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Pharmacognostic and phytochemical investigation of *Ensete superbum* (Roxb.) Cheesman pseudostem

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The present study investigates preliminary pharmacognostic, phytochemical and antioxidant property of *Ensete Superbum* (Roxb.) Cheesman pseudostem. Macroscopical and microscopical features of the pseudostem have been documented. Presence of phytochemicals such as steroids, terpenoids, alkaloids, flavanoids, tannins and sugar were tested by chemical tests and TLC method. Total phenolic and total flavonoid content were determined using Folin-Ciocalteu reagent and complementary colorimetric methods (aluminum chloride method and 2, 4-dinitrophenylhydrazine method, respectively). Antioxidant was evaluated by using DPPH free radical scavenging activity, H_2O_2 and reducing power by $FeCl_3$. Pharmacognostic studies revealed presence of epidermis, hypodermis, vascular bundles, phloem fibres, sclereids ground tissue and stomata. Methanol extract of pseudostem showed highest concentration of phenolics and flavonoids and it also showed significant anti-oxidant activity ($P< 0.05$) when compared with standard. TLC fingerprint of plant extract is useful in characterisation of plant extract for standardization.

Keywords: Antioxidant, *Ensete superbum* (Roxb.) Cheesman, Pseudostem, Wild banana.

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Introduction

Nature is an inexhaustible source of metabolites and various plants sources are utilised for this purpose[1]. Botanical identification and phytochemical characterization of plants having medicinal properties for health benefits is an important path towards standardization[2]. Several types of reactive species are generated in the body as a result of metabolic reactions in the form of free radicals or non-radicals. These species called prooxidants may either be oxygen derived or nitrogen derived. They attack macromolecules including protein, DNA, lipid, etc. causing cellular or tissue damage[3]. An antioxidant is a molecule that inhibits oxidation of other molecules. Recent clinical and animal studies have identified traditional herbal medicines, dietary foods and other nutritional intervention as a viable method to curtail the progression of oxidation damage caused by free radicals[4].

Ensete superbum (Roxb.) Cheesman belonging to the family Musaceae is monocarpic, non-stoloniferous perennial shrub found in Western Ghats, Northeastern hills of India and Northern Thailand[5,6]. It is known as *Banakadli* in Hindi, *Kal vazhai* in Malayalam and wild banana in English. It is one of the less studied but highly exploited medicinal species of India and has been listed as rare, endangered, threatened and conservation concern species[7,8]. Phytochemical analysis of the seeds revealed presence of alkaloids, steroids, phenolics, glycoside and sugars[6,9]. Various colour pigments like chroman derivatives (contain non-steroidal phytosterol) isolated from seeds of *E. Superbum* and evaluated on the basis of physical and spectral data, can be further validated by HPLC and HPTLC as a marker compound for elaborate antifertility studies[10,11]. These compounds find use in medicine, cosmetics and as food additive to reduce cholesterol. In Ayurvedic system of medicine, pseudostem and seed of *E. superbum* are used for the treatment of various human ailments like debility, diabetes, kidney stone, leucorrhoea, measles, stomach ache and easy delivery[12,13]. It has been reported to possess no toxicity (LD_{50} = 3235.9 mg/kg), corrosion inhibitor, antiviral, antivariola, antivaccinia, anti-implantation, antifertility, cardiovascular, respiratory, cholinergic and hypoglycemic

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activities[14-17]. With the scientific perspective regarding standardization of pseudostems, present work was designed to explore the pharmacognostic parameter, identification of main classes of compounds by TLC and antioxidant activity of pseudostem of *E. superbum*.

Materials and Methods

Collection and identification of plant material

The fresh pseudostem of *E. superbum* was collected in July 2013, from Vadodara, Gujarat, India. It was identified and authenticated by Dr. P S Nagar, Botany Department, The M.S. University of Baroda and a voucher specimen (PG/KB/HDT-1-2013) was retained in the Pharmacy Department for further reference.

Reagents and chemicals

DPPH was purchased from Himedia Laboratories Pvt Ltd, Mumbai, India and ascorbic acid from Loba Chemie, Mumbai. Other solvents and chemicals were of analytical grade. Pre-coated silica gel $60F_{254}$ TLC plates were purchased from Merck, Darmstadt, Germany.

Morphological and microscopic investigation

The macroscopic features of the fresh pseudostems were determined by utilising protocol of various methods published[18]. Anatomical sections, surface preparations of the fresh pseudostems and powdered samples for the microscopy were carried out according to methods reported by Trivedi *et al*[19]. The peripheral (most dividing) position of dried pseudostems was used for the macroscopic studies, whereas for microscopical characterization both peripheral as well as center position of dried pseudostems were taken. Coarsely powder materials from whole pseudostems were used for the physico-chemical analysis, preparation of extract for biological analysis and TLC analysis.

Fluorescence analysis

Many powder drugs give fluorescence when exposed to ultra violet radiation[20], hence fluorescence character of powder obtained from *E. superbum* pseudostem was evaluated.

Proximate and toxic element analysis

Moisture content, ash value and extractive value of powder obtained from *E. superbum* pseudostem were analyzed. For toxic elemental analysis, 5 g powder was ignited in muffle furnace to obain total ash. Then 100 mg of ash was dissolved in 10 mL of 1 N HCl and the solution was filtered and diluted to 50 mL with distilled water. The solution was further used for determination of elements by absorption spectroscopy[21].

Extract preparation and phytochemical investigation

For phytochemical screening, 100 g powder was subjected to successive solvent extraction by hot percolation (Soxhlet) with petroleum ether, benzene, chloroform, ethyl acetate, acetone, methanol and water (500 mL each). After complete extraction, solvents were removed by evaporation under reduced pressure to obtain solid residues. The percentage yield, color, consistency and response to various reagents of successive solvent extracts were determined[32].

HPTLC fingerprinting

A Camag (Muttenz, Switzerland) HPTLC system including a Linomat V sample applicator, a Camag twin-trough plate development chamber, Camag TLC Scanner 3 and WinCATS integration software was used. Aluminium backed HPTLC plates 10 × 10 cm with 0.2 mm layer of silica gel 60 F254 (E. Merck), prewashed with methanol were used. The chromatogram was developed up to 80 mm under chamber saturation conditions[22]. The TLC studies were performed using different solvent systems and finally ethyl acetate : methanol : water (10 : 1.35 : 1 v/v); chloroform : methanol (4.5 : 0.5 v/v); hexane : ethyl acetate (9 : 1 v/v); chloroform : glacial acetic acid : methanol : water (64 : 32 : 12 : 8 v/v) were found to be suitable mobile phase for proper separation of the phytoconstituents. Anisaldehyde-sulphuric acid, dragendorffs reagent, natural product-polyethylene glycol reagent, vanillin sulphuric acid reagent and ferric chloride were used as spraying agent for detection of various classes of compounds. The plate was then dried with an air dryer and scanned with a Camag TLC scanner 3 using a multiple wavelength (254, 366 and 580 nm).

Antioxidant assays

DPPH radical scavenging assay

The stable free radical-scavenging activity was determined by the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay[23]. In this method the bleaching rate of a stable free radical, DPPH is monitored at a characteristic wavelength in the presence of the methanol extract of *E. superbum* pseudostem. The absorbance was recorded at 517 nm and EC_{50} values

denote the concentration of sample, which is required to scavenge 50 % of DPPH free radicals.

FRAP assay

The FRAP (Ferric reducing antioxidant power) assay was carried out according to the procedure of Benzie and Strain[24]. The absorbance of the reaction mixture was recorded at 593 nm after 4 minutes. The standard curve was constructed using iron (II) sulfate solution (100–2000 µg/mL) and the results were expressed as µg/mL Fe (II) of methanol extract.

Hydrogen peroxide scavenging capacity

The ability of methanol extract to scavenge hydrogen peroxide was determined[25]. Absorbance of hydrogen peroxide at 230 nm was determined 10 minutes later against a blank solution containing the phosphate buffer without hydrogen peroxide.

Total polyphenolics and flavonoids content

Total polyphenolics content of methanol extract determined using Folin Ciocalteau reagent method was measured as gallic acid equivalent and expressed as mg/g of dry extract[26]. Total flavonoid contents of methanol extract was measured by aluminium chloride and 2, 4-dinitro phenyl hydrazine colorimetric method[27]. It was quantified according to the standard curve prepared for quercetin (by aluminium chloride method) and naringin (by 2, 4-dinitro phenyl hydrazine method). The concentration of flavonoids was reported as mg quercetin and naringin equivalents per gram of sample, respectively.

Statistical analysis

All results were reported as mean ± SEM (n = 3). The variation in a set of data has been estimated by performing Bonferroni's multiple comparisons post-test to measures one-way ANOVA using non-parametric methods in Graph pad prism.

Results and Discussion

Despite the availability of various sophisticated analytical techniques for standardization of plants drugs, identification and evaluation by means of pharmacognostic and physico-chemical parameters is still more reliable, accurate and inexpensive methods[18]. World Health Organisation also recognised the macroscopic and microscopic determination of the plants as first step for establishing identity and purity[21].

Morphological and microscopic investigation

The salient pharmacognostical characteristic of the pseudostem of *E. superbum* are, as seen in the transectional views of the pseudostem; two epidermal layers and the hypodermal regions of both surfaces and the central zone contains the phloem and xylem, disposed bicollaterally. It was found as a non-stoloniferous plant with a stout pseudostem, 1.2-1.8 m tall, with an enormous swollen base (2-2.4 m circumference), narrowing to 1 m below the leaves (Plate 1a). The major morphological identification parameters (Plate 1b-c) observed on pseudostem was similar to earlier reports of *Ensete* species[5-6]. Microscopic characterization of the pseudostem is summarized in Plate 2. For powder microscopy, separate slides were prepared with glycerin, phlorogluinol + HCl and iodine. The powder was green in colour and contained paracytic stomata, cortex, xylem vessels, phloem fiber and sclereids (Plate 2).

Plate 1—a) Whole plant of *Ensete superbum*, b) Pseudostem of *Ensete superbum* and c) Transverse section of Pseudostem of *Ensete superbum.*

Fluorescence analysis

When powder was examined under UV light, it appeared green in color. After treatments with various reagents, the behavior was observed and recorded (Table 1). The change in various fluorescence colours after treatments with various reagents, strongly suggested the presence of various fluorescence compounds related with phenolics and flavonoids classes[20].

Proximate and toxic element analysis

Analysis of moisture content, ash values, and extractives values are shown in Table 2. Quantitative analysis of toxic elements revealed presence of lead (0.599 ppm), which was found within the limit as prescribed in WHO guideline. However, copper, iron and arsenic were not detected.

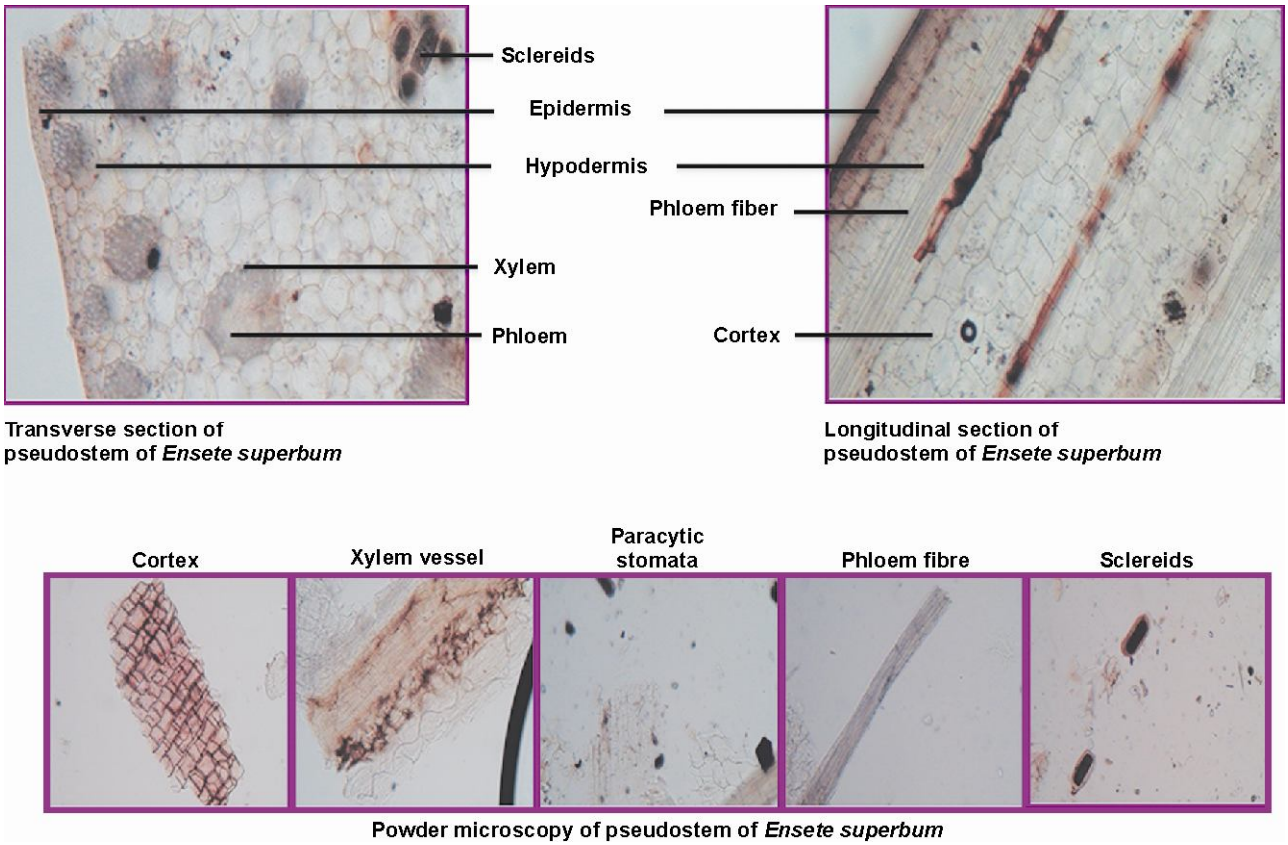

Plate 2—Microscopical features of *Ensete superbum* pseudostem.

Table 1—Fluorescence analysis of *E. superbum* pseudostem powder

S. No.	Treatment	Day light	Ultra violet light	
			254 nm	366 nm
1.	Dry powder	Light brown	Brown	Brown
2.	Powder + conc. HNO_3	Yellowish brown	Brown	Blackish brown
3.	Powder + conc. HCl	Reddish brown	Blackish brown	Blackish brown
4.	Powder + glacial acetic acid	Red	Dark brown	Light brown
5.	Powder + 40 % NaOH	Dark brown	Blackish brown	Greenish brown
6.	Powder + 5 % $CuSO_4$	Greyish brown	Brown	Brown
7.	Powder + chloroform	Brown	Dark brown	Light brown
8.	Powder + dragendorff's reagent	Dark brown	Brown	Blackish brown
9.	Powder + fehling solution A	Brown	Light brown	Greenish brown
10.	Powder + 2 % resorcinol	Brown	Light brown	Brown
11.	Powder + conc. H_2SO_4	Black	Light brown	Blackish brown

Phytochemical investigation

Various extracts were prepared by Soxhlet apparatus by successive solvent extraction method. Percentage yield, color and consistency of various extract are shown in Table 3. The extraction yield (% w/w) of *E. superbum* pseudostem in petroleum ether, toluene, chloroform, ethyl acetate, acetone, methanol and water were about 0.26±0.012, 0.13±0.007, 0.27±0.003, 0.10±0.006, 0.13±0.006, 3.64±0.268 and 7.48±0.047, respectively.

Preliminary phytochemical screening revealed presence of sterol and flavanoids in petroleum ether and toluene extract, alkaloids, sterol and flavanoids in chloroform, ethyl acetate and acetone extract, alkaloids, carbohydrates, glycosides, flavanoids, sterol, tannins and protein in methanol extract and carbohydrates, glycosides, flavanoids, tannins and protein in water extract (Table 4).

Table 2—Proximate analysis of *E. superbum* pseudostem powder

S. No.	Parameter	% W/W	S.D.
1.	Total ash	8.3	± 0.18
2.	Acid insoluble ash	1	± 0.12
3.	Water soluble ash	4.5	± 0.09
4.	Alcohol soluble extractive	7.56	± 0.06
5.	Water soluble extractive	3.79	± 0.05
6.	Loss on drying	6	± 0.10

Table 3-Extractive values of *E. superbum* pseudostem powder in different solvents

S. No.	Solvents	Colour	Consistency	% Yield (Mean ± SEM)
1	Petroleum ether	Dark brown	Sticky material	0.26 ± 0.012
2	Toluene	Brown	Non-sticky	0.13 ± 0.007
3	Chloroform	Dark green	Non-sticky	0.27 ± 0.003
4	Ethyl acetate	Brown	Non-sticky	0.10 ± 0.006
5	Acetone	Brown	Non-sticky	0.13 ± 0.006
6	Methanol	Brown	Sticky material	3.64 ± 0.268
7	Water	Dark brown	Non-sticky	7.48 ± 0.047

HPTLC studies

Various classes of compound of successive solvent extracts have been identified by HPTLC fingerprinting method (Table 5). Various spot were derivatized by different spray reagents in order to confirm their chemical class and to calculate their R_f.

Antioxidant assays

DPPH radical scavenging assay

The radical scavenging effects of all the extracts are represented in Fig. 1. It was observed that the methanol extract was most efficient as an antioxidant.

FRAP assay

The antioxidant activity of all the extracts is shown in Fig. 2. It was observed that methanol soluble factor was most likely responsible for reducing potential of the extracts.

Hydrogen peroxide scavenging capacity

The scavenging ability of all the extracts using hydrogen peroxide assay is shown in Fig. 3 and compared with ascorbic acid as standard. The methanol extract showed scavenging of hydrogen peroxide in an amount dependent manner.

Total Polyphenolics and flavonoids content

Flavonoids and phenolics are a group of compounds naturally present in most of the plants. Flavonoids and phenolics from the plant sources are currently widely studied as components that have the potential to provide multiple health benefits[23]. Epidemiological and clinical studies have provided

Table 4—Preliminary phytochemical screening of various successive solvent extract of *E. superbum* pseudostem powder

S. No.	Chemical class	Test	Petroleum ether	Toluene	Chloroform	Ethyl acetate	Acetone	Methanol	Water
1.	Alkaloids	Dragendorffs	-	-	+	+	+	+	-
		Mayer's	-	-	+	+	+	+	-
2.	Carbohydrates (sugars)	Molisch	-	-	-	-	-	+	+
3.	Glycosides	Brontagers	-	-	-	-	-	-	-
		Kedde	-	-	-	-	-	+	+
4.	Sterols	Salkowski	+	+	+	+	+	+	-
		Liebermann Burchard	+	+	+	+	+	+	-
5.	Tannin	Ferric chloride	-	-	-	-	-	+	+
6.	Flavanoids	Lead acetate	+	+	+	+	+	+	+
7.	Protein	Millons	-	-	-	-	-	+	+

Table 5—Major classes of compound by TLC screening of various successive solvent extract of *E. superbum* pseudostem powder.

S. No.	Chemical class	Detections	Solvent system	R_f						
				Pet. ether	Toluene	Chloroform	Ethyl acetate	Acetone	Methanol	Water
1.	Carbohydrates	Anisaldehyde sulphuric acid reagent	A	-	-	-	-	-	0.65	0.35 0.74
2.	Alkaloids	Dragendorff's reagent	B	-	-	0. 50	0. 56	0. 53	0.47	-
3.	Flavanoids	Natural product-poly ethylene glycol reagent	C	0.14 0.24 0.33 0.48 0.56	0.37 0.48	0.41 0.25	0.27	0.27	0.60 0.72	-
4.	Saponins	Vanillin sulphuric acid reagent	D	-	-	-	-	-	0.76 0.89	0.65
5.	Terpenoids		C	0.05 0.07 0.15 0.23 0.33 0.38	0.05 0.07 0.16 0.23	0.05 0.07 0.15 0.23	0.05 0.07 0.15	0.05 0.07	-	-
6.	Phenolics	Ferric chloride	C	-	-	-	-	-	0.78	0.79

Where A-ethyl acetate : methanol : water (10 : 1.35 : 1); B- chloroform : methanol (4.5 : 0.5); C-hexane : ethyl acetate (9 : 1); D- chloroform : glacial acetic acid : methanol : water (64 : 32 : 12 : 8)

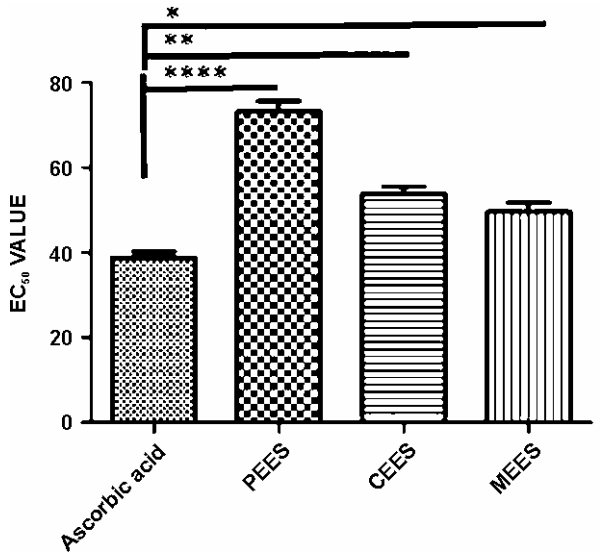

Fig 1—Antioxidant by DPPH, expressed as Mean ± SEM, n=3. One-way ANOVA, followed by Bonferroni's Multiple Comparison Test [****P <0.0001, **P < 0.01, *P < 0.05; F=53.02; df (3, 8) = 11].

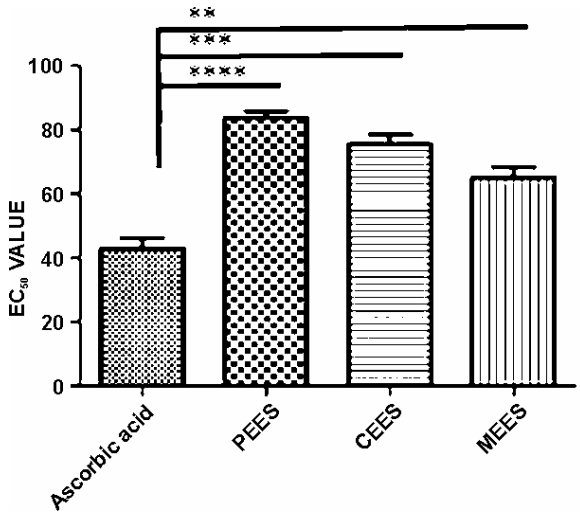

Fig 2—Antioxidant by FRAP, expressed as Mean ± SEM, n=3. One-way ANOVA, followed by Bonferroni's Multiple Comparison Test [****P <0.0001, ***P < 0.001, **P < 0.01; F=35.20; df (3, 8) = 11].

evidence of a potential role for flavonoids and phenolics in lowering the risk of various diseases[26]. It has been proved that phenolic and flavonoids compounds present in the plants are mainly responsible for antioxidant activity[27]. The flavonoids and phenolics phytochemicals exhibit redox properties which play a crucial role in determining the antioxidant properties[23]. The amount of total polyphenolics content determined in methanol extract is shown in Table 6 and Fig. 4. The amount of total polyphendics in methanol extract is shown in Table 6 and Fig. 5-6. In our study, we found that *E. superbum* revealed the presence of higher content of flavonoids and phenolics. This is directly related with its most significant antioxidant activity for extracts used in the present studies.

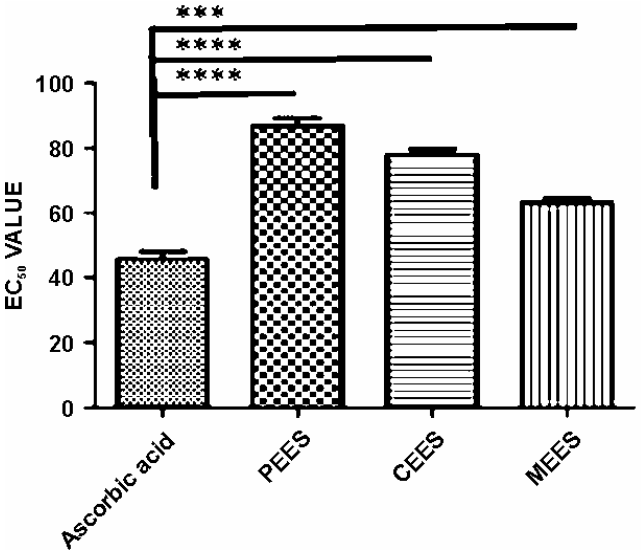

Fig 3—Antioxidant by hydrogen peroxides assay, expressed as Mean ± SEM, n=3. One-way ANOVA, followed by Bonferroni's Multiple Comparison Test [****P <0.0001, ***P < 0.001; F=78.16; df (3, 8) = 11].

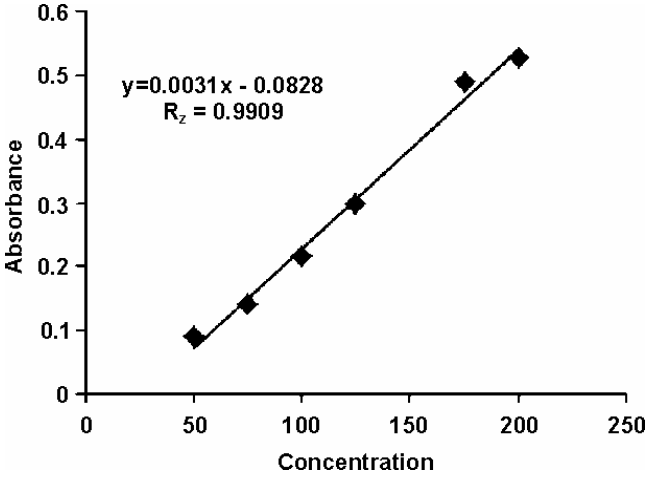

Fig 4—Calibration curve of gallic acid.

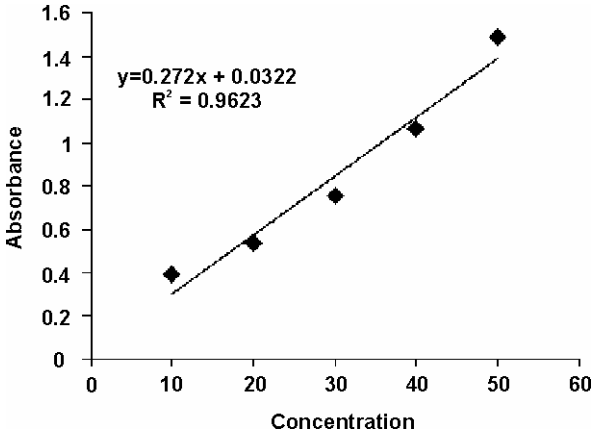

Fig 5—Calibration curve of quercetin.

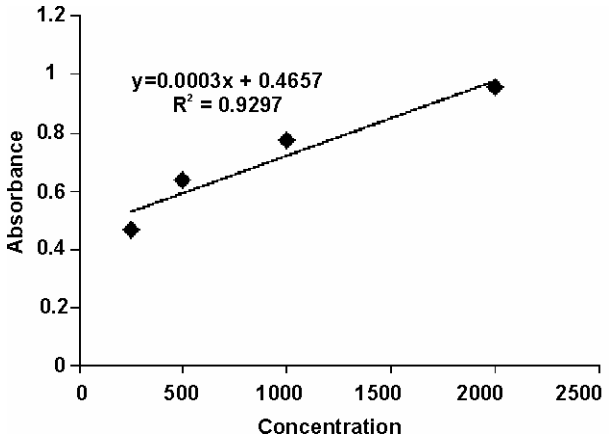

Fig 6—Calibration curve of naringin.

Table 6—Total phenolics and flavonoids content in methanol extract of *E. superbum* pseudostem

S. No.	Concentration of extract	Absorbance	Total content (% w/w)	Calibration
Total phenolic content				
1.	1 mg/mL	0.01	3.06	Fig. 6
Total flavonoids content (aluminium chloride colorimetric Method)				
2.	1.5 mg/mL	0.25	0.54	Fig. 7
Total flavonoids content (2,4-dinitro phenylhydrazine colorimetric method)				
3.	5 mg/mL	0.82	4.73	Fig. 8

Conclusion

The present investigation yielded a set of qualitative and quantitative parameters that can serve for quality control studies of pharmaceutical preparations from the pseudostem of *E. superbum* and as reference to ascertain the identity and to determine the quality and purity of the plant materials for future studies. There is scope in future for evaluation of pharmacological potential and chemical markers based identification.

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