PHARMACOGNOSTICAL, PRELIMINARY PHYTOCHEMICAL AND *IN VITRO* ANTIOXIDANT EVALUATION OF THE AERIAL PARTS OF *CYPHOSTEMMA SETOSUM* (ROXB.)

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ABSTRACT

The objective of the investigation presented here is to explore the pharmacognostical nature, phytochemical characterization and *in vitro* antioxidant activity of *Cyphostemma setosum* (Roxb.). *Vitis setosa* (Roxb.) synonymous to *C. setosum* (Roxb.), is a herbaceous plant known for its folkloric treatment of ailments and belongs to the family *Vitaceae.* The aerial parts of the plant were collected and the pharmacognostic studies including anatomy of the aerial parts of the pant and powder microscopy, were performed. Microscopical examination of the transverse section of the leaf identified the presence of multicellular trichomes and tetracyclic stoma in the epidermal layers of the leaf. The shade dried crude plant drug powder was tested for ash values, extractive values and loss on drying. The crude drug was extracted with 80 % ethanol and fractionated with solvents of increasing polarity. The preliminary phytochemical chemical tests were done on various extracts of the powdered dried aerial parts of *C. setosum* (Roxb.). The phytochemical tests revealed the presence of alkaloids, tannins, flavonoids, carbohydrates, proteins, gums and quinones. These observations will be useful in evaluation of pharmacognostical and phytochemical standards to ensure the purity and quality of this plant. The chloroform, ethyl acetate and aqueous fractions of ethanolic extract of the leaves of *V. setosa* (Roxb.) were tested for antioxidant activity using *in vitro* models like nitric oxide radical scavenging study and 1,1-diphenyl, 2-picryl hydrazyl (DPPH) free radical scavenging activity. The results revealed that the fractions tested exhibit concentration dependent antioxidant activity. The ethyl acetate fraction predominant antioxidant activity than the other two fractions exhibited, while the chloroform fraction exhibited showed a minimal or less antioxidant activity when compared with standard.

Keywords: Pharmacognosy, Phytochemistry, *In vitro* antioxidant activity and *Cyphostemma setosum* (Roxb.)

INTRODUCTION

Vitis setosa (Roxb.), synonymous to *Cyphostemma setosum* (Roxb.), is a prostate herb that belongs to one of the 910 species of Vitaceae family which are categorized under 14 genera[1-2]. This plant is called as "Pulinaralai" in Tamil and is widely distributed in the Western ghats of Tamil Nadu, Karnataka, Andhra Pradesh, and Assam of India, Rangamati district of Bangladesh and in Sri Lanka[2]. The phytochemical constituents of this plant have been reported for both ethanolic and methanolic extracts[3-4]. Antimicrobial activity[4] and antioxidant activity[5] were proven by *in vitro* models with the methanolic extract of shade-dried and finely powdered aerial parts of *C. setosum* (Roxb.). An *in vivo* study performed on mice with ethanolic extract of the leaves of *C. setosum* (Roxb.) confirmed the folkloric claim of antinociceptive activity[6]. Pylorus ligation and ethanol-induced *in vivo* models in Wistar albino rats exhibited the antiulcer activity of methanolic extract of *Cissus setosa* Roxb. synonymous to *C.setosum* (Roxb.)[7]. Very limited information is available for this folkloric herbaceous plant *C. setosum* (Roxb.), so a detailed anatomy, preliminary phytochemical study and *in vitro* antioxidant activity was explored for the chloroform, ethyl acetate and water fractions of ethanolic extracts of aerial parts. The *in vitro* antioxidant activity of *V. setosa* (Roxb.) was proved using nitric oxide scavenging and inhibition of DPPH radical models with fractions of ethanolic extract.

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MATERIALS AND METHODS

The leaves and aerial parts of *C. setosum* (Roxb.) were collected from Singaperumal Kovil, Kanchipuram, Tamil Nadu, India and identified and authenticated by Dr. P. Jayaraman, Director, Plant Anatomy Research Center, (PARC), Tambaram, Chennai. The registered number of the authentication certificate[8] is PARC/2018/3735.

ANATOMICAL STUDIES

The required samples of different organs were cut and removed from plant and fixed in FAA (formalin - 5mL + acetic acid - 5 mL + 70 % ethyl alcohol – 90 mL). After 24 h of fixing, the specimens were dehydrated with graded series of tertiary butyl alcohol (TBA) as per the schedule given by Sass, 1940. Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58 - 60 °C) until TBA solution attained super saturation. The specimens were cast into paraffin blocks. The paraffin embedded specimens were sectioned with the help of rotary microtome. The thickness of the sections was 10 - 12 µm. Dewaxing of the sections was done by customary procedure[9]. The sections were stained with toluidine blue. Since toluidine blue is a polychromatic stain, the staining results were remarkably good; and some cytochemical reactions were also obtained[10]. The dye rendered pink color to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to mucilage and blue to the protein bodies. Wherever necessary, sections were also stained with safranin and fast green and iodine-potassium iodide (IKI) for starch. For studying the stomatal morphology, venation pattern and trichome distribution, paradermal sections (section taken parallel to the surface of leaf) as well as clearing of leaf with 5 % sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey's maceration fluid were prepared[11]. Glycerin mounted temporary preparation were made for macerated/cleared materials. Powdered materials of different parts were cleared with sodium hydroxide and mounted in glycerin medium after staining. Different cell components were studied and measured. Microscopic descriptions of tissues are supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon Labphoto - 2 microscopic unit. For normal observations, bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the scale-bars[12-21].

EVALUATION OF ANALYTICAL PARAMETERS

Ash values[22]

Determination of total ash

About 2 g of dried crude drug was weighed accurately in a tared silica crucible and incinerated at a temperature not exceeding 450 °C until free from carbon. It was then cooled in desiccator and weighed. The percentage of ash was calculated with reference to the air dried drug.

Determination of water-soluble ash

The total ash was boiled for five minutes with 25 mL of water. The insoluble matter was collected in an ash less filter paper, washed with hot water and ignited for 15 minutes at a temperature not exceeding 450 °C. The weight of the insoluble matter was subtracted from the weight of the ash and the difference in the weight of the ash represents the water-soluble ash. The percentage of water-soluble ash was calculated with reference to the dried drug.

Extractive values[22]

Determination of alcohol soluble extractive

About 5 g of the powder was macerated with 100 mL of alcohol of the specified strength in a closed flask for 24 h, shaking frequently for 6 h and allowed to stand for 18 h. It was filtered rapidly, and 25 mL of the filtrate was evaporated to dryness at 105 °C and weighed. The percentage of alcohol soluble extractive was calculated with reference to the dried drug.

Determination of water-soluble extractive

About 5 g of the powder was macerated with 100 mL of distilled water in a closed flask for 24 h, shaking frequently for 6 h and allowed to stand for 18 h. It was filtered rapidly, and 25 mL of the filtrate was evaporated to dryness at 105 °C and weighed. The percentage of water-soluble extractive was calculated with reference to the dried drug.

Determination of chloroform soluble extractive

About 5 g of the powder was macerated with 100 mL of chloroform in a closed flask for 24 h, shaking frequently for 6 h and allowing to stand for 18 h. It was filtered rapidly, and 25 mL of the filtrate was evaporated to dryness at 105 °C and weighed. The percentage of chloroform soluble extractive was calculated with reference to the dried drug.

Loss on drying

Loss on drying is the loss of mass expressed as percent w/w and was determined by the following procedure: About 2 g of drug was weighed and transferred to a dry stoppered weighing bottle. The weight of the bottle and the drug was taken accurately. After removing the stopper, the bottle containing drug was placed in an oven for 1 h at 120 °C. After 1 h, the bottle was removed and cooled in a desiccator and weighed by replacing the stopper which was continued until difference between two successive weighing was not more than 0.25 % of constant weight.

Preliminary phytochemical analysis

The leaves of the plant were shade dried, coarsely powdered, and extracted with 80 % V/V ethanol by cold maceration method for 72 h. The total extract thus obtained was fractionated successively with solvents of increasing polarity viz, petroleum ether, ethyl acetate, chloroform and water[23]. The total ethanolic extract fractions viz., petroleum ether, ethyl acetate, chloroform and water fraction of the powder were subjected to the following chemical tests for identification of phytochemical constituents.

Test for alkaloids - Mayer's test - A pinch of dried extract was taken, and 2 mL of dilute hydrochloric acid was added, mixed, filtered and one or two drops of Mayer's reagent was added. Formation of white precipitate indicates the presence of alkaloids[24].

Test for alkaloids - Dragendorff's test - A pinch of dried extract was taken and treated with 2 mL of 2 % V/V of acetic acid, mixed thoroughly and filtered. Then two drops of Dragendorff's reagent was added to the filtrate. Formation of orange brown precipitate indicates the presence of alkaloids[24].

Molisch's test for carbohydrates - The substance was treated with alpha naphthol and concentrated sulphuric acid. Formation of violet color indicates the presence of carbohydrates[24].

Test for glycosides - A pinch of extract was taken in a watch glass and 2 drops of alcohol was added to dissolve the extract. An equal quantity of anthrone was added and mixed thoroughly and dried. Then one drop of concentrated sulphuric acid was added and spread in a thin film with a glass rod in a watch glass and heated over the water bath. Formation of dark green color confirms the presence of glycosides[24].

Borntraeger's test for anthraquinone glycosides - A pinch of the extract was boiled with dilute sulphuric acid, filtered while hot and filtrate was extracted with solvents like benzene. It was shaken well, and the organic layer was separated and to this equal volume of dilute ammonia was added. Formation of rose-pink color in ammonia layer indicates the presence of anthraquinone glycoside[24].

Legal's test for cardiac glycosides - The substance was hydrolysed for few hours in a water bath. The hydrolysate was added with 2 mL of pyridine, sodium nitro prusside solution and was made alkaline with sodium hydroxide solution. The color change from yellow to orange indicates the presence of cardiac glycoside[24].

Kellerkillani's test for cardiac glycosides - About 1 g of powder was boiled with 70 % ethanol for 3 minutes and filtered. The filtrate was treated with lead acetate solution, shaken well, and filtered. The clear filtrate was treated with equal volume of chloroform and the chloroform layer was evaporated. The residue was dissolved in 3 mL of glacial acetic acid and to this solution 2 drops of ferric chloride was added. The contents were transferred to test tube containing 2 mL of concentrated sulphuric acid. Formation of reddish-brown layer acquiring bluish green color after standing indicates the presence of cardiac glycoside[24].

Fehling's test for sugar - The extract was treated with equal quantity of Fehling's solution A and B and it was heated on a water bath. Formation of brick red precipitate indicates the presence of sugar[24].

Benedict's test for sugar - The substance was treated with Benedict's reagent and heated in a water bath. Formation of reddish-brown precipitate indicates the presence of sugar[24].

Liebermann's Burchard test for steroids - The extract was dissolved in 2 mL of chloroform and 10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid were added. Formation of green color indicates the presence of phytosterols[24].

Salkowski test for steroids - The extract was dissolved in 2 mL of chloroform and an equal volume of concentrated sulphuric acid was added slowly through the sides of the test tube. The chloroform layer turns reddish violet color and the lower layer turns a yellowish color with green fluorescence, which indicates the presence of phytosterols[24].

Test for tannins[24] - A pinch of dried extract was dissolved in ethanol, mixed thoroughly and filtered. The filtrate was treated with the following reagents.

1. Lead acetate solution- formation of white precipitate shows the presence of tannins

2. Ferric chloride solution- formation of deep blue color shows the presence of tannins
3. Aqueous gelatin solution- formation of white precipitate shows the presence of tannins

Foam test for saponins - 1 mL extract was diluted with distilled water to 20 mL and shaken in a graduated cylinder for 15 min. Formation of foam for about 1 cm layer indicates the presence of saponins[24].

Millon's test for proteins - The extract was dissolved in 1 mL of ethanol, filtered and the filtrate was treated with Millon's reagent. Formation of red color indicates the presence of proteins[24].

Biuret test for proteins - The extract was dissolved in 1 mL of ethanol, filtered and the filtrate was added with 40 % V/V sodium hydroxide and copper sulphate solution. Formation of violet color indicates the presence of proteins[24].

Ninhydrin test for proteins - The extract was dissolved in 1 mL of ethanol, filtered and the filtrate was treated with ninhydrin reagent. Formation of purple color indicates the presence of proteins[24].

Xanthoprotein test for proteins - The extract was dissolved in 1 mL of water, filtered and the filtrate was treated with 1 mL of concentrated nitric acid. The content was made alkaline to litmus paper by adding ammonia solution. Formation of orange color indicates the presence of proteins[24].

Test for terpenoids - A pinch of dried extract was taken in a dried test tube. A piece of tin foil and 0.5 mL of thionyl chloride was added and heated gently. Formation of pink color indicates the presence of terpenoids[24].

Test for flavonoids[24]

1. **Shinoda test:** A pinch of dried extract was dissolved in ethanol, mixed thoroughly and filtered. To the filtrate, magnesium metal pieces and concentrated hydrochloric acid were added and heated gently. Appearance of magenta color indicates the presence of flavonoids.
2. A pinch of dried extract was treated with sodium hydroxide solution. Formation of yellow color indicates the presence of flavones and yellow to orange color indicates the presence of flavanones.
3. The extract was treated with concentrated sulphuric acid. Yellow to orange color indicates the presence of flavones and orange to crimson color indicates the presence of flavanones.

Test for anthocyanins[24]

1. The extract was treated with sodium hydroxide solution. Formation of blue - violet color indicates the presence of anthocyanins.
2. The extract was treated with concentrated sulphuric acid. Formation of yellowish orange color indicates the presence of anthocyanins.

Test for quinones - The extract was treated with sodium hydroxide. Formation of blue green or red color indicates the presence of quinones[24].

IN VITRO ANTIOXIDANT STUDY

Based on the results obtained from the preliminary phytochemical screening of the various fractions of ethanolic extract, the chloroform, ethyl acetate and water fractions were selected for further screening. Several concentrations ranging from 25-1000 µg mL^{-1} of the various fractions of ethanolic extract of *V. setosa* namely, chloroform fraction of ethanolic extract of *V. setosa* (CEVS), ethyl acetate fraction of ethanolic extract of *V. setosa* (EAEVS) and water fraction of ethanolic extract of *V. setosa* (WEVS) were tested for their antioxidant activity in different *in vitro* models.

Nitric oxide scavenging activity

Nitric oxide was generated from sodium nitroprusside and measured by Griess reaction. Sodium nitroprusside (5 mM) in standard phosphate buffer saline solution (0.025 M, pH: 7.4) was incubated with different concentrations of CEVS, EAVS and WEVS (25-1000 µg mL^{-1}) and ascorbic acid as reference standard (5 - 1000 µg mL^{-1}) and the tubes were incubated at 25 °C for 5 h. This control (ascorbic acid) was used without the test compounds but with equivalent amount of buffer added in an identical manner. After 5 h, 0.5 mL of incubated solution was removed and diluted with 0.5 mL of Griess reagent (1 % sulphanilamide, 2 % O-phosphoric acid and 0.1 % naphthyl ethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthyl ethylene diamine was read at 546 nm. All the determinations were performed in 6 replicates[25,26]. Percentage inhibition of nitric oxide radical was calculated by using the formula:

$$\text{Percentage inhibition (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

DPPH free radical scavenging activity

The free radical scavenging activity was measured in terms of hydrogen donating or radical scavenging ability

using the stable radical DPPH. About 0.1 mM solution of DPPH in methanol was prepared – called here after as DPPH solution. 1 mL of DPPH solution was added to 3 mL of the different concentration of CEVS, EAVS and WEVS (25-1000 µg mL^{-1}) separately in different test tubes. 1 mL of DPPH solution was added to standard vitamin E solution of varying concentrations ranging from 5-1000 µg mL^{-1} separately in different test tubes. This control solution (a solution without the test compound, but with an equivalent amount of methanol) used was also allowed to react with 1 mL of the DPPH solution separately in different test tubes. These mixtures were shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm using a spectrophotometer. The IC_{50} value (50 % of inhibitory concentration in µg mL^{-1}) of the crude extract and its fractions were compared with that of vitamin E, which was used as the standard. When the DPPH radical is scavenged, the color of the reaction mixture changes from purple to yellow with decreasing of absorbance at wavelength 517 nm. Decrease in absorbance of the reaction mixture indicates higher free radical scavenging activity[27].

The percentage inhibition of DPPH radical was calculated using the formula:

$$\text{Percentage inhibition (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

RESULTS AND DISCUSSION

Results

Transverse section (TS) of leaf

The leaf consists of a thick, conical adaxial part and broad semicircular abaxial part (Fig. 1). The midrib is 700 µm in vertical plane and 900 µm in horizontal plane. The epidermal layer of the adaxial cone consists of small rectangular or cubical thin walled cells. The abaxial part of the midrib has small squarish cells (Fig. 2). The adaxial cone consists of a thick mass of collenchyma cells. The ground tissue of the abaxial midrib consists of small air chambers, partition filaments and small parenchyma cells (Fig. 2).

The vascular system of the midrib consists of abaxial band of three independent bundles and adaxial set of two vascular bundles (Fig. 2, Fig. 3). The vascular bundles are conical and collateral. The bundles have radial rows of wide circular thick-walled xylem elements and three or four short lines of phloem elements (Fig. 3). The phloem elements are wide, cubical and thick walled. The protoxylem elements of both abaxial and adaxial bundles are directed towards the center of the midrib.

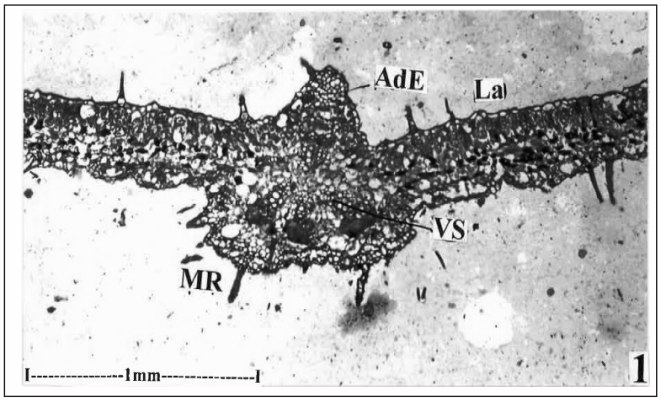

Fig. 1: Transverse section (T.S) of the leaf showing the adaxial epidermis (AdE), lamina (La), midrib (MR) and vascular strand (VS)

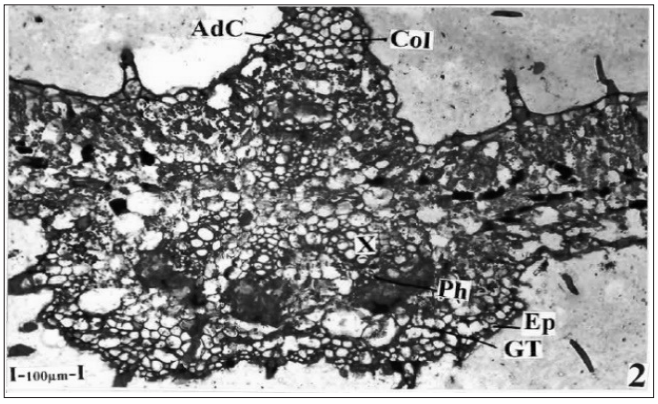

Fig. 2: Enlarged image of midrib showing the adaxial cone (AdC), collenchyma (Col), xylem(X), phloem (Ph), epidermis (Ep) and ground tissue (GT)

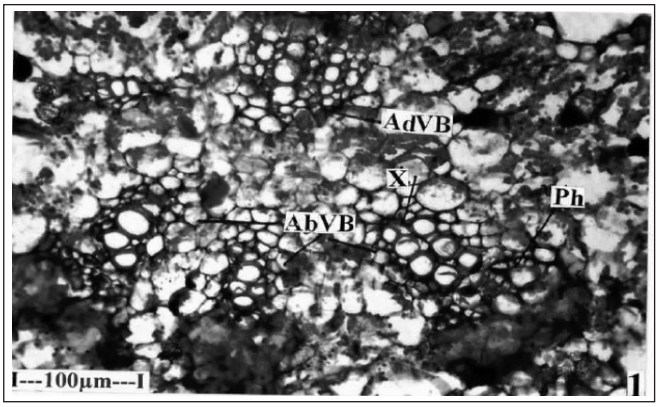

Fig. 3: Enlarged view of vascular bundles showing abaxial vascular bundle (AbVB), adaxial vascular bundle (AdVB), phloem (Ph) and xylem(X)

Leaf margin

The marginal part (Fig. 4) of the lamina is conical and thinner as compared to lamina further away from the margin. The leaf margin is 90 µm thick. The leaf margin is slightly bent down. The epidermal cells of the leaf margin are small and thick walled. There is no differentiation of the mesophyll cells into palisade and spongy parenchyma. There is a small group of wide parenchyma cells at the marginal part.

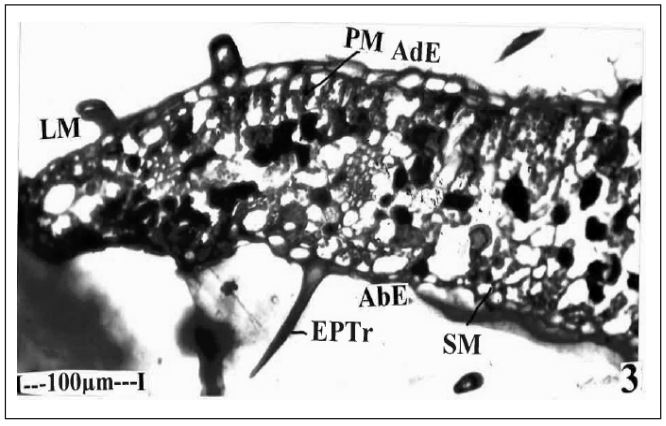

Fig. 4: T. S. of leaf margin (LM) showing palisade mesophyll (PM), adaxial epidermis (AdE), epidermal trichome (EpTr) and spongy mesophyll (SM)

Lamina

The lamina has dorsiventral differentiation of the mesophyll. The lamina is 250 µm thick. The adaxial epidermis consists of small squarish thin walled parenchyma cells. The cuticle is very thin. The abaxial epidermal cells are further smaller and the epidermal layer is slightly undulate. There are long straight multicellular uniseriate unbranched non glandular trichomes both on the adaxial and abaxial epidermal layers (Fig. 5). The cells of the trichomes are vertically elongated and thick walled. The trichomes are slightly buried in the epidermal layer.

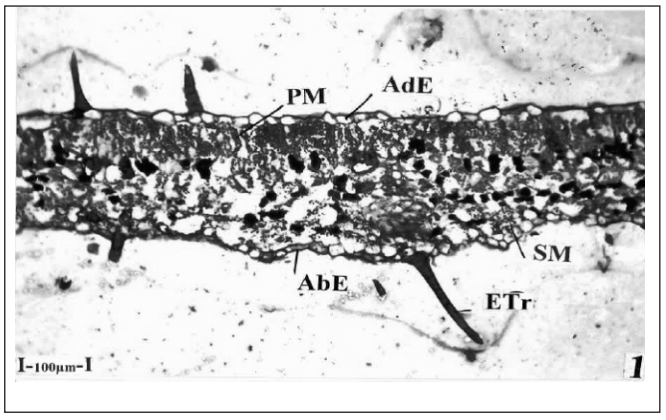

Fig. 5: T. S. of lamina showing abaxial epidermis (AbE), adaxial epidermis (AdE) and epidermal trichome (ETr)

The trichomes are 100 – 200 µm long and 25 µm thick.

Crystals

Calcium oxalate crystals (Fig. 6) are abundant in the leaf. The crystals are druses which are spherical spiny bodies. The druses occur in vertical line. The vertical parenchyma cells adjoining the filances bear the druses. There is one crystal in each parenchyma cell. The druses are 20 - 25 µm thick.

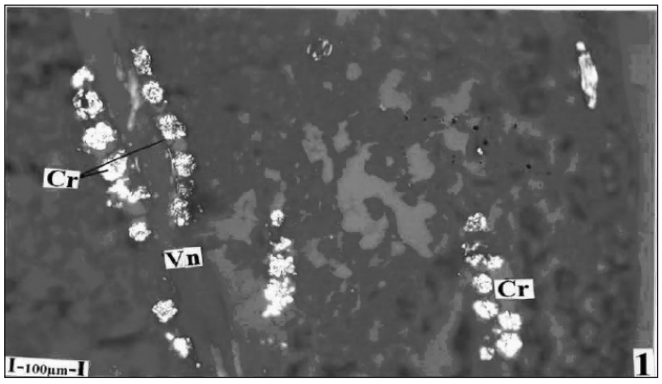

Fig. 6: Image showing the distribution of crystals(Cr) in the leaf vein (Vn)

Epidermal cells and stomata

Paradermal sections were prepared for the study of epidermis and stomata. The epidermal cells are polygonal in outline with straight thin anticlinal walls. The epidermal trichrome from where they arise in the epidermis is marked by wide circular thick-walled cell; this cell is surrounded by radiating triangular epidermal cells. The entire structure is called Cicatrix (Fig. 7).

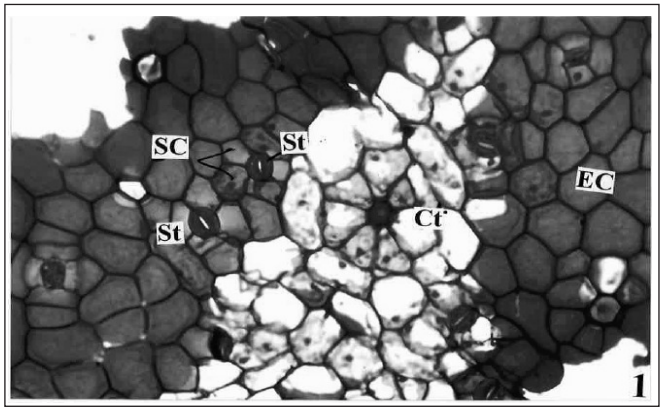

Fig. 7: Paradermal section of the leaf showing cicatrix (Ct), epidermal cells (EC), subsidiary cells (SC) and Stoma (St)

Stomata

The stomata are sparsely distributed in the epidermis. The guard cells are 50 * 60 µm in size. Each stoma is surrounded by four rectangular subsidiary cells. Two

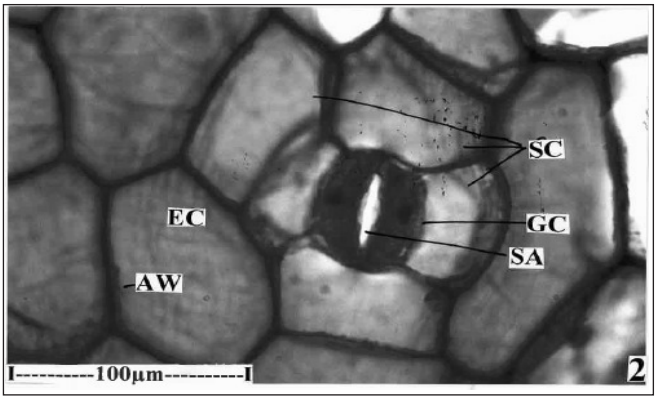

Fig. 8: Stoma enlarged image showing stomatal aperture (SA), guard cell (GC) with two polar & lateral subsidiary cell (SC) surrounded by epidermal cells (EC) with anticlinal wall (AW)

subsidiary cells are at the polar part of the stoma and the other two are at lateral part of the guard cells. The stoma is called tetracyctic stoma. (Fig. 8)

Powder microscopy

The powder preparation of the leaf shows the following inclusions as seen under the microscope

(i) Epidermal peelings of the leaf are frequent in the powder. They appear in surface view exhibiting the epidermal cells, epidermal trichomes and stomata.

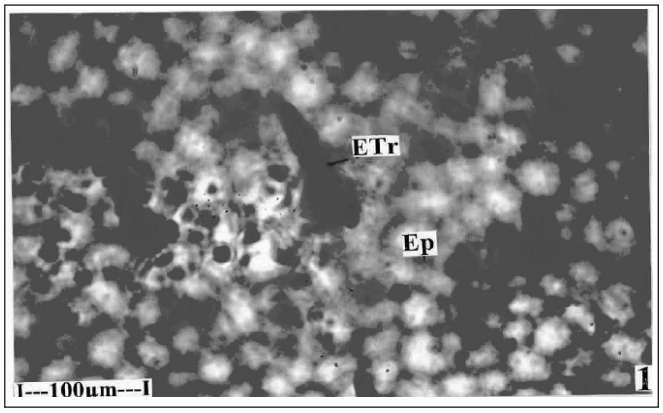

Fig. 9: Surface view of the laminar epidermal cells (EP) and epidermal trichome (ETr)

a. Epidermal cells are polygonal in outline with small area and thin straight anticlinal wall. (Fig. 9)

b. Epidermal trichomes: Long, conical multicellular, uniseriate, unbranched non glandular trichomes are common in the epidermal pealing (Fig. 9). The cells of the epidermis from which the trichomes originate are circular, thick walled and darkly stained. This cell is surrounded by many radiating epidermal cells. This trichome bearing epidermal apparatus is called cicatrix (Fig. 10)

(ii) Adaxial epidermal pealing shows various types of outline and straight anticlinal walls (Fig. 10). The adaxial epidermis has no stomata (apostomatic)

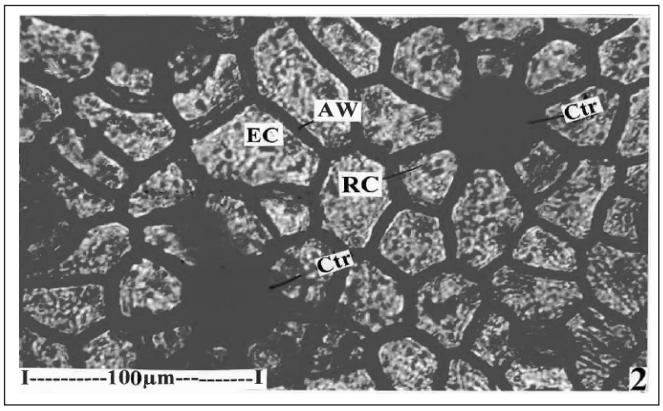

Fig. 10: Epidermal cells (EC) and Cicatrix (Ctr) enlarged image showing the anticlinal wall (AW) and rosette cell (RC)

(iii) Abaxial epidermal pealing shows polyhedral cells with thick straight anticlinal walls. Stomata are sparsely distributed in the epidermis (Fig. 11). The stomata are broadly elliptical with thick walls. The stomata are tetracytic type.

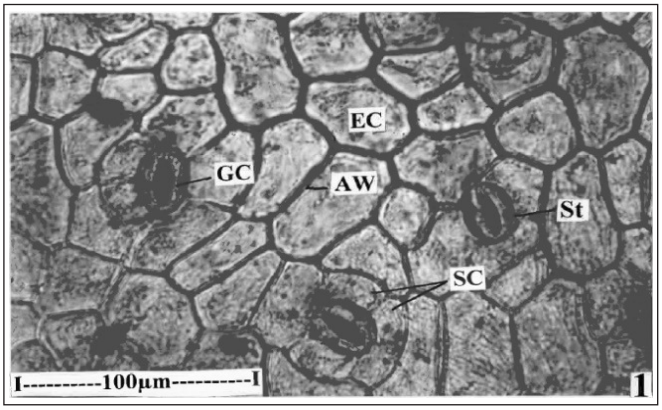

Fig. 11: Surface view of the epidermal cell (EC) showing stomata (St), guard cell (GC), subsidiary cells (SC) and anticlinal wall (AW)

(iv) Different types of parenchyma cells are abundant in the powder. Some of the cells are elongated and rectangular (Fig. 12). Some are squarish and some are long and rectangular (Fig. 12). The parenchyma cells have druse cell contents.

(v) Fibers: There are two types of fibers in the powder. Some of the fibers are thin, long and wavy with thick walls and narrow lumen (Fig. 13). These narrow fibers are 650 – 950 µm long and 10 µm thick. There are some wide fibers which possess wide lumen, thin walls and sometimes possess cell inclusion (Fig. 13).

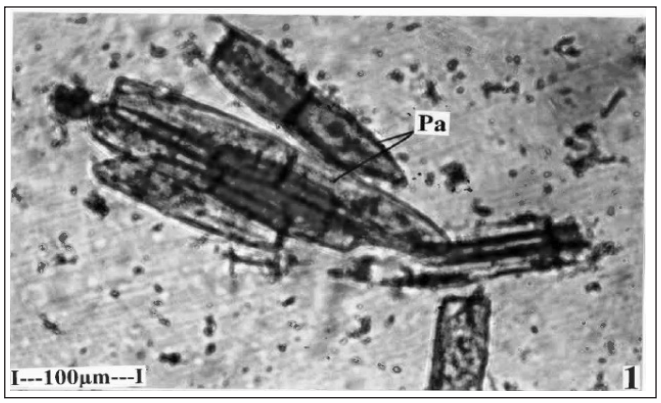

Fig. 12: Powder microscopic image of crude drug showing a cluster of parenchyma cells (Pa)

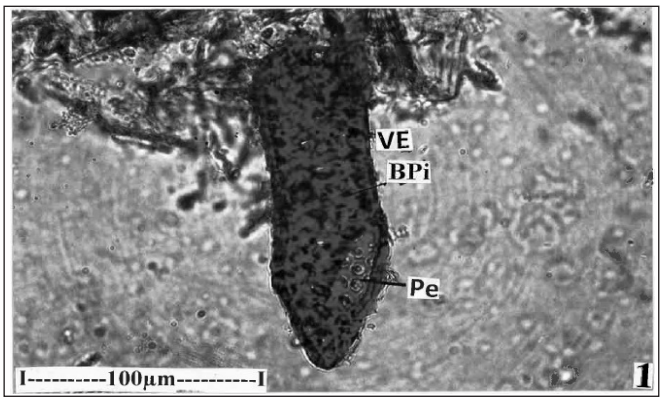

Fig. 14: Powder microscopy of crude drug showing short, wide cylindrical vessel element (VE) with oblique perforation (Pe) and bordered pits (BPi)

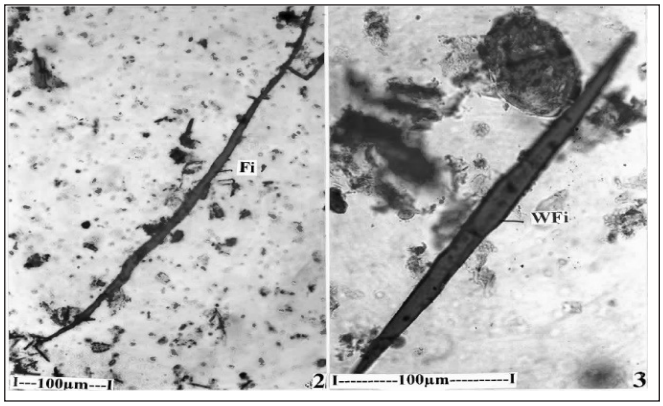

Fig. 13: Powder microscopic image of crude drug showing long, narrow fibers (Fi) and wide straight fibers (WFi)

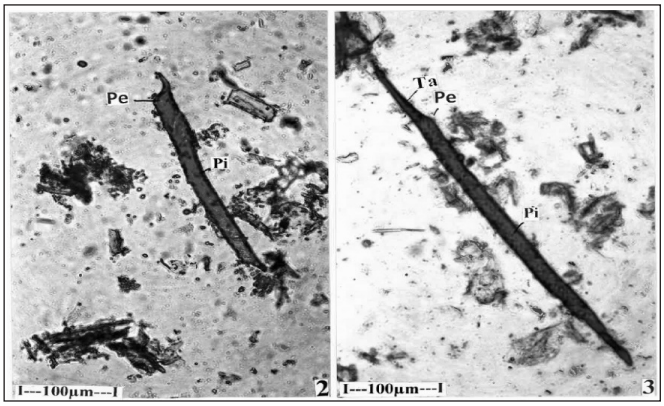

Fig. 15: Powder microscopy of crude drug showing short narrow vessel element and long narrow tailed vessel element with pits (Pi) and perforation (Pe)

Some of the wide fibers are separate with one or two cross walls. The wide fibers are 250 µm long and 20 µm wide.

(vi) Vessel elements: Vessel elements of different types are often met with in the powder. Some of the vessel elements are short, thick, and wide with elliptical oblique perforations and circular wide bordered pits on the lateral walls. This type of vessel element is 150 µm long and 40 µm wide (Fig. 14). Long, thin, and narrow fiber like vessel elements are also seen (Fig. 15). These types of vessel elements have long thin pointed tail. The element is 550 µm long and 30 µm wide. Some narrow vessel members are 350 µm long and 20 µm wide (Fig. 15).

EVALUATION OF ANALYTICAL PARAMETERS

Ash value[22]

Ash values are helpful in determining the quality and purity of a crude drug, especially in the powdered form. The ash content of a crude drug is generally taken to be the residue remaining after incineration. It usually represents the inorganic salts naturally occurring in the drug and adhering to it, but it may also include inorganic matter fielded for the purpose of adulteration. The total ash

Table I: Estimation analytical parameters

Sr. No	Parameters	Values (% w/w)
1.	**Ash values**	
	Total ash	6.42
	Water-soluble ash	0.87
2.	**Extractive values**	
	Water-soluble extractive value	9
	Alcohol-soluble extractive value	18
	Chloroform-soluble extractive value	18
3	**Loss on drying**	1.14

and the water-soluble ash determined for the powdered drug of *C. setosum* (Roxb.) is listed in Table I.

Extractive value[22]

Extractive value of crude drugs is useful for their evaluation, especially when the constituents of a drug cannot be readily estimated by any other means. These values are indicative of approximate measures of their chemical constituents and the nature of the constants present in crude drugs. The water-soluble, alcohol soluble and the chloroform soluble extractive values determined for the powdered drug of *C. setosum* (Roxb.) are listed in Table I. The extractive values of the dried crude drug of *C. setosum* (Roxb.) was found to be less than 18 % approximately.

Loss on drying

From the loss on drying study listed in Table I, it is evident that the powdered drug of *C. setosum* (Roxb.) can hold a moisture of ~1 % w/w.

Preliminary phytochemical screening of ethanol extracts fractions

The ethanolic extract of *C. setosum* (Roxb.) was fractionated with petroleum ether, ethyl acetate, chloroform and water. These fractions were tested for various phytochemicals and the results are presented in Table II. The presence of flavonoid is indicated in ethyl acetate and water fraction. The presence of alkaloid and quinones is indicated in aqueous fraction[23,24].

Table II: Preliminary phytochemical screening of ethanol extracts fractions

Sr. No.	Phytochemical	Petroleum ether (PEVS)	Chloroform (CFVS)	Ethyl acetate EAVS	Water (WVS)
1	Alkaloid	-	-	-	+
2	Glycoside	-	-	-	-
3	Tannin	-	+	+	+
4	Flavonoid	-	-	+	+
5	Carbohydrates	+	+	+	+
6	Proteins	+	+	-	+
7	Steroids	-	-	-	-
8	Gums	-	-	-	+
9	Terpenoids	-	-	-	-
10	Quinones	-	-	-	+

Nitric oxide scavenging activity

The nitric oxide scavenging activity of CEVS, EAEVS, WEVS and ascorbic acid was expressed as % inhibition. The scavenging of nitric oxide by EAEVS, WEVS and ascorbic acid (reference standard) were concentration dependent, whereas CEVS does not showed potential nitric oxide scavenging activity. The IC_{50} value of CEVS, EAEVS, WEVS and ascorbic acid were found to be 852 µg mL^{-1}, 66.71 µg mL^{-1}, 83.74 µg mL^{-1}, and 42.12 µg mL^{-1} respectively. The values are shown in Table III. Fig. 16 provides a graphical representation of the nitric oxide scavenging activity.

Table III: Nitric oxide radical scavenging study results

Sr. No.	Concentration (µg mL^{-1})	% inhibition			
		CEVS	EAEVS	WEVS	STD
1	50	24.38 ± 0.53*	46.45 ± 1.28**	41.52 ± 0.34**	60.32 ± 0.86**
2	100	31.89 ± 2.34**	59.03 ± 1.58**	54.67 ± 0.91**	71.32 ± 1.98**
3	200	42.48 ± 1.08**	68.56 ± 1.36**	64.04 ± 1.90**	81.02 ± 0.56*
4	400	38.91 ± 1.03*	77.45 ± 1.83**	70.57 ± 1.27*	85.97 ± 0.22**
5	800	44.59 ± 0.75*	88.07 ± 2.27**	78.99 ± 1.55**	91.26 ± 0.72**
6	1000	67.56 ± 1.20*	91.29 ± 0.93*	84.63 ± 0.90**	96.54 ± 1.24**
7	IC_{50}	852 µg mL^{-1}	66.71 µg mL^{-1}	87.34 µg mL^{-1}	42.12 µg mL^{-1}

*Values are mean ± SEM of 6 parallel measurement.** $P< 0.05$ and * $P< 0.01$ as compared against standard (ANOVA followed by Dunnet's 't' test). CEVS - chloroform fraction of ethanolic extract of V. setosa, EAEVS - ethyl acetate fraction of ethanolic extract of V. setosa, WEVS - water fraction of ethanolic extract of V. setosa. STD – ascorbic acid*

DPPH free radical scavenging activity

The potential decrease in the concentration of DPPH radical due to the scavenging ability of CEVS, EAEVS,

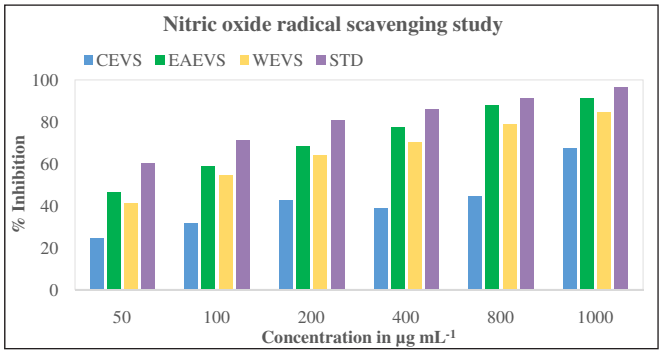

Fig. 16: Graphical representation of nitric oxide radical scavenging study of *V. setosa*

CEVS - Chloroform fraction of ethanolic extract of V. setosa, EAEVS - Ethyl acetate fraction of ethanolic extract of V. setosa, WEVS - Water fraction of ethanolic extract of V. setosa, STD – Standard (Ascorbic acid)

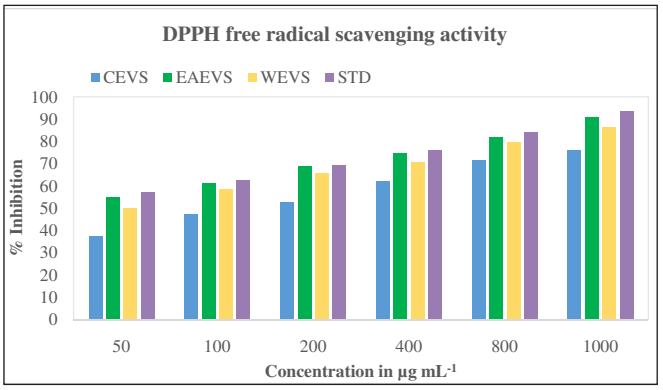

Fig. 17: Graphical reperesentation of DPPH free radical scavenging activity results of *V. setosa*

CEVS - Chloroform fraction of ethanolic extract of V. setosa, EAEVS - Ethyl acetate fraction of ethanolic extract of V. setosa, WEVS - Water fraction of ethanolic extract of V. setosa, STD – Standard (Vitamin E)

WEVS and ascorbic acid was expressed as % inhibition. The DPPH radical % inhibition exhibited by CEVS, EAEVS, WEVS and ascorbic acid was and found to be about 76.09 % ± 0.88 %, 90.71 % ± 1.42 %, 86.31 % ± 2.62 % and 93.72 % ± 0.90 %, respectively, at higher dose of 1000 µg mL^{-1}. The IC_{50} value of CEVS, EAEVS, WEVS and ascorbic acid was found to be 144.7 µg mL^{-1}, 45.4 µg mL^{-1}, 51.35 µg mL^{-1}, and 43.8 µg mL^{-1}, respectively. The scavenging activity was increasing in a dose-dependent manner. The results of DPPH scavenging assay are listed in Table IV. Fig. 17 provides the graphical representation of the inhibition of DPPH radical.

DISCUSSION

The objective of the current study was to standardize the plant drug by using anatomical evaluation, phytochemical estimations and to evaluate the *in vitro* antioxidant activities of *V. setosa* (Roxb.) synonymous to *C. setosum* (Roxb.). The anatomy of the plant was studied by performing the microscopical evaluation of the fresh leaf and shade dried crude dry powder. The transverse section of the leaf revealed the presence of tetracyctic stoma covered by 4 subsidiary cells. The stoma is present only in abaxial epidermis and is not present in adaxial epidermis. Calcium oxalate crystals are present abundantly in the leaf. Power microscopy revealed the presence of different types of parenchyma cells, vessel elements and two types of fibers. The analytical parameters ash value, extractive value and the loss on drying reported here shall be used as a standardization measure for the plant collection at different seasons in future. The phytochemical screening of *C. setosum* (Roxb.) indicated the presence of alkaloids, carbohydrates, proteins, tannins, flavonoids, gums and quinones[24].

Table IV: DPPH free radical scavenging activity results

Sr. No	Concentration (µg mL^{-1})	% inhibition			
		CEVS	EAEVS	WEVS	STD
1	50	37.54 ± 0.54*	54.72 ±0.45*	49.91 ± 1.21*	57.18 ± 1.64**
2	100	47.13 ± 1.49*	61.21 ± 1.07*	58.66 ± 2.28*	62.36 ± 0.77*
3	200	52.72 ± 2.05**	68.86 ±2.86**	65.87 ± 0.59*	69.24 ± 2.72*
4	400	62.09 ± 1.67*	74.89 ± 1.27*	70.72 ± 0.37*	76.12 ± 2.16**
5	800	71.54 ± 0.54*	81.92 ±2.34*	79.76 ± 5.53**	84.12 ± 1.69*
6	1000	76.09 ± 0.88*	90.71 ±1.42	86.31 ± 2.62*	93.72± 0.90
7	IC_{50}	144.7 µg mL^{-1}	45.4 µg mL^{-1}	51.35 µg mL^{-1}	43.8 µg mL^{-1}

*Values are mean ± SEM of 6 parallel measurement.**P< 0.05 and *P< 0.01 as compared against standard (ANOVA followed by Dunnet's 't' test).CEVS - chloroform fraction of ethanolic extract of V. setosa, EAEVS - ethyl acetate fraction of ethanolic extract of V. setosa, WEVS - water fraction of ethanolic extract of V. setosa. STD – vitamin E*

The nitric oxide scavenging and DPPH free radical scavenging models were used to demonstrate the *in vitro* antioxidant activities of *C. setosum* (Roxb.).

Oxidative damage of biomolecules like lipids, proteins and nucleic acids in the aerobic cells is associated with

aging and a variety of pathological events, including atherosclerosis, carcinogenesis, ischemia reperfusion injury and neurodegenerative disorders. This oxidative damage is caused by increased production of reactive oxygen/nitrogen species (ROS/RNS) and decreased capacity of antioxidant defenses in the body leads to oxidative stress. This ROS/RNS is counteracted by the antioxidants produced in the human body. These antioxidants were available from endogenous and exogenous origin. Endogenous sources of antioxidant defenses include superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase enzymes, which catalyze free radical quenching reactions internally. Exogenous sources of antioxidants like ascorbic acid (vitamin C), vitamin E and β-carotene are obtained from foods.

The hepatic damages and malignant adverse effects of synthetic antioxidants including butylated hydroxy anisole and butylated hydroxytoluene currently used against oxidative stress, had created the urge to search for substitute synthetic antioxidants with naturally occurring antioxidants from plants as they are considerably safer, easily accessible, and affordable[25]. The objective of the current study was to standardize the plant drug by using anatomical evaluation, phytochemical estimations and to evaluate the *in vitro* antioxidant activities of *V. setosa* (Roxb.) synonymous to *C. setosum* (Roxb.). The nitric oxide scavenging and DPPH free radical scavenging models were used to demonstrate the *in vitro* antioxidant activities of *C. setosum* (Roxb.).

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reduction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrate ions that can be estimated by use of Greiss reagent. Scavengers of nitric oxide compete with the oxygen, leading to reduced production of nitric oxide. Significant nitric oxide scavenging activity was observed for ethyl acetate and aqueous fractions of ethanolic extracts of *C. setosum* (Roxb.)[26,27]. The chloroform fraction demonstrated a minimal nitric oxide scavenging activity when compared to other two fractions of ethanolic extracts of *C. setosum* (Roxb.).

DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical supplied by antioxidants to become a stable diamagnetic molecule hydrazine. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. The significant decrease in the concentration of the DPPH radical is due to the scavenging ability of ethyl acetate and aqueous fractions of ethanolic extracts of *C. setosum* (Roxb.) while the chloroform fraction exhibited a minimal or less DPPH radicals scavenging activity[26,27].

The *in vitro* antioxidant activity of fractions of ethanolic extract of *C. setosum* (Roxb.) was found to be concentration dependent and is demonstrated in two *in vitro* models used for the study.

CONCLUSION

In the present work, the prostate herb *C.setosum* (Roxb.), vernacularly called as 'Pulipirandai' in Tamil, has been evaluated for its anatomy, analytical parameters of qualification or standardization and *in vitro* antioxidant activity. From the study, it was proved by *in vitro* models that the chloroform, water, and ethyl acetate fractions of ethanolic extract of dried powdered leaves of *C. setosum* (Roxb.) possess antioxidant activity. Further, the isolation of active constituents and a detailed study on *C. setosum* (Roxb.) is necessary to be done for the development of novel drugs in the arena of anticancer, anti-inflammatory, antiandrogenic, antiarthritic, and anti-coronary activity.

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