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ORIGINAL ARTICLE

# Evaluation of Diosgenin Content in *Costus speciosus* Germplasm Collected from Eastern Ghats of India and Identification of Elite Chemotypes

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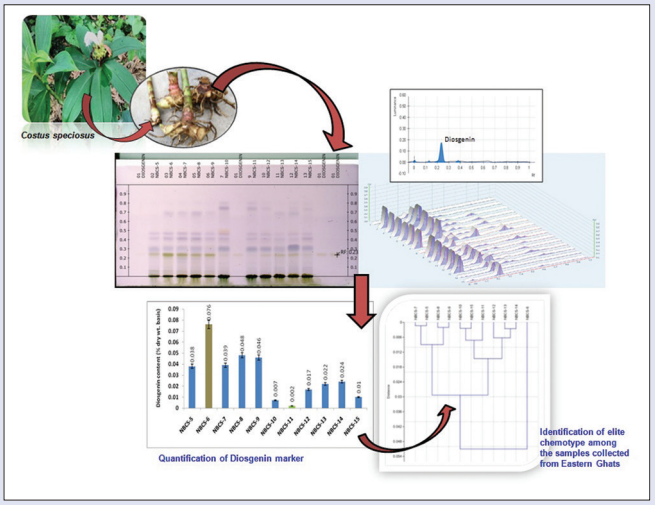
## ABSTRACT

**Background:** *Costus speciosus* known as "insulin plant" for its anti-diabetic potential. It has commercial significance due to the presence of industrially viable metabolite diosgenin. **Objective:** The identification of elite chemotypes of *C. speciosus* (Rhizome) through high-performance thin-layer chromatography (HPTLC) from the Eastern Ghats (India). **Materials and Methods:** A validated HPTLC method for the quantification of diosgenin was developed in accordance with the International Conference on Harmonization Guidelines. **Results:** In total, 11 populations of species were collected from their natural habitat with all the Global Positioning System (GPS) coordinates. The method was developed on HPTLC pre-coated silica gel 60 $F_{254}$ plates under a binary solvent system of n-hexane and ethyl acetate (7:2 v/v). The linearity was established at concentration range of 0.1–0.9 µg/spot having regression equation, 0.010× + 0.002. The limit of detection and limit of quantification were 0.907 and 2.751 with a regression coefficient of 0.999. The diosgenin content varies significantly ($P < 0.05$) from 0.002% to 0.076% and NBCS-06 from Patiya, Bhubaneswar, was identified as elite chemotype. **Conclusion:** The validation data confirm that developed HPTLC method was precise, accurate, robust, reproducible, and reliable in nature. The study resulted in the identification of elite chemotype of *C. speciosus* through validated HPTLC method from the Eastern Ghats of India. It will promote site-specific commercial cultivation of high metabolite yielding germplasm for good quality raw material to meet the industrial demand and in turn income generation of local inhabitants. The developed method will also aid in the regulation of quality standard and batch consistency of diosgenin-containing formulations in industry.

**Key words:** Chemotypes, *Costus speciosus*, diosgenin, Eastern Ghats, high-performance thin-layer chromatography

## SUMMARY

- 11 populations of *Costus speciosus* were collected from the Eastern Ghats of India
- The diosgenin content varies significantly ($P < 0.05$) from 0.002% to 0.076%
- NBCS-06 from Patiya, Bhubaneswar, was identified as elite chemotype
- High-performance thin-layer chromatography method was linearly calibrated for quantification of diosgenin marker.

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## INTRODUCTION

*Costus speciosus* (Koen) J. E. Sm. (Family: *Zingiberaceae*) commonly known as Keukand is an erect herbaceous plant with white fragrant flowers in terminal clusters. The plant flowers during the months of July and August and the species is widely distributed throughout India.[1] In Ayurveda, the rhizome is indicated as bitter, astringent, acrid, cooling, aphrodisiac, purgative, anthelmintic, depurative, febrifuge, expectorant, tonic and useful in burning sensation, constipation, leprosy, worm infection, and skin diseases. It has potential antidiabetic action due to which also known as "insulin plant," besides this, the species also exhibits various pharmacological effects such as anti-hyperlipidemic, anti-oxidant, antifungal, anti-tuberculosis, hepatoprotective, anti-stress, and oestrogenic activity.[2] In some parts of Asia, it is used to treat boils, constipation, diarrhea, dizziness, headache, ear, eye, nose pain, and used to stop vomiting. In Japan, the rhizome extract is used to control syphilis.[3] Phytochemistry on rhizome revealed the presence of diosgenin, prosapogenin B of dioscin, diosgenone, cycloartanol,



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25-en cycloartenol, octacosanoic acid, spirostanol glycoside (steroidal saponins), and furostanol glycoside 26-O-β-glucosidase.[4-6] Methyl ester of para-coumaric acid, an antifungal agent and 6-methyl dihydrophytylplastoquinone and dihydrophytylplastoquinone are also reported in *C. speciosus*.[7]

Diosgenin, a steroidal sepogenin is commercially exploited in pharmaceutical industries as a natural precursor for the synthesis of steroidal hormones, namely oral contraceptives, sex hormones, and other steroids.[8] The use of natural precursor is economically beneficial due to the high market demand of steroids. *Dioscorea* species are well known and major natural source of diosgenin but due to over-exploitation, this poses a threat to the biodiversity. The Quantification of diosgenin in various medicinal species has already been done through high-performance layer chromatography and High-performance thin-layer chromatography (HPTLC);[9] however, chemotypic variation of diosgenin content in *C. speciosus* was not reported yet. In the ancient Indian literature, it is suggested that the active principles are liable to fluctuate with variation in plant maturity (growth stage), season, and geographic conditions. A classical Ayurveda text also mentioned the impact of season, altitude, soil, and period of harvesting on the content of active principles in the plants. Considering the fact of the existing variation in the metabolite content, the study aimed to investigate the variation in diosgenin content among the natural populations, existing in the unexplored geographical zones of the Eastern Ghat (India). The present work records the existing variability in diosgenin content through a validated HPTLC method for the identification of elite chemotypes of *C. speciosus.* This will also aid in site-specific commercial cultivation of species to meet the industrial demand of good quality material and economic benefit to local inhabitants engaged in agricultural practices.

# MATERIALS AND METHODS

## Chemicals and reagents

Solvents and chemicals (A. R grade) were procured from SD Fine Chemicals, Mumbai, India, and HPTLC precoated silica gel 60 $F_{254}$ (20 cm × 20 cm) plates were procured from Merck, India. Marker compound diosgenin was purchased from ChromaDex Inc., the USA for quantification studies.

## Plant material

The rhizome samples were collected in the month of September from different locations of Eastern Ghat, Orissa, India. Plant material was identified and authenticated by Dr. Sharad Srivastava, Principal Scientist, Pharmacognosy Division, CSIR-NBRI, Lucknow. The herbarium specimen was prepared for each sample and deposited in the institute repository with the voucher number. Rhizomes were washed, shade dried, and powdered (40 mesh) using an electric grinder.

## Extraction protocol

The coarsely powdered rhizomes of each collection (2 g) were macerated with methanol for 24 h at room temperature (25°C ± 2°C). Extraction was repeated thrice, filtered, and pooled filtrate was dried in the rotatory evaporator (Buchi, USA) under standard conditions of temperature (55°C ± 2°C) and pressure (40 mbar) and finally lyophilized (Labconco, USA) to dry residue. The extractive yield of collected sample was calculated on percentage dry weight basis.

## High-performance thin-layer chromatography

### *Preparation of working solutions*

The stock solution of standard, namely diosgenin (0.1 mg/ml) and each *Costus* samples (10 mg/ml) were freshly prepared in methanol. For calibration, a stock solution of 0.1 mg/ml diosgenin was diluted in same solvent to obtain different working dilutions in concentration, ranging from 0.1 to 0.9 µg/spot. The solutions were filtered through a 0.45 µm Millipore membrane filter (Pall, USA) before application. The dilutions were prepared freshly on the day of analysis.

## High-performance thin-layer chromatography conditions

High-performance thin-layer chromatography was used for separation of the components present in the extracts, both qualitatively and quantitatively. For quantitative analysis, 10 µl of each *Costus* sample(s), having the working concentration of 10 mg/ml was applied using 100 µl sample syringe (Hamilton, Switzerland) on pre-coated plates with silica gel 60 $F_{254}$ of 0.2-mm thickness as 6 mm-wide bands. The spotting of sample/diosgenin in the form of bands were positioned 10 mm from the bottom and 15 mm from the side of the plate, using CAMAG Linomat V automated TLC applicator with nitrogen flow providing a delivery speed of 150 nl/s. HPTLC plate was developed in a CAMAG twin trough glass chamber, pre-saturated with the mobile phase. After development, the plate was air-dried and then derivatized with anisaldehyde-sulfuric acid reagent. The marker compounds were quantified using a CAMAG TLC Scanner equipped with CAMAG vision CAT software at 440 nm. The scan conditions were slit width, 4 mm × 0.45 mm and absorption-reflection mode [Table 1].

## Method validation

HPTLC method validation includes the evaluation of linearity, sensitivity, precision, selectivity, and robustness parameters according to the guidelines of the international conference on harmonization (ICH).[10]

## Linearity

Different dilutions were spotted in triplicate on a TLC plate to obtain concentrations of 0.1, 0.3, 0.5, 0.7, and 0.9 µg of diosgenin per spot.

**Table 1:** GPS information of the collected samples of *Costus speciosus* from Eastern Ghat

| Sample code | Locations | Longitude (E) | Latitude (N) | Alt. (ft) | Soil type | LWG number | Extractive value (mg) |
|---|---|---|---|---|---|---|---|
| NBCS - 05 | Raigada | 83°24'58.76" | 19°10'16.35" | 733 | Red, gravel soil | 308,005 | 162 |
| NBCS - 06 | Patiya, Bhubaneswar | 85°49'59.76" | 20°21'31.77" | 100 | Red, gravel soil | 308,006 | 80 |
| NBCS - 07 | Kailashpurgati, Rayagada | 83°32'49.17" | 19°12'51.57" | 1867 | Red, gravel soil | 308,007 | 102 |
| NBCS - 08 | Bhubaneswar | 85°49'28.34" | 20°17'45.81" | 151 | Red, gravel soil | 308,008 | 161 |
| NBCS - 09 | Madhopur | 84°24'59.80" | 20°47'10.80" | 347 | Red, gravel soil | 308,009 | 140 |
| NBCS - 10 | Anandapur | 86°07'29.63" | 21°12'53.46" | 159 | Red, gravel soil | 308,010 | 102 |
| NBCS - 11 | Duburi, Jajpur | 85°58'58.12" | 20°59'39.77" | 254 | Red, gravel soil | 308,011 | 74 |
| NBCS - 12 | Sambalpur | 83°58'52.20" | 21°28'00.74" | 532 | Red, gravel soil | 308,012 | 82 |
| NBCS - 13 | Sambhalpur University | 83°53'01.33" | 21°28'49.30" | 586 | Red, gravel soil | 308,013 | 130 |
| NBCS - 14 | Attabira, Bargada | 83°46'32.60" | 21°22'09.32" | 536 | Red, gravel soil | 308,014 | 73 |
| NBCS - 15 | Deogarh, Shambhalpur | 84°22'36.03" | 21°52'33.87" | 839 | Red, gravel soil | 308,015 | 135 |

LWG: Code of Herbarium specimen deposited at CSIR-NBRI; GPS: Global positioning system; NBCS: Code used for *costus speciosus* sample

The data of peak area versus concentration were subjected to the linear least-square regression equation. The slope, intercept, and correlation coefficient for the calibration curve were determined. The results were expressed as percentages of the total area of the diosgenin present in *C. speciosus* sample(s) based on the calibration curve of standard. The diosgenin content was estimated in each population and expressed on a dry weight basis (%).

## Sensitivity

The sensitivity of the method was determined with respect to the limit of detection (LOD) and the limit of quantification (LOQ) and is calculated from the standard deviation (SD) of the response and slope of the calibration curve.

## Stability

Reproducibility of the method was determined by analyzing the standard of a single concentration (0.3 µg/spot) over five times on the same day. The relative SD (RSD) was used to evaluate the reproducibility of the method within the limit of standard. The developed method was also validated for selectivity, specificity, and resolution of the analyte.

## Precision

Inter-day and intra-day studies were carried out to test the precision of the method and were expressed as RSD (%). Intra-day repeatability was tested by injecting the sample solutions of a population three times a day. Similarly, inter-day repeatability was assessed for thrice a day over three consecutive days.

## Accuracy

The accuracy (standard addition method) of the methods was determined by analyzing the percentage recoveries and the mean RSD (%) of the standard diosgenin in the collected sample extract. The sample was spiked with three different concentrations: 50, 75, and 100 µg. The spiked samples were recovered in triplicate and then analyzed by the developed HPTLC method.

## Statistical analyses

The observations of each sample were performed in triplicate. The data were recorded as a mean ± SD and analysis of variance (ANOVA) was used to test the statistical significance ($P < 0.05$) of diosgenin content within the population (XLSTAT, Microsoft Inc., USA). The elite chemotypes were identified through cluster analysis using PAST 2.15 software (Oslo, Norway).

# RESULTS

## Morphological description of collected germplasm

The germplasm of *C. speciosus* was collected from 11 different natural locations of Eastern Ghat, covering the Orissa state of India with variable altitude of 100–1867 ft. Common morphological features observed for all the collected germplasm as perennial, rhizomatous, succulent herbs with erect stems up to 1.5–3.0 m in height, arising horizontally from rhizome. Leaves were long lanceolate, elliptic or obovate, sub-sessile with cuspidate form of leaf apex. Color of leaves was dark green (NBCS - 5, 7, 11, 12, 13, 14, and 15) to light green (NBCS–6, 8, 9, 10), upper surface glabrous while lower surface sparsely hairy. Arrangement of leaves on stalk was spiral. Inflorescence was dense capitates spike with large bright red bract in subterminal position and bracts were ovate or mucronate. The rhizomes were collected during the flowering season as during the reproductive phase, the syntheses of metabolites are at its high [Figure 1].

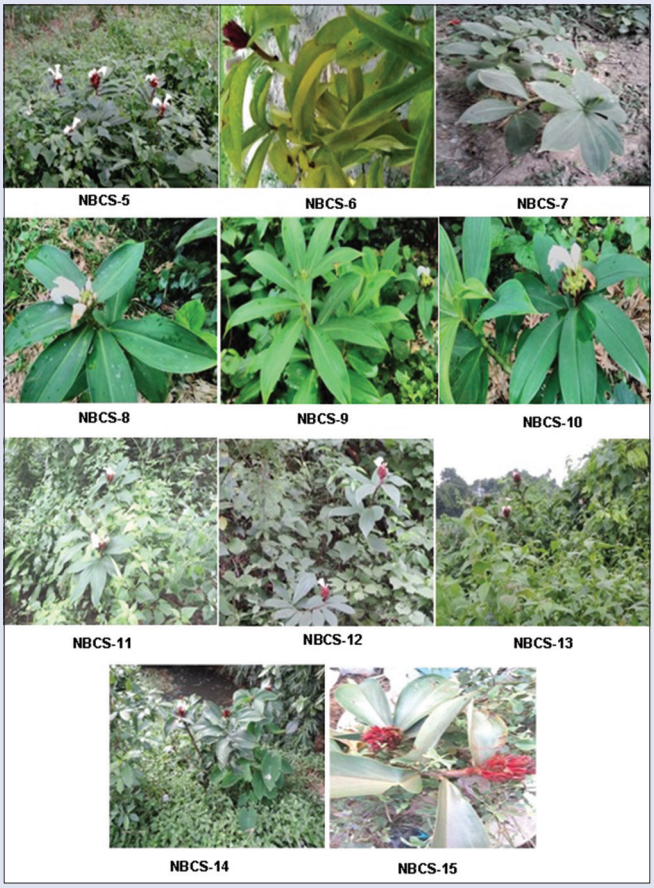


**Figure 1:** *Costus speciosus* populations in their natural habitats showing the morphological characters

## High-performance thin-layer chromatography quantification of marker compound

The extractive yield (Methanolic) of *C. speciosus* within the population significantly ($P < 0.05$) varies from 73 to 163 mg [Table 1]. Optimization of HPTLC condition(s), namely, selection of mobile phase, absorption maxima and slit dimensions was done to provide an accurate, precise, and reproducible method for the quantification of diosgenin. The method development was initiated with separation of diosgenin (extract) in various solvent systems by hit and trial method and finally a binary solvent system of n-Hexane: Ethyl acetate (7: 2 v/v) was selected based on the separation of unknown markers from diosgenin. The absorption spectrum of diosgenin was observed at 440 nm [Figure 2] after scanning the entire ultraviolet (UV) range of 200–800 nm. In sample, diosgenin was identified at $R_f$ 0.23 ± 0.05 [Figure 3a]. The saturation time for the development of chromatogram was optimized to 20 min for a good resolution of the diosgenin markers and the total run time was 30 min at room temperature (27°C ± 2°C). Purity of the bands in the samples was confirmed by comparing band spectra of samples with the corresponding band spectra of standards at the start, middle, and end position of the bands peak [Figure 3b].

The quantification of diosgenin within the population revealed that the content varies from 0.002% to 0.076% on dry weight basis; maximum content was recorded in NBCS–06 from Patiya, Bubhneshwar. The minimum content was observed in NBCS–11 from Dubri, Jajpur [Figure 4]. One-way ANOVA reflects that there is a significant difference in diosgenin content among the population ($P < 0.05$, fcal > fcritical).

## Method validation

The linearity of the developed method was achieved for standard diosgenin in the concentration range of 10–90 ng spot − 1 with statistically significant regression coefficient of 0.999. The LOD (3.3:1) and LOQ (10:1) values were within the limit of acceptance. Other statistical parameters of regression are in accordance with the ICH guidelines, as shown in Table 2 and thus confirming the linearity of the developed method. Apart from this, the residual plot of the calibration curve [Figure 5] for standard diosgenin (area vs. concentration) showed the positive random pattern, indicating that a linear model provides a decent fit to the data. The stability of method was evaluated by repeated ($n$ = 5) analysis of the standard at single level (0.3 µg/spot) and a lower value of SD (0.000404) reveal that the method is stable under chromatographic conditions. For specificity validation, the UV spectra of the samples were analyzed and found to be superimposed over the reference standard with the same retention factor. The peak purity of these compounds was also assessed by comparing the spectra at three points' namely peak start, peak middle, and peak end positions [Table 3]. The precision of method was analyzed by inter-day and intra-day repeatability studies at two levels using a fixed concentration (100 and 300 ng/spot) of the standard solution. Results expressed in terms of mean RSD (%) and SD were within the limits of the ICH guidelines (2005), i.e., not more than 5% [Table 4] and reflect that the method is precise and

**Table 2:** Statistical parameters for the calibration of standard diosgenin ($n$=3)

| Parameter | Value |
|---|---|
| Linearity (µg/spot) | 0.1-0.9 |
| Regression equation | 0.010x+0.002 |
| Regression coefficient | 0.9998 |
| $R_f$ | 0.23 |
| Average | 0.0065 |
| SD | 0.003 |
| SE | 0.0001 |
| LOD (µg/spot) | 0.907 |
| LOQ (µg/spot) | 2.751 |

LOD: Limit of detection; LOQ: Limit of quantification; SD: Standard deviation; SE: Standard error

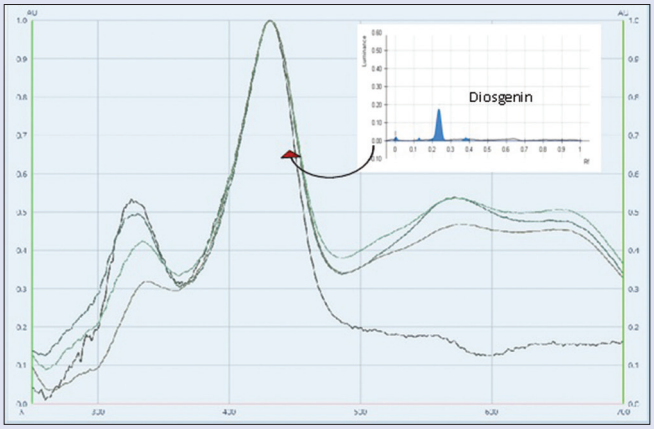


**Figure 2:** Absorption spectra of standard diosgenin signify the $\lambda_{max}$ at 440 nm

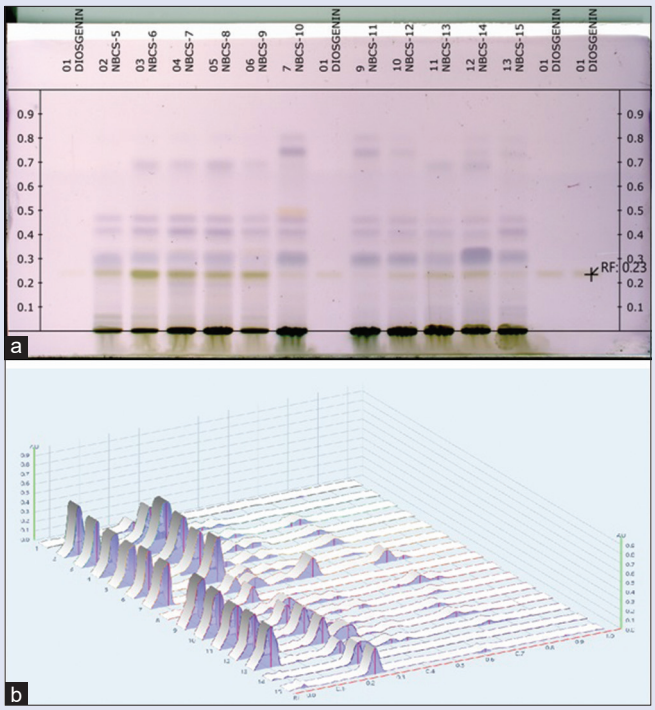


**Figure 3:** (a) High-performance thin-layer chromatography chromatogram of *Costus speciosus* for major marker diosgenin after derivatization with detecting reagent in visible range. (b) Overlay spectra of *Costus speciosus* and diosgenin as reference standard at 440 nm. Tracks from bottom to top follow the order: 2. NBCS-05, 3. NBCS-06, 4. NBCS-07, 5. NBCS-08, 6. NBCS-09, 1,8,14 and 15: Diosgenin. 7. NBCS-10, 9. NBCS-11, 10. NBCS-12, 11. NBCS-13, 12. NBCS-14 and 13. NBCS-15

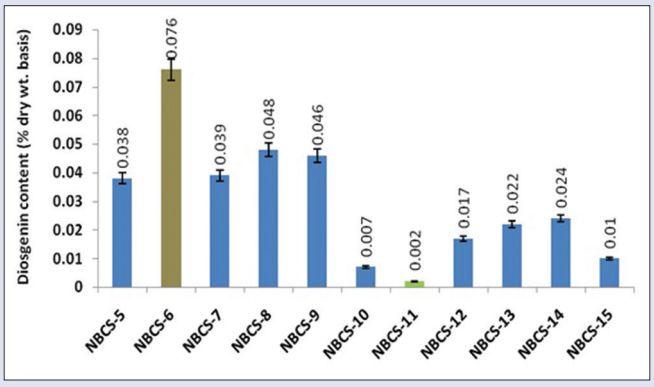


**Figure 4:** Densitometric quantification of diosgenin in 11 different populations of *Costus speciosus*

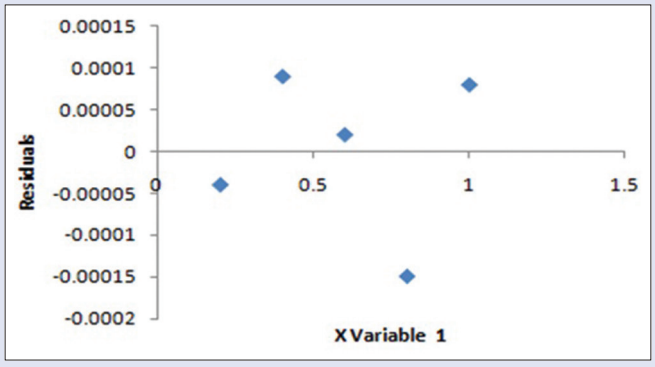


**Figure 5:** Residual plot of regression analysis for the calibration of diosgenin content

reproducible for the quantification of diosgenin under the developed protocols. Accuracy was tested through standard addition method by spiking the sample at three different levels of 50%, 75%, and 100%. The recovery of the analyte [Table 5] showed a variation from 98.77% to 101.97%, which is within the acceptance limit of 95%–105% and hence, the method was found to be accurate and precise. The method was also found to be robust when tested by slight changes in mobile-phase composition and saturation time.

## Chemotypic studies

This program was originally designed as a follow-up to PALSTAT, a software package for paleontological data analysis written by P.D. Ryan, D.A.T. Harper and J.S. Whalley (Ryan *et al.* 1995) of 11 samples based on diosgenin content, UPGMA (correlation, unweighted pair group method using arithmetic mean) dendogram results in the bifurcation of population into two groups. NBCS-6 stands out from the rest of the population as elite chemotype having high diosgenin content [Figure 6]. The distance was calculated between the pair of populations based on the diosgenin content. The samples studied are from the same phytogeography, i.e., from Eastern Ghats and thus, the existing variation may be due to change in local climatic conditions, soil profile, rainfall, etc. However, to conclude this substantial study, it is needed in near future to record the effect of biotic and abiotic factors on the production of secondary metabolites. In addition, the effect of the altitude on the content of metabolite was also checked, and correlation (Karl Pearson) statistics revealed that the two variables were negatively correlated (−0.06). This suggested that altitude was not the limiting factor for the production of diosgenin in the natural population of *C. speciosus*. The variation in secondary metabolite content is well recorded in various medicinally important medicinal plants, namely *Gloriosa superba, Coleus forskohlii, Bergenia* species.[11-13]

## DISCUSSION AND CONCLUSION

*C. speciosus* is well known as "insulin plant" for its anti-diabetic action and has commercial significance for its industrially viable metabolite diosgenin. Diosgenin has relevance from dated back for its use as natural precursor in the synthesis of steroidal hormones. Hence, it is quintessential to develop an accurate, easy and time-saving method for the quantification of diosgenin in *C. speciosus* and HPTLC is choice of preference for the same. The desired objective was achieved by the use of n-Hexane: Ethyl acetate 7: 2 (v/v) as mobile phase. This mobile phase was also found to be the best for the simultaneous analysis of diosgenin; the $R_f$ values were 0.23 ± 0.05, respectively. The statistical data suggest that the developed HPTLC method was linear, precise, accurate, and reliable having reproducibility as well as robustness and quality control of herbal formulations containing this plant as an ingredient densitometric scanning reveals the significant variation in diosgenin content, ranging from 0.002% to 0.076% in 11 populations on dry weight basis. Collected from phytogeographical zones of Eastern

**Table 3:** Peak purity test for the standard diosgenin

| Standards | *r* (s, m) x | | *R* (s, m) y | |
|---|---|---|---|---|
| | Standard track | Sample track | Standard track | Sample track |
| Diosgenin | 0.9875 | 0.9885 | 0.9980 | 0.9987 |

s: Refers to start; m: Refers to middle; x: Correlation of spectrum at the start of peak with spectrum at the center of the peak; y: Correlation of spectrum at center of the peak with spectrum at the end of the peak

**Table 4:** Precision studies (interday and intraday) of HPTLC method for the quantification of diosgenin

| Code | Amount (ng per spot) | Inter day | Intra day | | |
|---|---|---|---|---|---|
| | | | Day 1 | Day 2 | Day 3 |
| Diosgenin | 100 | 0.0307 | 0.0309 | 0.029 | 0.0255 |
| | | 0.0315 | 0.0295 | 0.0288 | 0.0268 |
| | | 0.0299 | 0.0289 | 0.0276 | 0.0268 |
| | SD | 0.0008 | 0.001 | 0.0007 | 0.0007 |
| | Mean | 0.0307 | 0.029767 | 0.028467 | 0.026367 |
| | RSD (%) | 2.60 | 3.45 | 2.66 | 2.85 |
| | 300 | 0.0055 | 0.0054 | 0.0049 | 0.0045 |
| | | 0.0051 | 0.0053 | 0.0052 | 0.0047 |
| | | 0.0054 | 0.0051 | 0.005 | 0.0048 |
| | SD | 0.0002 | 0.0001 | 0.0001 | 0.0001 |
| | Mean | 0.005 | 0.005 | 0.005 | 0.005 |
| | RSD (%) | 3.90 | 2.90 | 3.03 | 3.27 |

*n*=3; values are mean. SD: Standard deviation; RSD: Relative standard deviation; HPTLC: High performance thin layer chromatography

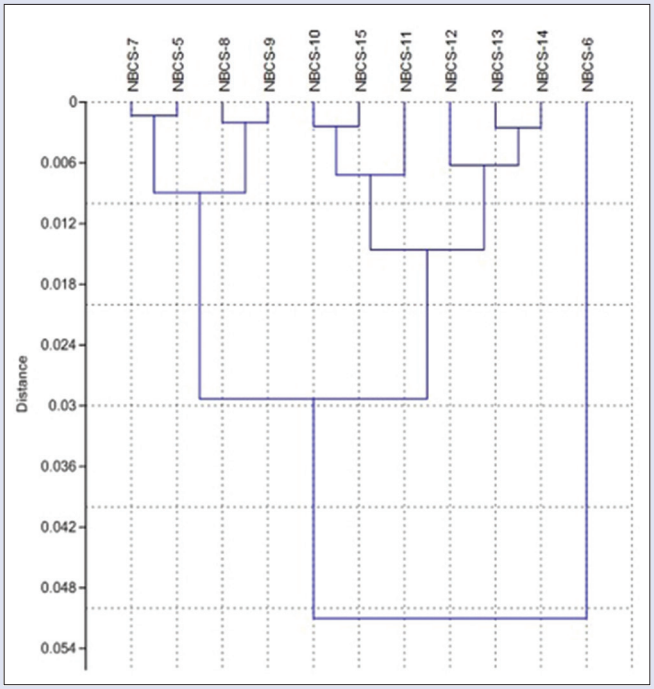


**Figure 6:** UPGMA dendogram of *Costus speciosus* population from the Eastern Ghats of India

**Table 5:** Recovery studies of standard diosgenin

| Code | Amount of standard present (µg) | Amount of standard added (µg) | Theoretical value (µg) | Practical value (µg) | Recovery (%) | Mean recovery (%) | SD | Mean RSD (%) |
|---|---|---|---|---|---|---|---|---|
| Diosgenin | 0.959 | 50 | 50.959 | 51.959 | 101.9623619 | 100.327 | 1.597 | 1.592 |
| | 0.959 | 75 | 75.959 | 75.025 | 98.77038929 | | | |
| | 0.959 | 100 | 100.959 | 101.21 | 100.2486158 | | | |

*n*=3; values are mean. SD: Standard deviation; RSD: Relative standard deviation

Ghats and the correlation coefficient value is 0.999. The results of the analysis were validated in terms of accuracy and precision. NBCS-06 from Patiya, Bhubaneswar was identified as elite chemotype for the particular geography. The purity of the marker components in the sample extracts was also confirmed by absorption spectra. HPTLC profile of the rhizomes of the plant will assist in standardization for quality, purity, and sample identification.

The targeted molecule is well known steroidal sapogenin and is found in several plants, namely *Dioscorea* spp., *Trigonella* spp., *Costus* spp., and *Smilax* spp. Diosgenin is originated by the hydrolysis of saponin dioscin. It is evident that several HPTLC methods were used to quantify the diosgenin in various species such as *Fenugreek*, *Tribulus terristris*, *Gokshuradi guggulu*, and various Ayurvedic formulations.[14] An HPTLC quantification of isolated Diosgenin was published[15] with a similar solvent system. However, we find that the slight variation leads to more precise, accurate, and sharp peaks in our samples. As the developed method was not claimed as novel for the identification of diosgenin, both the methods can be equally accurate for the study depending on the nature of the sample, processing of material, handling of instrumentation, etc., Moreover, the focus of our study is to record the variation of diosgenin in a natural population of *C. speciosus* for the identification of elite chemotype(s) to promote the commercial cultivation of species and to provide the good quality material to the herbal drug industry.

This will promote the site-specific commercial cultivation of high metabolite yielding germplasm to meet out the industrial demand of good quality raw material and income generation of local inhabitants and unexplored phytogeographical region of India. However, this study is limited to the Eastern Ghats only and we are working for the identification of elite chemotypes from other geography which may lead to identifying for better sources because of cost-effectiveness and advantage in some aspects, it is frequently used in the pharmaceutical industry in the standardization of plant materials. The developed method will also aid in the regulation of quality standard and batch-to-batch consistency of diosgenin containing formulations in industry.

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## Conflicts of interest

There are no conflicts of interest.

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