacognostical, Phytochemical, PIXE

INTRODUCTION

Herbal medicines are the synthesis of therapeutic experiences of generations of practicing physicians of indigenous systems of medicine for over hundreds of years. They are known to be the oldest healthcare products that have been used by mankind all over the world in the form of folklore medicines or traditional medicines or ethnic medicines[1].

There are evidences of herbs having been used in the treatment of diseases and for revitalizing body system in almost all ancient civilization. Ayurveda, the science of life, has provided important and rationale basis for the various ailments[2]. The genus *Clerodendrum* has more than 500 species and is very extensively distributed in tropical and subtropical regions of

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the world and it is comprised of small trees, shrubs and herbs[3].

Phyto-medical importance of various species of *Clerodendrum* genus has been reported in various indigenous systems of medicines and also in folk medicines. This genus is being used specifically in Indian, Chinese, Thai, Korean, Japanese systems of medicine for the treatment of various diseases such as syphilis, typhoid, cancer, jaundice and hypertension[4]. The major groups of chemical constituents present in the *Clerodendrum* genus are phenolics, flavonoids, terpenoids and steroids. The isolated flavonoids like hispidulin and cleroflavone posses potent antioxidant, antimicrobial, antiasthmatic, antitumor and CNS binding activities. The presence of phenolic compounds like serratagenic acid, acteoside, indolizino and verbascoside are responsible for antioxidant, antimicrobial, antiproliferative, antihypertensive and anticancer activities.

Traditionally the leaf extract of *Clerodendrum serratum* (L.) Moon has been used for the treatment of rheumatism, asthma, anorexia, fever, jaundice, diarrhoea and other inflammatory diseases[5]. The leaf decoction (4-5 leaves) are used in jaundice twice a day and improves high blood pressure[6]. The leaf of *Clerodendrum viscosum* Vent. is traditionally used to manage disease states like convulsion, diabetes, scorpion sting, tumor, malaria and skin diseases[7]. In Thai medicine, the leaves are known to be diuretic and used for the treatment of intestinal infections and kidney dysfunction. Fresh leaf juice is widely used as antidandruff, antipyretic, laxative and vermifuge[8].

The process of standardization can be achieved by stepwise pharmacognostical studies in which the identification of plant drugs are more important. Hence pharmacognostical study gives the scientific information regarding the purity and quality of the plant drugs[9]. The objective of the present study is to evaluate various pharmacognostical standards which include macroscopy, microscopy, determination of leaf constants, ash values, extractive values, detection of starch grains, preliminary phytochemical screening and trace metal analysis of the leaves of *C. serratum* and *C. viscosum*.

MATERIAL AND METHODS

Plant Material Collection and Identification

C. serratum and *C. viscosum* were collected respectively from Utkal University campus, Vani Vihar, Bhubaneswar and Naharkanta village of Khordha district, Odisha, India. Identification of voucher specimen was authenticated by Dr. K.B. Satapathy, P.G. Department of Botany, Utkal University, Vani Vihar Bhubaneswar and voucher specimen (SVN-537, SVN-536) was deposited in the departmental herbarium. After authentication, the leaves were collected in bulk quantity.

Drying and Pulverization

The collected leaves of *C. serratum* and *C. viscosum* were shade dried and pulverized to obtain a coarse powder and stored in air tight container for future use.

Extraction of Powdered Plant Material

The powdered leaves were successively extracted[10] with petroleum ether (60-80ºC), chloroform and methanol for 48 hours by soxhlation and the solvent was evaporated under reduced pressure in a rotary evaporator. The extracts were kept in desiccator for further use. The yield of petroleum ether, chloroform and methanol extracts of *C. serratum* were 2.62, 1.89, 11.52% w/w and *C. viscosum* were 2.71, 2.12, 12.16% w/w respectively.

Macroscopy

The freshly collected leaves were evaluated for organoleptic properties/macroscopical characters such as shape, size, colour, odour, taste and texture.

Microscopy

Microscopy of plant material was performed to distinguish it from the allied drugs and adulterant. The thin transverse sections of both the leaves were cut, treated with chloral hydrate solution with gentle warming and stained with phloroglucinol and concentrated HCl (1:1). They were then mounted with glycerine water and

observed under compound microscope. Photomicrographs were obtained during observation of the section in appropriate field[11] in Figure 1. (A, B) & Figure 9. (A, B).

Determination of Leaf Constants[12]

Stomatal Number

A piece of the middle portion of the leaf was cleared by boiling with chloral hydrate solution. Then upper and lower epidermis were peeled out separately by means of forcep and mounted on a slide with glycerin water. A square of 1 mm was drawn arranging camera lucida in microscope by using stage micrometer. Then the slide with epidermis was placed on the stage of microscope and epidermal cells and stomata were traced out in the square. Then the number of stomata present in the area of 1 sq. mm was counted in Figure 2, 10.

Vein-Islet Number

A piece of the leaf was cleared by boiling in chloral hydrate solution for about 30 minutes. By using 16 mm objective, a line was drawn equivalent to 1mm as seen through microscope and a square was constructed on this line. The cleared leaf was placed on the slide and the veins included within the square were traced off, completing the outlines of those islets which overlap two adjacent sides of the square. Then the number of vein-islets in the square was counted in Figure 3, 11.

Veinlet Termination Number

For the determination of veinlet termination number, similar procedure was followed as that of vein-islet number. The veinlet terminations within the square were only taken into account in Figure 5, 13.

Palisade Ratio

A piece of the leaf was cleared by boiling in chloral hydrate solution. By using 4 mm objective, the outlines of four cells of epidermis were traced. Then palisade layer was focused down and sufficient cells were traced off to cover the tracings of the epidermal cells. Then palisade cells under four epidermal cells were counted and the average number of cells beneath a single epidermal cell was calculated in Figure 4, 12.

Detection of Starch Grains[13]

In order to detect the starch grains, a small amount of the powder was mounted in dilute iodine solution and observed under the microscope in Figure 6, 14.

Determination of Physicochemical Parameters[14]

Ash Values

Ash values like total ash, acid-insoluble ash, water soluble ash were determined using standard procedure.

Total Ash

About 2 gm of the leaf powder was accurately weighed in a tared silica dish previously ignited and weighed. It was then incinerated gradually by increasing the heat; not exceeding dull red heat, until free from carbon (Temp. 450^{o}C). It was then cooled and weighed. The percentage of ash was calculated with reference to the air-dried plant material.

Acid-Insoluble Ash

The ash was boiled for 10 minutes with 25 ml of dilute HCl and the insoluble matter was collected in a gooch crucible (Whatmann filter paper). It was washed with hot water, ignited and weighed. The percentage of acid-insoluble ash was calculated with reference to the air-dried plant material.

Water Soluble Ash

The total ash was boiled for 5 minutes with 25 ml of water. The insoluble matter was collected in a gooch crucible. It was washed with hot water, ignited and weighed. The percentage of water soluble ash was calculated with reference to the air-dried plant material.

Extractive Values

Alcohol Soluble Extractive

About 5 gm of leaf powder was macerated with 100 ml of alcohol in a closed flask for 24 hrs, with occasional shaking during 6 hrs and was allowed to stand for 18 hrs. It was then filtered rapidly taking precaution against loss of alcohol.

A volume of 25 ml of filtrate was then evaporated to dryness in a tared flat-bottomed shallow dish. It was dried at 105ºC, cooled and weighed. The percentage of alcohol soluble extractive was calculated with reference to the air-dried plant material.

Water Soluble Extractive

About 5 gm of leaf powder was macerated with 100 ml of water in a closed flask for 24 hrs, with occasional shaking during 6 hrs and was allowed to stand for 18 hrs. It was then filtered rapidly taking precaution against loss of water. A volume of 25 ml of filtrate was then evaporated to dryness in a tared flat-bottomed shallow dish. It was dried at 105ºC, cooled and weighed. The percentage of water soluble extractive was calculated with reference to the air- dried plant material.

Preliminary Phytochemical Screening

Petroleum ether, chloroform and methanol extracts of leaves of *C. serratum* and *C. viscosum* were subjected to preliminary phytochemical screening by following standard procedure[15,16]. The extracts were subjected to different chemical tests to detect the presence of various active phytocompounds like alkaloids, flavonoids, carbohydrate, saponins, triterpenoids, steroids, tannins, glycosides, phenols, phytosterols, proteins and quinones.

Trace Metal Analysis

Inorganic element composition of leaves of *C. serratum* and *C. viscosum* was determined using PIXE technique.

Proton induced X-ray emission (PIXE) is a widely used nuclear analytical technique based on the measurements of characteristic X-rays which are induced by the energetic proton beam (MeV energy scale) directed onto the surface of a sample held under vacuum/air[17]. This technique is non-destructive, accelerator-based, simultaneous multi elemental and suitable for quantitative elemental characterization of a wide range of complex materials, including biological samples, especially containing lower-middle Z (atomic number) elements[18]. Additional advantages of this technique encompass its reliability, high sensitivity (below ppm low level detection) for several elements and its appropriateness for routine analytical work. While most of the other techniques (ICP-OES, AAS, GF-AAS) demand extensive (multi-step) and destructive sample preparation procedures but in PIXE analysis, minimal sample preparation is required; hence external sources of contamination resulting in erroneous results can be avoided.

Instrumentation

At the Ion Beam Laboratory of the Institute of Physics (Department of Atomic Energy, Government of India), Bhubaneswar, India, PIXE technique was carried out using the 3 MV horizontal Tandem Pelletron accelerator (9SDH-2, NEC, USA) for elemental analysis of the leaf samples[19]. The samples were made into pellets and mounted on a multifaced target holder which was placed at 45^0 to the beam direction. The target holder was mounted on an insulated stand and was surrounded by a cylindrical electron suppressor held at negative potential with respect to the target. Inside the PIXE chamber, the proton beam was collimated to a diameter of 2 mm on the target in vacuum (10-6 Torr)[20].

Integrated charge on the thick sample was measured using a current integrator, which was connected to the target holder. Proton beams (3 MeV) with 20 nA beam current was used to bombard the targets. To detect the characteristic X-rays emitted from the targets, a Si(Li) detector (Ortec, SLP-06165-opt-0.5) with a resolution of 165 eV at 5.9 keV with a thin beryllium window (0.0127 mm), placed at 90° to the beam direction, was used. The X-rays exit the scattering chamber through a 95 lm Mylar window before entering the detector.

During data collection, no extra/other X-ray absorbers were used between the detector and incoming X-rays. Spectra were recorded using a multi-channel analyser (MCA) calibrated with 55Fe X-ray source.

Sample Preparation

The leaves were washed carefully with running tap water to remove dirt materials and shade

dried. The dried samples were powdered using mortar and pestle. For PIXE analysis, pellets (13 mm diameter) were prepared for which 150 mg of powdered samples were mixed with 150 mg of high pure graphite powder (1:1; Alfa Aesar, purity 99.9999 %).

Thick targets were prepared by pressing the above mixture in a hydraulic press[21]. In a similar fashion, pellets were also made from a certified Standard Reference Material (SRM) i.e. Pine Needle (NIST-1575) obtained from the National Institute of Standards & Technology (NIST), USA. Prior to irradiation of the samples, pellets of NIST–SRM were irradiated as standards for calibration, quantification and verification of results in Figure 7, 8 & 15.

Data Analysis

GUPIX-2000 software package[22] was used to carry out the PIXE spectral analyses. GUPIX is versatile software package for fitting PIXE spectra for thin, thick, intermediate and layered specimens. This package has provision to convert the X-ray peak intensities into elemental concentrations using a standardization technique involving fundamental parameters and pre-determined instrumental constants.

This provides a non-linear least square fitting of the spectrum, together with subsequent conversion of the fitted X-ray peak intensities into elemental concentrations which were expressed on dry weight basis (mg element per kg d. wt. test sample). The certified and measured values of elemental concentrations of ‘‘NIST Pine Needle Standard’’ (NIST-1575) are presented in Table 6.

RESULTS & DISCUSSION

The pharmacognostical investigations and preliminary phytochemical screening of leaves of both the plants were performed. The leaves of *C. serratum* are green in colour. These are obovate, oblong and glabrous. Leaves are slightly bitter in taste with characteristic odour. The leaves of *C. viscosum* are green in colour. Leaves are ovate and glandular beneath. These are bitter pungent in taste with disagreeable odour. The microscopy of the leaves of *C. serratum* and *C. viscosum* shows the presence of cuticle, epidermis, trichomes, stomata, palisade layer, spongy parenchyma, collenchyma, vascular bundles and parenchymatous cells, which are shown in Table 1. The results of the leaf constants of *C. serratum* and *C. viscosum* such as stomatal number, vein-islet number, veinlet termination number and palisade ratio are presented in Table. 2.

Simple starch grains are observed in the leaves of both *C. serratum* and *C. viscosum*, stained in blue colour with dilute iodine solution. The physicochemical parameters such as ash values and extractive values of the leaves of *C. serratum* and *C. viscosum* were determined and the results are shown in Table 3. The results of preliminary phytochemical screening of pet ether, chloroform and methanol extracts of *C. serratum* and *C. viscosum* are recorded in Table 4, 5. In trace metal study, the respective K and Ca values of 20,500 ± 4.33, 18,400 ± 5.01 µg g^{-1} for *C. serratum* and 20,100 ± 4.21, 18,200 ± 4.76 µg g^{-1} for *C. viscosum* are found. These values for K and Ca are higher in case of *C. serratum* than *C. viscosum*.

The trace elements like Ni, As and Pb are not detected in case of *C. serratum* and the trace elements such as Ni, As, Se and Pb are not detected in case of *C. viscosum*. The K and Ca values in both *C. serratum* and *C. viscosum* are found to be much higher than the other trace elements present. The results of trace metal analysis of the leaves of *C. serratum* and *C. viscosum* are presented in Table 7, 8. The improvement in the standardization of herbal drugs has led to the development of effective quality medicines from plants.

The evaluation of physical constants of drug is an important parameter in detecting adulteration. In the present study, the leaves of *C. serratum* and *C. viscosum* were evaluated qualitatively by studying various morphological, microscopical features, physicochemical parameters, preliminary phytochemical screening and trace metal analysis. These are performed according to WHO guidelines. These parameters are useful for standardization and isolation of new phytoconstitutents from the leaves.

Table 1: Microscopical features of *C. serratum* and *C. viscosum* leaves

Microsopical Features	***C. serratum***	***C. viscosum***
Leaf	Dorsiversial	Dorsiversial
Lamina	Comparatively Very thin	Thick
Cuticle	Thinner	Thinner
Epidermis cell	Polygonal, slightly elongated	Polygonal
Trichomes	Non glandular, comparatively less	Glandular and non glandular
Stomata	Diacytic	Anomocytic
Pallisade Layer	Uniserriate	Uniserriate
Spongy Parenchyma	3 to 4 layered	5 to 7 layered
Mid-rib	plano-convex	plano-convex dorsal surface is more convex than ventral surface.
Collenchymas	Compact	Comparatively less compact
Vascular Bundle	Collateral	Collateral
Mid rib parenchymatous cell	20-25 % portion of mid rib occupied. large in size	30-35 % portion of mid rib occupied

Table 2: Leaf constants *C. serratum and C. viscosum*

Leaf constants	***C. serratum***	***C. viscosum***
Stomatal Number	22 Upper surface 31 lower surface	31 Upper surface 43 lower surface
Vien-islet Number	9-10	9-11
Veinlet	10-12	11-13
Termination Number		
Palisade ratio	10	13

Table 3: Physicochemical parameters of *C. serratum* and *C. viscosum* leaves

Physicochemical parameters	***C. serratum***	***C. viscosum***
Total Ash	12.54±0.32	15.59±0.37
Acid-insoluble ash	3.62±0.25	4.63±0.18
Water soluble ash	5.65±0.62	6.51±0.42
Alcohol soluble extractive	6.49±0.28	14.43±0.53
Water soluble extractive	22.45±0.37	12.65±0.71

Table 4: Preliminary phytochemical screening for the leaf extracts of *C. serratum*

Phytochemical constituents	**Pet. ether extract**	**Chloroform extract**	**Methanol extract**
Alkaloids	-	-	+
Flavonoids	+	-	+
Carbohydrate	+	+	-
Saponins	+	-	+
Triterpenoids	+	-	+
Steroids	+	+	-
Tannins	-	-	+
Glycosides	-	-	+

'+' denotes Present, '-' denotes Absent

Table 5: Preliminary phytochemical screening for the leaf extracts of *C. viscosum*

Phytochemical constituents	Pet. ether extract	Chloroform extract	Methanol extract
Alkaloids	+	+	+
Carbohydrate	-	+	+
Flavonoids	-	-	-
Glycosides	+	+	+
Phenols	+	-	-
Phytosterols	-	+	-
Saponins	+	+	-
Steroids	+	+	-
Sterols	-	-	+
Triterpenoids	-	+	+
Proteins	-	+	-
Quinones	-	+	-
Tannins	-	-	+

'+' denotes Present, '-' denotes Absent

Table 6: Certified and measured values of elemental concentrations (µg/g) of standard, Pine Needle (NIST-1575)

Elements	Certified values	Measured values
Potassium (K)	3700 ± 0.02	3680 ± 0.02
Calcium (Ca)	4100 ± 0.02	4110 ± 0.03
Chromium (Cr)	2.60 ± 0.2	2.599 ± 0.34
Manganese (Mn)	675 ± 15	678.53 ± 10.87
Iron (Fe)	200 ± 10	219.33 ± 12.80
Cobalt (Co)	0.1 ± 0.00	0.101 ± 0.004
Nickel (Ni)	3.5 ± 0.00	3.503 ± 0.12
Copper (Cu)	3.0 ± 0.3	2.98 ± 0.33
Rubidium (Rb)	11.70 ± 0.1	11.70 ± 0.31
Strontium (Sr)	4.80 ± 0.2	4.80 ± 0.40

Table 7: PIXE based estimation of elemental concentration in *C. serratum* leaf

Elements	*C. serratum*
Potassium(K)	20500 ± 4.33
Calcium(Ca)	18400 ± 5.01
Vanadium (V)	01.62 ± 0.1
Chromium (Cr)	8.78 ± 2.56
Manganese(Mn)	130.51 ± 1.32
Iron(Fe)	741.28 ± 4.86
Cobalt(Co)	0.13 ± 0.03
Nickel (Ni)	Nd
Copper(Cu	08.81± 1.45
Zinc (Zn)	122.85 ± 1.02
Arsenic(As)	Nd
Selenium(Se)	0.30 ± 0.32
Bromine(Br)	04.56 ± 0.34
Rubidium(Rb)	11.06 ± 1.56
Strontium(Sr)	77.60 ± 0.65
Lead(Pb)	Nd

Nd- Not detected

Table 8: PIXE based estimation of elemental concentration in *C. viscosum* leaf

Elements	*C. viscosum*
Potassium(K)	20100 ± 4.21
Calcium(Ca)	18200 ± 4.76
Vanadium (V)	1.43 ± 0.07
Chromium (Cr)	8.56 ± 0.11
Manganese(Mn)	120.69 ± 1.02
Iron(Fe)	740.53 ± 5.45
Cobalt(Co)	0.14 ±0.061
Nickel (Ni)	Nd
Copper(Cu	8.57 ± 0.12
Zinc (Zn)	116.41 ± 1.07
Arsenic(As)	Nd
Selenium(Se)	Nd
Bromine(Br)	04.88 ± 0.09
Rubidium(Rb)	10.61 ± 0.56
Strontium(Sr)	67.7 ± 3.45
Lead(Pb)	Nd

Nd- Not detected

Microscopy of *C. serratum* leaf

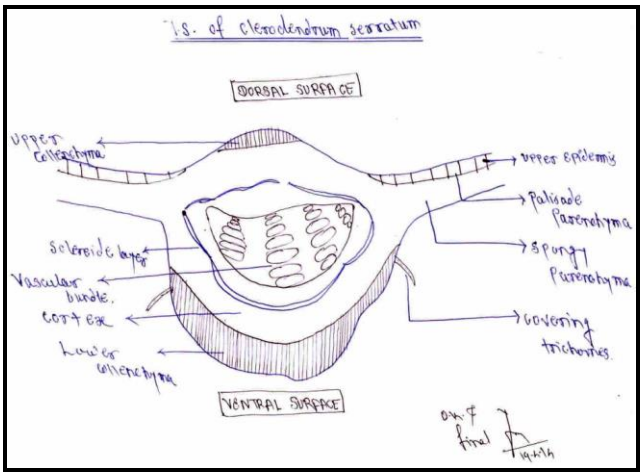

Figure 1A: Schematic diagram of *C. serratum* leaf

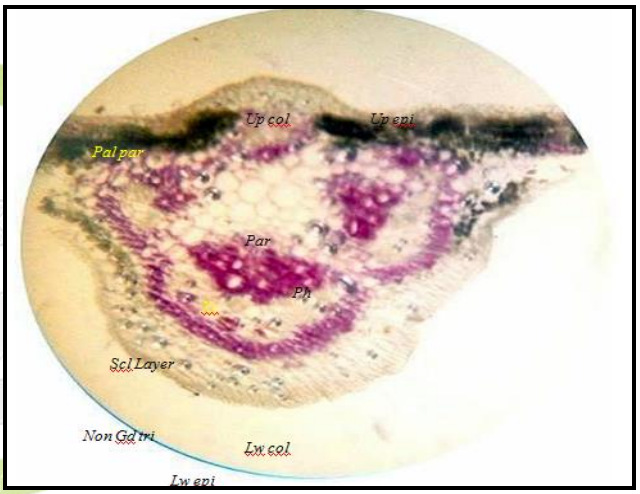

Figure 1B: Transverse section of *C. serratum* leaf stained in Phloroglucinol + Conc. HCl (1:1)

Leaf Constants of *C. serratum*

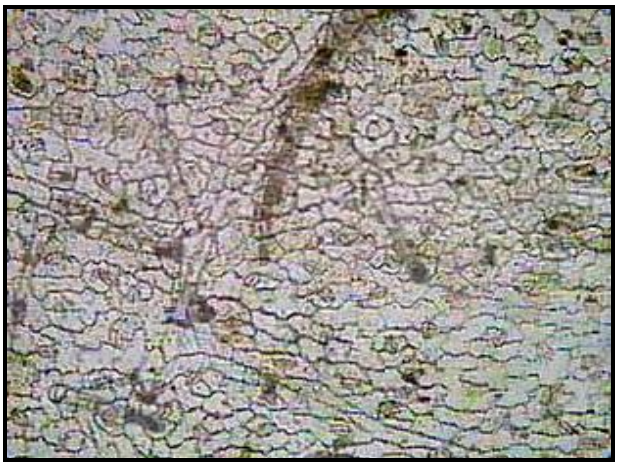

Figure 2: Stomatal number

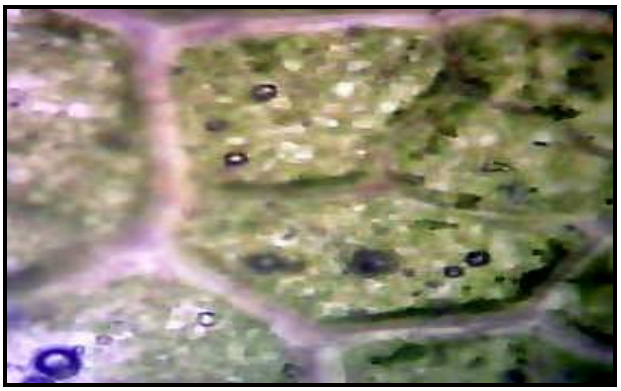

Figure 3: Vein-islet number

Figure 4: Palisade ratio

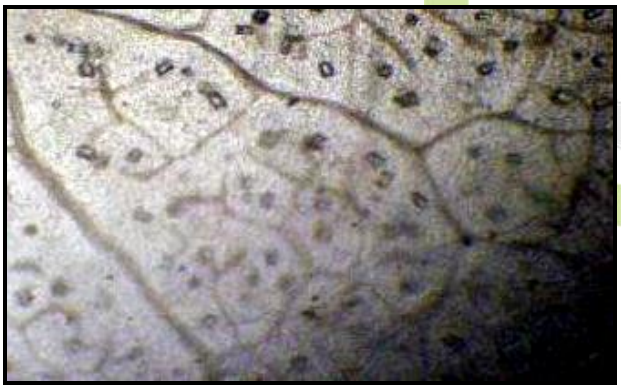

Figure 5: Vein let termination number

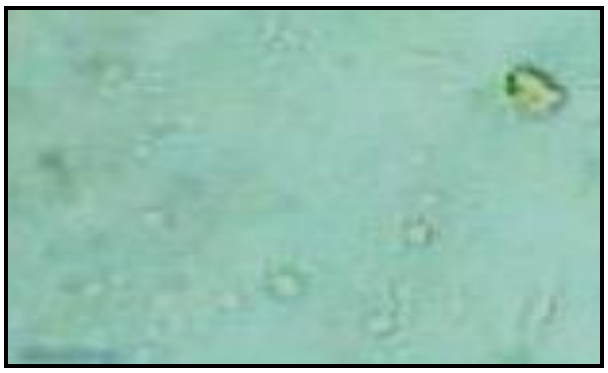

Figure 6: Observation of starch grains in *C. serratum* leaf

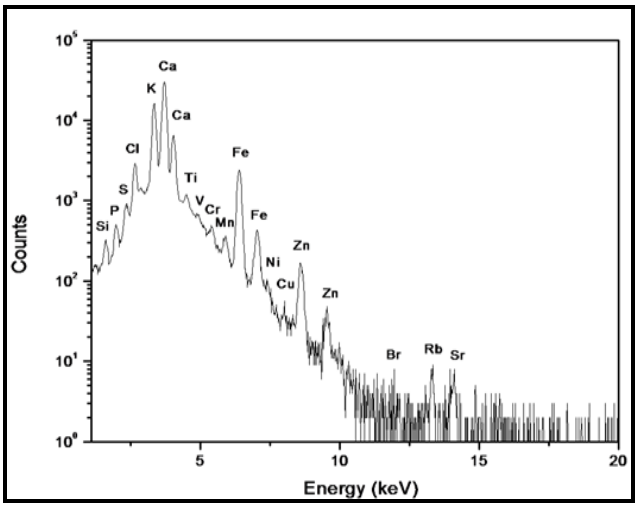

Figure 7: Representative PIXE spectra of NIST Pine Needle (1575)

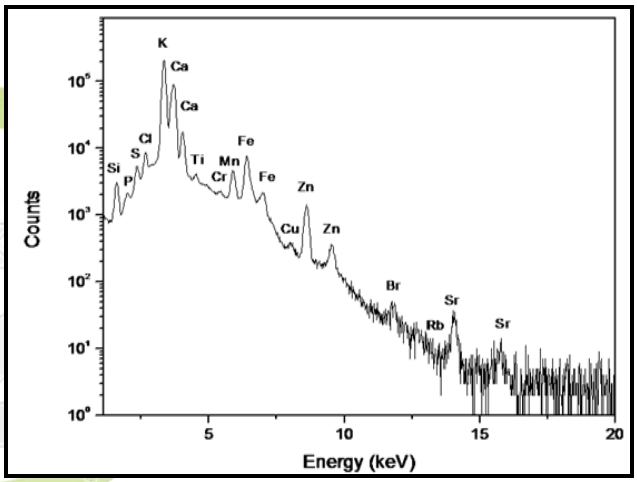

Figure 8: PIXE spectra of *C. serratum* leaf

Microscopy of *C. viscosum* Leaf

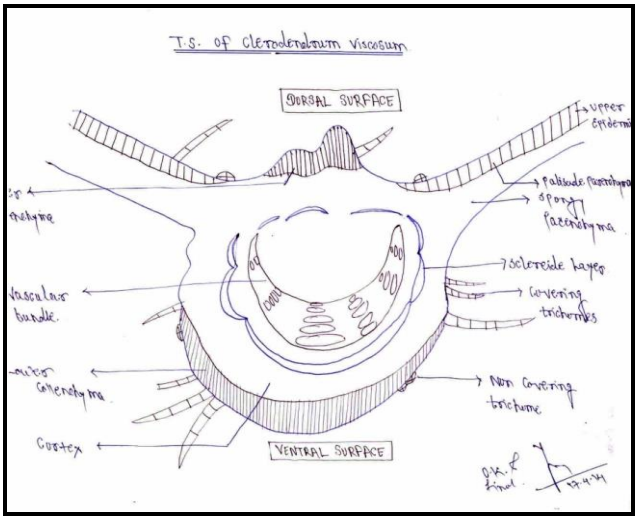

Figure 9A: Schematic diagram of *C. viscosum* Leaf

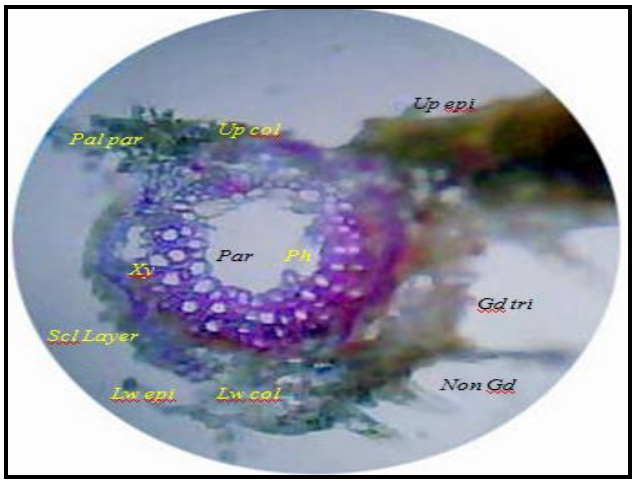

Figure 9B: Transverse section of *C. viscosum Leaf* stained in Phloroglucinol + Conc. HCl (1:1)

(*Up* epi- Upper epidermis; *Pal par*- Pallisade parenchymas; Xy-Xylem; *Ph*-Phloem; *Per fib*-Pericyclic fibre; *Par*- Parechymas; *Scl Layer*-Sclereide layer; *Lw col*- Lower collenchymas; *Non Gd tri*-Non Glandular trichomes; *Gd tri*-Glandular trichomes; *Lw epi*-Lower epidermis)

Leaf Constants of *C. viscosum*

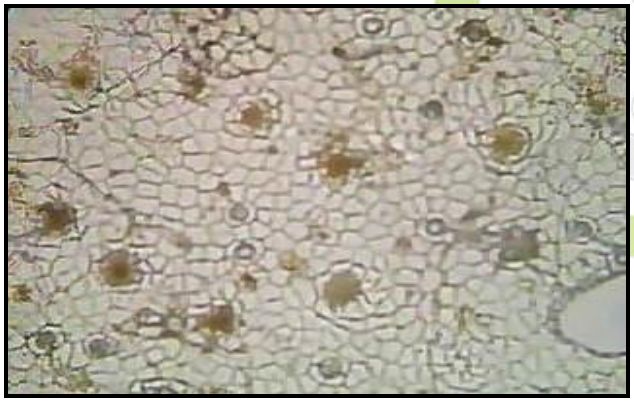

Figure 10: Stomatal number

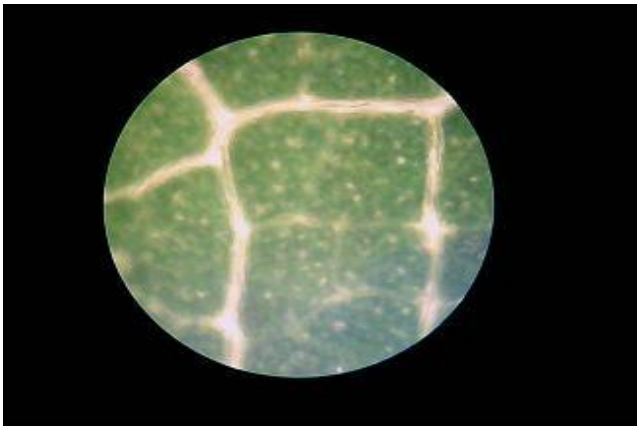

Figure 11: Vien-islet number

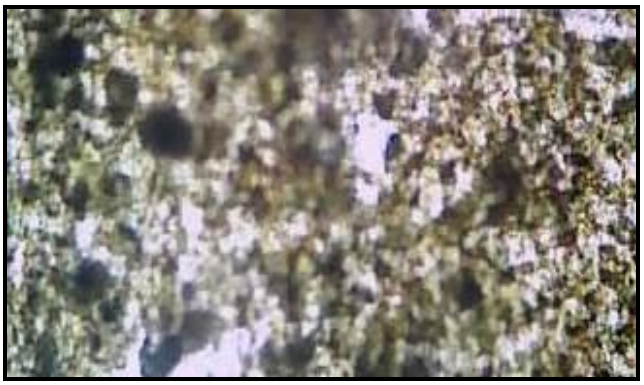

Figure 12: Palisade ratio

Figure 13: Veinlet termination number

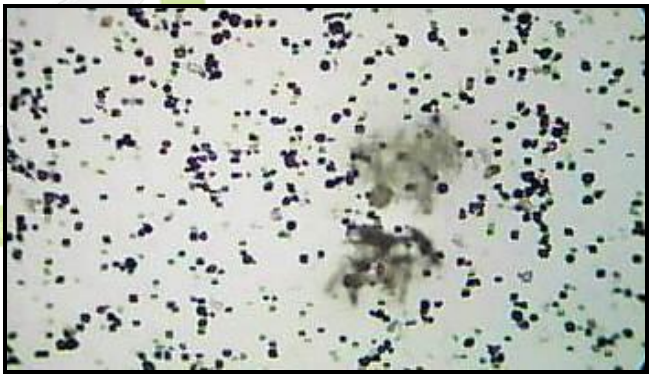

Figure 14: Observation of starch grains in *C. viscosum* leaf

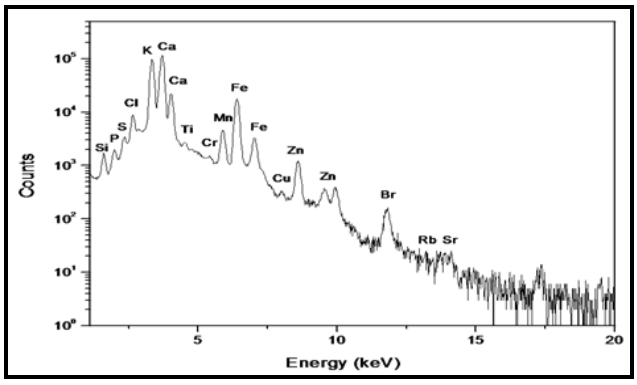

Figure 15: PIXE spectra of *C. viscosum* leaf

CONCLUSION

The pharmacognostical evaluation of the leaves of *C. serratum* and *C. viscosum* serves as a basis for proper identification, physical evaluation and monograph of these plants. Further, the preliminary phytochemical study aids in providing valuable information about the chemical composition of the plant material.

ACKNOWLEDGEMENT

The authors are thankful to the HOD, University Department Pharmaceutical Sciences, Utkal University, Vani Vihar and Director, Institute of Physics (Department of Atomic Energy, Government of India), Bhubaneswar, Odisha, India for providing facilities to carry out the work.

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