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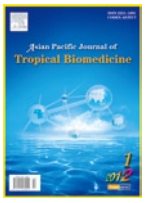

Document heading

Development of standardization parameters of *Costus speciosus* rhizomes with special reference to its pharmacognostical and HPTLC studies

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ABSTRACT

Objective: To study sailent diagnostic pharmacognostical characters of the rhizomes of *Costus speciosus* along with their phytochemical analysis, physicochemical parameters fluorescence characteristics and HPTLC studies. **Methods:** Fresh rhizome and dried powder sample of the rhizomes were studied macro- morphologically and microscopically. Preliminary phytochemical investigation of plant material and WHO recommended parameters for the standardization were also performed. HPTLC method for the quantification of diosgenin in the ethanolic extract of *Costus speciosus* rhizomes was also established. **Results:** The rhizome shows a special type of periderm called "Storied Cork". It consists of several, normally oblong, tangentially stretched, thin walled phellom cells. There are numerous vascular bundles scattered throughout the cortical zone. Each bundle has a cluster of four or five narrow angular xylem elements and a smallest of phloem elements. Stelar portion has less distinct endodermis and pericyclic layers and central scattered vascular bundles. Calcium oxalate crystals of rhomboidal, cuboidal and cylindrical in shape are common in the ground parenchyma cells. The prismatic crystals are 10 μm wide and the druses are 20-30 μm wide. The preliminary phytochemical screening of the rhizomes shows the presence of steroids, saponin glycosides and sugars. In HPTLC studies the Rf value of diosgenin was found to be 0.61. Linearity was found to be in the concentration range of 600 to 1800ng/spot and the correlation coefficient value is 0.9934. The results of analysis were validated in terms of accuracy and precision. The LOD and LOQ were found to be 5.69 ng and 17.25 ng/spot respectively. **Conclusions:** The pharmacognostical and HPTLC profile of the rhizomes of *Costus speciosus* will assist in standardization for quality, purity and sample identification.

1. Introduction

Sophisticated modern research tools for evaluation of the plant drugs are available but microscopic method is one of the simplest and cheapest methods to start with for establishing the identity of the source materials.

Costus speciosus (Koen) Sm. (Zingiberaceae) (*C. speciosus*) is an erect plant, up to 2.7 meters high; root stock tuberous; stem sub woody at the base; leaves [(15-30)×(5.7-7.5)] cm sessile, oblong, spirally arranged. It is native to the Malay Peninsula of Southeast Asia, but it has naturalized in some tropical areas, including Hawaii. It is listed as a potential invasive plant in the Federated States of Micronesia, Guam, Republic of Palau, and in French Polynesia. It is known as Keukand, Keu, Kust (Hindi), Pakarmula (Gujrati), Penva, Pushkarmula (Marathi), Kustha (Sanskrit) and Kostam (Tamil) [1].

According to Ayurveda the rhizomes are bitter, astringent, acrid, cooling, aphrodisiac, purgative, anthelmintic, depurative, febrifuge, expectorant and tonic and useful in burning sensation, constipation, leprosy, worm infection skin disease, fever, asthma, bronchitis, inflammations and anemia [2]. The plant is also used as anti-diabetic agent[3]. Eremanthin isolated from *C. speciosus* posses hypoglycemic and antihyperlipidimic activities[4]. Its antioxidant[5, 6], antifungal[7], antituberculosis[8] hepatoprotective[9] and oestrogenic activities [10, 11] have also been reported. Recently our research group has been evaluated and reported the effect of alcoholic extracts of *C. speciosus* rhizomes on stress induced changes in brain neurotransmitters and enzyme monoamine oxidase levels in albino rats. The results obtained provide

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biochemical evidence for antistress activity of the tested extracts [12]. Costunolide isolated from *C. speciosus* inhibits proinflammatory cytokines and iNOS in activated murine BV2 microglia [13].

It has been reported that its rhizomes contains diosgenin[14], prosapogenin B of dioscin, diosgenone, cycloartanol, 25-en cycloartenol octacosanoic acid, spirostanol glycoside (steroidal saponins)[15] and Furostanol glycoside 26-O-β-glucosidase[16]. Methyl ester of para -coumaric acid, an antifungal principle of the rhizome of *C. speciosus* has also been reported[7]. Quinones like 6-methyl dihydrophytylplastoquinone and dihydrophytylplastoquinone also reported in plant [17].

C. speciosus has recently gained much importance as a commercial source of diosgenin; the latter being a suitable material for the synthesis of corticosteroids and oral contraceptives. Survey of this plant from different parts of India has been carried out by many workers, and the diosgenin content up to 3.37% in the rhizome has been reported[18]. Panda et al. indicated a clear relationship between the occurrence of diosgenin and biomass with altitude, age and development stages of the plant[19]. A study to correlate some microscopic character of the rhizomes with their physicochemical parameters to fix up their standards and differentiate from their possible substitutes and adulterants is an attempt in this direction.

Phytochemical evaluation is one of the tools for the quality assessment, which includes preliminary phytochemical screening; chemo profiling and marker compound analysis using modern analytical techniques. In the last two decades HPTLC has emerged as an important tool for the qualitative, semi-quantitative and quantitative phytochemical analysis of herbal drugs and formulations. This includes developing TLC fingerprint profiles and estimation of chemical markers and biomarkers. Diosgenin is present in *C. speciosus* rhizomes[14]. To develop the standardization parameters, for its HPTLC quantification diosgenin is taken as a standard marker and its concentration has been estimated in the ethanolic extract of the *C. speciosus* rhizomes.

2. Materials and methods

2.1. Collection, identification and authentication of specimen

The plant specimen for the proposed study were collected from Jawaharlal Nehru Krishi Vishwa Vidyalaya, Krishi Nagar, Jabalpur, M.P. Care was taken to select healthy plants and for normal organs. It was identified and authenticated by taxonomic division, National Herbarium of Cultivated Plants, National Bureau of Plant Genetic and Resources, New Delhi and the specimen voucher no. NHCP/NBPGR/2007/98/2225 dated 22/08/2007 was preserved in the department. The required sample of different organs were cut and removed from the plant and fixed in FAA (Formalin- 5ml + Acetic acid- 5ml + 70% Ethyl alcohol-90 ml). After 24 hrs of fixing, the specimens were dehydrated with graded series of tertiary - Butyl alcohol [20]. Infiltration of the specimens was carried by gradual addition of paraffin wax (Melting Point 58-60℃) until TBA solution attained supersaturation. The specimens were cast in to paraffin blocks.

2.2. Sectioning

The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of sections was 10-12 μm. Dewaxing of the sections was by customary procedure[21]. The sections were stained with Toluidine blue as per the method published by O'Brien *et al*. [22]. Since Toluidine blue is a polychromatic stain, the staining results were remarkably good; and some cytochemical reactions were also obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. Wherever necessary sections were also stained with Safranin and Fast green and IKI (for Starch.)

2.3. Photomicrographs

Micrographic descriptions of tissues are supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon Labphot 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 birefringment property, under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the scale-bars. Descriptive terms of anatomical features are as given in the standard Anatomy book[23].

2.4. Powder characteristics

Preliminary examination, behavior of powder with different chemical reagents and microscopical examination was also carried out [24].

2.5. Fluorescence analysis

Fluorescence characteristics of powdered material were recorded under ultraviolet light as per the method mention by Chase and Pratt[25], and Kokaski *et al*. [26].

2.6. Physicochemical parameters

The various physicochemical parameters such as total ash, acid insoluble ash, water soluble ash, sulphated ash, moisture content (Loss on drying), water content, Foreign organic matter, extractive values (Petroleum ether, chloroform, alcohol and water) have been studied. In addition with their total aerobic bacterial count, total yeast & mould count, total enteric bacterial count and test for pathogens (*Escherichia coli*, *Salmonella* species, *Pseudomonas aeruginosa*, and *Staphylococcus aurus*) have been studied as per official method prescribed in Indian Pharmacopoeia, 1996[27] and WHO guidelines on quality control methods for plant materials (WHO/QCMMPM guidelines, 1992)[28].

2.7. Preliminary phytochemical analysis

For the preliminary phytochemical analysis, 100 g of powdered rhizomes was extracted with hexane, chloroform, and ethanol successively, using soxhlet apparatus. The aqueous extract was prepared by cold maceration technique. The extracts were concentrated under vacuum using rotary vacuum evaporator, dried and weighed. Each extract was tested for presence of different phytoconstituents *viz*. triterpenoids, steroids, alkaloids, sugars, tannins, glycosides, flavanoids, proteins and amino acids[29].

2.8. Chromatographic studies

Thin layer chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC), of the alcoholic extract of *C. speciosus* was done.

2.8.1. Preparation and application of test sample

About 100 mg ethanolic extract was dissolved in ethanol and as small as spots (not more than 4 mm) were placed on to the starting lines on pre-activated TLC plates through capillaries. The starting lines were parallel to and about 15 mm above the lower edge. The distance was marked to on the mobile phase; it was usually 10-15 cm long and not more than 5mm wide to improve the results of separation.

2.8.2. Solvent system

Firstly, TLC was performed in pure solvents based on trial and error method. Then different combinations of solvent system were tried for best separation of constituents [30]. By optimization of the solvent system, Toluene: Ethyl acetate: GAA: Formic acid (2:1:1:0.75) was found the best system for the resolution of various component and taken the same for HPTLC studies.

2.8.3. Development of chromatogram

The spots were allowed to dry and placed the plates as vertical as possible into the chamber, ensured that the points of application were above the surface of the mobile phase. Closed the chamber and developed the chromatogram at room temperature. Allowed the solvent to ascend the specified distance. The plates were removed and the position of the solvent front was marked and allowed the solvent to evaporate at room temperature.

2.8.4. Observation and interpretation of chromatogram

The spots produced were observed in daylight, under short and long-wavelength of ultraviolet light. Spots were also developed in iodine chamber and sprayed with specified reagent usually with anisaldehyde sulphuric acid and heated in electric oven for 30 minutes. R_f values of spots were calculated.

2.9. HPTLC method for estimation of diosgenin in ethanolic extract of C. speciosus

2.9.1. Equipment

A CAMAG TLC system comprising of a Linomat-5 applicator and CAMAG TLC scanner and single pan balance of Shimadzu model was used, for the present study.

2.9.2. Chemicals

Analytical grade Toluene, Ethyl acetate, glacial acetic acid, formic acid was obtained from Qualigens (Mumbai, India.). Stationary phase used was silica gel GF254, 20x10 cm TLC plate were obtained from E. Merk Ltd (Mumbai, India).

2.9.3. Sample preparation

The ethanolic extract was filtered with the help of Millipore filter and concentrated to 10 mL.

2.9.4. Preparation of calibration curve for diosgenin

300 μg/mL of working standard of diosgenin prepared in methanol was taken for the study. The calibration curve from 600-1 800 ng/spot was prepared and checked for reproducibility, linearity and validating the proposed method. The correlation coefficient, coefficient of variance and the linearity of results were calculated.

2.9.5. Method specification

Silica gel GF254 precoated plates (10×10 cm) were used with Toluene: ethyl acetate: GAA: Formic acid (2:1:1:0.75) as solvent system. Sample was spotted on precoated TLC plates by using Linomat 5 applicator. Ascending mode was used for development of thin layer chromatography. TLC plates were developing up to 8 cm. The plate was air dried and scanned under 194nm. The contents of diosgenin in the ethanolic extract of *C. speciosus* was determined by comparing area of the chromatogram of ethanolic extract of *C. speciosus* with calibration curve of the working standard of diosgenin.

2.9.6. Validation of method

The developed method was validated in terms of linearity, accuracy, precision, limit of detection, limit of quantification, robustness and ruggedness.

3. Results

3.1. Macro-morphological studies

The rhizomes are tuberous having length from 10-30 cm and diameter from 1.5 to 3.5 cm is usually unbranched subcylindrical and are covered by a brownish epidermis or cork. At intervals on the upper and lower surface are found small circular scars 4 to 6 mm in diameter (Figure 1b). The drug is odourless and insipid.

3.2. Microscopical studies

3.2.1. Anatomy

The rhizome is circular in cross sectional view with undulate outline and finely fissured surface. The tissue

system can be divided in to outer zone of Periderm and zone of cortex and central Stelar region (Figure 2).

Table 1.
Fluorescence characteristics of *C. speciosus* rhizome powder under ultraviolet light

Treatment	Fluorescence
Powder as such	Grayish
Powder mounted in nitrocellulose	Green
Powder treated with 1N-NaOH in methanol	Yellow
Powder treated with 1N-NaOH in methanol, Dried and mounted in nitrocellulose	Yellow
Powder treated with 1N-HCl	Yellowish brown
Powder treated with 1N-HCl, dried and mounted in nitrocellulose	Yellowish green
Powder treated with HNO_3 (1:1)	Yellow
Powder treated with H_2SO_4 (1:1)	Blackish

3.2.2. Periderm

It is special type of periderm called "Storied Cork". It consists of several, normally oblong, tangentially stretched, thin walled Phellom cells; the all walls are suberised. The epidermis is broken and disappeared. The periderm is 400-500 μm thick. It occurs all round the rhizome. The cells are in regular radial files and homogenous (Figure 3a).

3.2.3. Cortex

Inner of the periderm, the parenchymatous is the cortex. There are numerous vascular bundles scattered throughout the cortical zone (Figure 3a & b). The cortical bundles are small, circular and diffuse in distribution, (Figure 3b). Each bundle has a cluster of four or five narrow angular xylem elements and a smallest of phloem elements; the bundles are surrounded by a few, small, angular parenchyma cells, (Figure 4a).

3.2.4. Stelar

Stelar portion has less distinct endodermis and pericyclic layers and central scattered vascular bundles (Figure 4c). The stellar bundles are slightly larger and circular, they have wider angular xylem elements surrounded by phloem elements; thick walled sclerenchyma cells may ensheath partially each vascular bundle, (Figure 4b). The ground tissue is homogenous and parenchymatous.

Table 2
Physicochemical Values of *Costus* speciosus.

S. No.	Parameters	Result ± SD
Organoleptic characteristics	Appearance	Powder
	Colour	Buff Brown
	Odour	No characteristic
	Taste	No taste
	Loss on drying	3.55± 0.22
pH values	pH of 1% aqueous solution	7.33 ±0.08
	pH of 10% aqueous solution	6.97 ±0.05
Ash values (w/w)	Total ash	4.32±0.36
	Water soluble ash	1.68±0.07
	Acid insoluble ash	0.989±0.02
	Sulphated ash	4.53±0.87
	Alcohol soluble matter (%)	8.84±1.25
	Water soluble matter (%)	11.45±2.28
Successive extractive values	Hexane extractive value	1.55±0.02
	Chloroform extractive value	2.17±0.07
	Methanol extractive value	7.36±0.97
	Aqueous extractive value	8.94±0.99
	Moisture content (%)	3.16±0.36
	Foreign organic matter (FOM) (%)	1.20±0.04
Microbiological analysis	Total aerobic bacterial count	100 CFU/ g
	Total yeast and mould count	<100 CFU/ g
	Total enteric bacterial count	<10 CFU/ g
Test for pathogens	i)	*E. coli*
	ii)	*Salmonella* species
	iii)	*Pseudomonas aeruginosa*
	iv)	*Staphylococcus aurous*

3.2.5. Powder characteristics

Calcium oxalate crystals are common in the ground

Figure 1. Photograph of *C. speciosus*
(a): Plant of *Costus* speciosus; (b): Rhizome of *Costus* speciosus

parenchyma cells. Prismatic types of crystals are abundant in the cortical parenchyma (Fig. 5 a). They are rhomboidal, cuboidal and cylindrical in shape. In the stelar region the crystals are druses. They are large, occur in cluster or may be solitary (Figure 5b). The prismatic crystals are 10 μm wide and the druses are 20-30 μm wide.

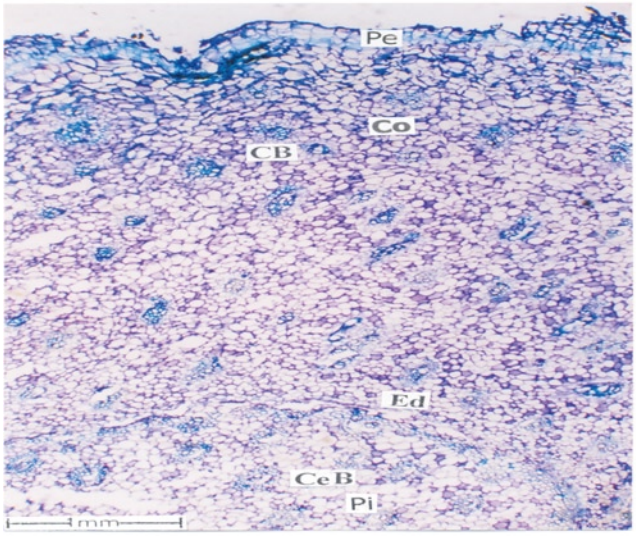

Figure 2. Tissue system of rhizome entire view (2X2.5).

3.2.6. Fluorescence analysis

Fluorescence characteristics of plant under ultraviolet light are presented in Table 1.

3.2.7. Physicochemical parameters

The various physicochemical parameters are presented in Table 2.

3.2.8. Preliminary phytochemical analysis

The chloroform extract of the rhizomes shows the presence of steroids. The alcoholic extract shows the presence of saponin glycosides. The water extract of the rhizomes shows the presence of some glycosides, saponins and reducing sugars, where as alkaloids and flavanoids are found to be absent in various extract (Table 3).

Table 3
Qualitative chemical analysis of various extract of *Costus* speciosus.

Type of constituents	Extracts			
	Hexane	Chloroform	Ethanol	Aqueous
Alkaloids	–	–	–	–
Phenolic compounds	–	–	+	+
Flavonoids	–	–	–	–
Saponins	–	–	++	+
Steroids	–	++	–	–
Triterpenoids	–	–	–	–
Proteins	–	–	–	+
Carbohydrates	–	–	+	++
Glycosides	–	–	+	+

Note: (+) indicates presence; (–) indicates absence.

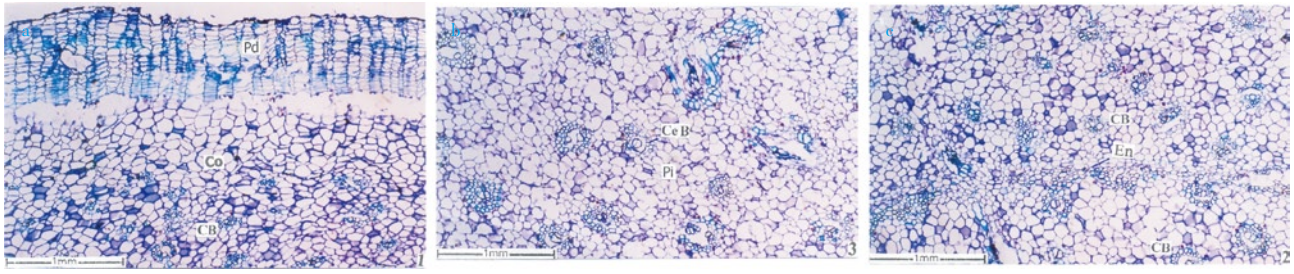

Figure 3. Different tissues zones of the rhizome.
(a): Periderm and cortex enlarged (4X2.5); (b): Cortical bundles and endodermis (4X2.5); (c): Central bodies in pith parenchyma

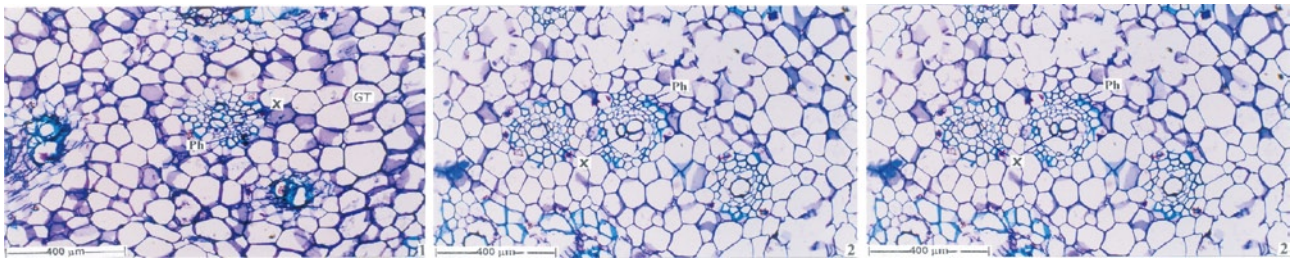

Figure 4. Structure of the vascular bundles
(a): Cortical bundles 10X2.5; (b): Central vascular bundles (10X2.5); (c): Central vascular bundles (10X2.5).

Table 4
Observation of TLC of alcoholic extract of *Costus* speciosus.

S. No.	Solvent system	No of sports	Rf Values	Visualization
1.	Chloroform : Methanol (8: 1.5v/v)	3	0.08, 0.4, 0.6,	In Iodine Chamber
2.	Chloroform: Ethyl acetate: Formic acid (7: 3: 0.5v/v)	4	0.08, 0.47, 0.50, 0.6,	In Iodine Chamber
3.	Toluene: Ethyl acetate, (7: 3 v/v)	6	0.04, 0.41, 0.48, 0.58, 0.6, 0.92,	In Iodine Chamber
4.	Toluene: Ethyl acetate: Formic acid: GAA, (2:1:1: 0.75 v/v)	10	0.04, 0.08, 0.35, 0.41, 0.48, 0.58, 0.6,0.77, 0.79 0.92,	In Iodine Chamber

Table 5
Responses obtained for diosgenin in preparation of calibration curve for HPTLC studies.

Track	Rf	Volume applied (μL)	Amount fraction (ng)	Area	X (calc)	Remark
1	0.61	10	–	1853.74	608.53	Diosgenin
2	0.61	2	600	1847.19	–	Diosgenin
3	0.61	2	600	1845.12	–	Diosgenin
4	0.61	3	900	2102.20	–	Diosgenin
5	0.61	3	900	2100.12	–	Diosgenin
6	0.61	4	1200	2527.00	–	Diosgenin
7	0.61	4	1200	2575.79	–	Diosgenin
8	0.61	5	1500	2803.20	–	Diosgenin
9	0.61	5	1500	2830.23	–	Diosgenin
10	0.61	6	1800	3187.49	–	Diosgenin
11	0.61	6	1800	3250.23	–	Diosgenin
12	0.61	7	2100	3417.54	–	Diosgenin
13	0.61	7	2100	3498.51	–	Diosgenin

Table 6
Results and Statistical data for recovery study of diosgenin.

Sr. No.	Disogenin in extract (ng)	STD added (ng)	Total added conc.	Actual Conc.	% Recovery	Mean
1.	608.53	50	658.53	651.38	98.99	
2.	608.53	100	708.53	705.75	99.68	99.66
3.	608.53	150	758.53	760.53	100.33	

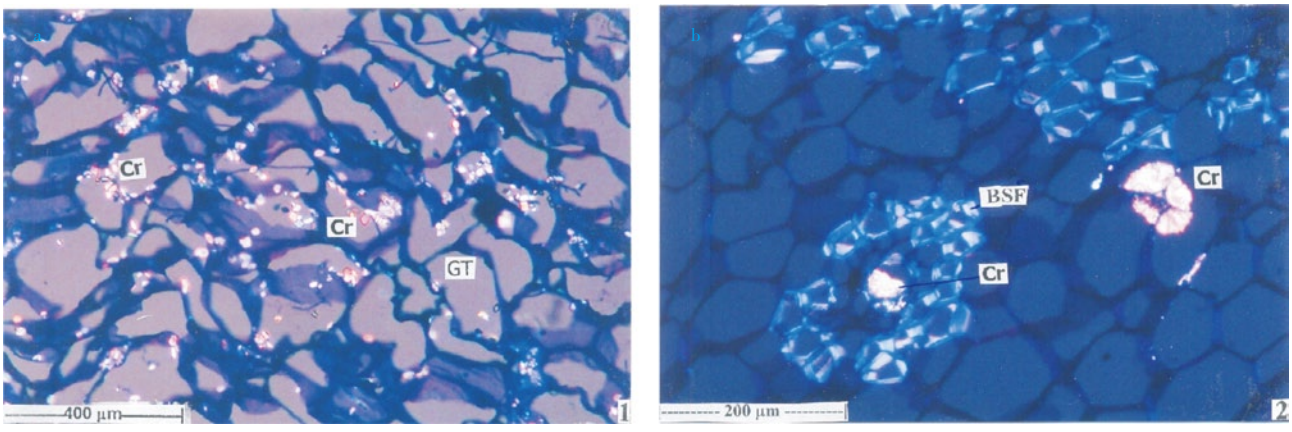

Figure 5. Crystal distribution in the rhizome (Under Polarized light Microscope)
(a): Prismatic crystals Scattered in the ground parenchyma (10X25); (b): Druses near sclerenchyma cells (10X25).

3.3. Chromatographic studies

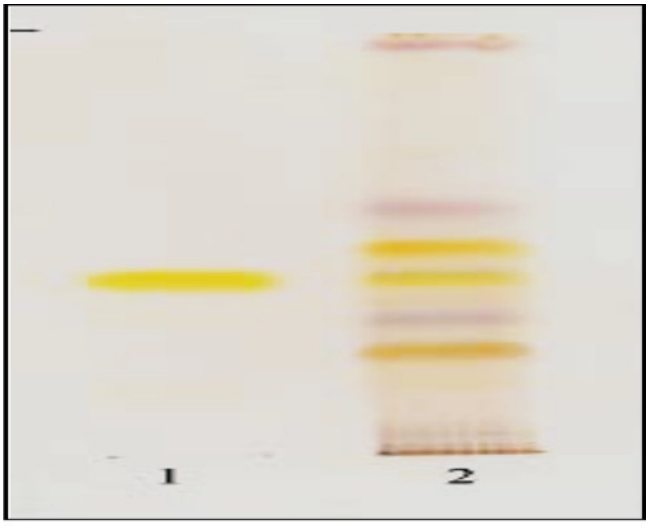

Figure 6. HPTLC plate of ethanolic extract of *Costus* speciosus rhizomes and diosgenin (marker).

3.3.1. TLC studies

The results of TLC studies are presented in Table 4.

Table 7
Validation Study Data for Diosgenin .

Sr No.	Parameter	Values
1.	Linearity range	600–1800ng
2.	Accuracy (mean recovery)	99.66%
3.	Precision (RSD)	1.10
4.	LOD	5.69 ng
5.	LQD	17.25 ng
6.	Ruggedness/Robustness (RSD between two experiments)	0.5892

3.3.2. HPTLC studies

A solvent system that would give dense and compact spots with significant Rf values was desired for quantification of Diosgenin in extract. The mobile phase consists of Toluene: ethyl acetate: GAA: Formic acid (2:1:1:0.75 v/v/v) gave R_f values (0.61±0.2) for diosgenin (Figure 6). The linear

regression data showed a good linear relationship over a concentration range 600-1800ng/spot. Standard Diosgenin showed single peak in HPTLC chromatogram (Figure 8). The calibration curve of diosgenin was obtained by spotting standard diosgenin on HPTLC plate. After development the plate was scanned at 194 nm. The calibration curve was prepared by plotting the concentration of diosgenin versus average area of the peak (Table 5, Figure 7). The ethanolic extract of *C. speciosus* was analyzed by the proposed method. The amount of diosgenin was computed from calibration curve. The developed method was validated as per ICH guidelines[31] and presented in table 7. The observed percentage recoveries were 99.66% for diosgenin, which shows that the method is free from interference Table 6.

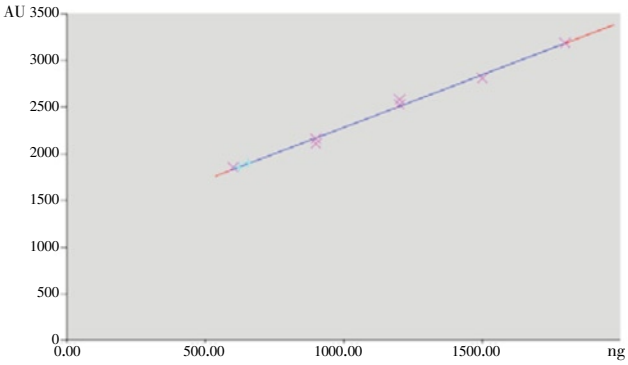

Figure 7. Calibration graph for diosgenin

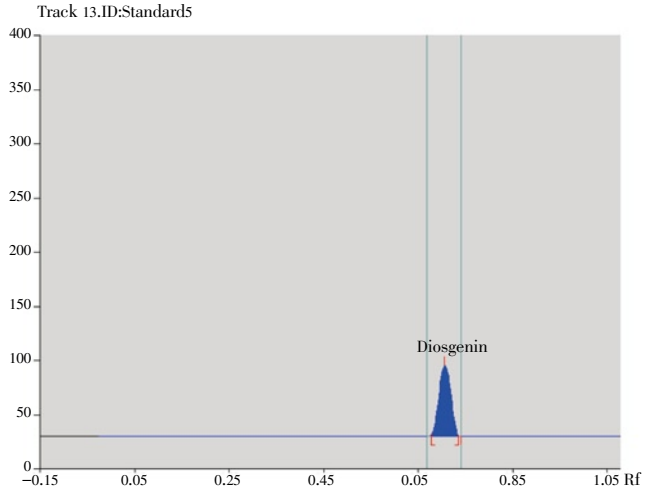

Figure 8. Peak response of diosgenin standard

4. Discussion

The present study establishes macro and microscopic characteristics, physicochemical values, fluorescence analysis of powder and phytochemical screening of *C. speciosus* rhizomes.

In recent year, there has been a rapid increase in the standardization of selected medicinal plants of potential therapeutics significance. It is due to their specific healing properties and potential action. Of course, in this view pharmacognostical standardization of *C. speciosus* is a substantial step. As the most cost effective aid of identification of a medicinal herb, the uses of microscopic characteristics have been the mainstay of classical pharmacognosy and remain a vital component of the modern monograph[32]. Macro and Micro standards can be identifying parameters to substantiate and authenticate the drug[33]. The constant physical evaluation of drugs is an important parameter in detecting adulteration or improper handling of drugs[34].

Standardization of natural products is a complex task due to their heterogeneous composition, which is in the form of whole plant, plant part/extracts obtained thereof. To ensure reproducible quality of herbal products, proper identification of starting material is essential[35].

The salient diagnostic pharmacognostical characters of the rhizomes of *C. speciosus* shows a special type of periderm called "Storied Cork". It consists of several, normally oblong, tangentially stretched, thin walled phellom cells. There are numerous vascular bundles scattered throughout the cortical zone. Each bundle has a cluster of four or five narrow angular xylem elements and a smallest of phloem elements. Stelar portion has less distinct endodermis and pericyclic layers and central scattered vascular bundles. Calcium oxalate crystals are common in the ground parenchyma cells. They are rhomboidal, cuboidal and cylindrical in shape. In the stelar region the crystals are druses. The prismatic crystals are 10 μm wide and the druses are 20-30 μm wide.

The preliminary phytochemical screening of the rhizomes shows the presence of steroids, saponin glycosides, phenolics and sugars.

The Rf value diosgenin was found to be 0.61. Linearity was found to be in the concentration range of 600 to 1800 and the correlation coefficient value is 0.9934. The results of analysis were validated in terms of accuracy and precision. The LOD was found to be 5.69ng. LOQ was found to be 17.25 ng/spot. The proposed HPTLC method provides a faster and cost effective quantitative control for routine analysis of diosgenin in *C. speciosus* extract.

After the present investigation it can be concluded that the pharmacognostical and HPTLC studies of the rhizomes of *C. speciosus* yielded a set of qualitative and quantitative parameters or standards, that can serve as an important source of information to ascertain the identity and to determine the quality and purity of the plant materials in future studies. These parameters also will be serving as standard data for quality control studies of pharmaceutical preparations from the rhizomes of *C. speciosus*.

Conflict of interest statement

We declare that we have no conflict of interest.

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