Available online on 15.11.2024 at http://jddtonline.info

Journal of Drug Delivery and Therapeutics

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Research Article

Extraction, Isolation, Identification and Estimation of Diosgenin by TLC Profiling and UHPLC-LC-SRM Analysis in Three *Costus* Species

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Article Info:

Article History:

Received 19 Aug 2024
Reviewed 05 Oct 2024
Accepted 01 Nov 2024
Published 15 Nov 2024

Cite this article as:

G M RD, Extraction, Isolation, Identification and Estimation of Diosgenin by TLC Profiling and UHPLC-LC-SRM Analysis in Three *Costus* Species, Journal of Drug Delivery and Therapeutics. 2024; 14(11):120-127 *DOI: http://dx.doi.org/10.22270/jddt.v14i11.6878*

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Abstract

Medicinal plants are utilized for treating many ailments, since ancient times due to their tremendous therapeutic properties. The plant derivatives are used as a raw material for the synthesis of drugs either as a natural or artificial synthetic drug. *Costaceae* family members are known to possess many medicinal properties few of them are mainly consumed to reduce the blood glucose level and treat many disorders. The sapogenin contained in the rhizome of three *Costus* species was extracted, identified, and characterized. Young rhizomes were harvested surface sterilized, hydrolyzed; extracted through a soxhlet apparatus and concentrated fractions were subjected to a preliminary phytochemical screening test for saponin, Thin Layer Chromatography, and the UHPLC-MS SRM analysis. The result showed that the maximum yield of the Diosgenin was obtained from the chloroform extract of *Costus speciosus*. The pharmaceutical analysis in all the three *Costus* species carried out with isolated saponin was compared with standard Diosgenin. The characterization of isolated diosgenin was done by UHPLC MS and SRM, based on the retention time of all three samples and the comparison with standard Diosgenin. Isolated Diosgenin was quantified through thin layer chromatography with n-hexane: ethyl acetate (7:3) as mobile phase at Rf value of 0.48. The amount of Isolated Diosgenin was estimated by comparing the peak area at a retention time Rt value of 8.5 in all the samples and compared with standard Diosgenin high amount of Diosgenin was found in *Costus speciosus* rhizome followed by *Costus igneus* and *Costus pictus*. The preliminary phytochemical screening and analytical methods involved during the present study were found satisfactory. Thus, the protocol can be used for the extraction of Diosgenin and further used in the synthesis of Drugs.

Keywords: *Costus pictus, Costus speciosus, Costus igneus*, rhizome, TLC, UHPLC-MS, SRM

INTRODUCTION:

Diosgenin a phyto-steroid, a bio-active steroidal sapogenin (5α-Spirosten-3β-ol), was first isolated from *Dioscorea takaco* in 1930's [1], belongs to a tri-terpene group and is widely utilized in pharmaceutical industries [2]. It is obtained mainly from yams for the synthesis of the steroidal hormone, either by hydrolysis of acids and strong bases, neither by enzymes action process. Diosgenin mainly obtained from *Dioscorea cayenesis*, *Dioscorea nipponoea*, *Dioscorea zingiberensis* [3], *Dioscorea collettii* [4], *Solanum incanum*, *Solanum xanthocarpum*, *Trigonella foenum graceum*, [5] *Costus speciosus, Costus igneus*, etc, [6]. Diosgenin belongs to the saponin group, saponin acts as a dietary element that has been used in complementary, traditional, and modern medicine against many diseases, which acts as a novel multi-target product [7]. It acts as a starting material for the production of corticosteroids, sexual hormones, oral contraceptives [8, 9,] and other associated steroidal drugs as a main and signal precursor in the manufacture of the synthetic steroid. Diosgenin being tested through clinical trials for Anti-oxidant agent [10], Anti-inflammatory[11], Anti-metastatic therapy [11], Anti-fungal [12], Anti-thrombin effect on megakaryocytic differentiation inducer of HEL cells [13], which induces cell cycle arrest cycloxygenase activity on Osteosarcoma cells [14] and thromoboxane synthase (TxS). Administration of Diosgenin at a dosage of 20 and 40 mg/kg b/w for 15 days shows the growth of mammary epithelial cells; reveals Estrogenic action and osteoporosis on ovariectomized (OVX) mouse [15,16]. It also regulates Hypercholesterolemia, improves lipid profile, and modulates anti-oxidative stress caused by lipoxygenase activities [17; 18; 19 20]. A significant change was noticed in the induction of differentiation on human erythroleukemia cell line studies on HEL, TIB, 180 [21] Diosgenin induces hypolipidemic, calcium metabolism, inhibits rector activated nuclear factor-Kappa B ligand-induced Osteoclastogenensis[22] by suppressing tumor necrosis factor. Diosgenin has been reported for its anti-cancerous, anti-proliferative, inhibition of k562 cells via cell cycle G2/M arrest, and apoptosis by disruption of Ca 2+homeostasis and mitochondrial dysfunction [23]. Diosgenin also shows action on human prostate cancer PC3 cells [24] human erythroleukemia cells by lipoxygenase activities it shows the greater ability of P53 medicated cell cycle G1 arrest and apoptosis in osteosacroma cells [25] when compared to two other plant steroids hecogenic and trigogenin [26]. It is well depicted

by induction by HeLa cells, programmed cell death through the Caspase pathway [27]. It is also reported to show many significant properties; thus used as chemopreventive or chemotherapeutic agents against cancer [28], known to suppress FAS expression in HER-2 over-expressing breast cancer cells, induction of apoptosis in HT-29 cells of bcl-2 by induction of caspase-3-protein expression. Diosgenin acts as a novel compound in the prevention of colon cancer, proliferation studies on rats, regulates gene expression; enhances apoptosis by cytokines [29]. Effective on cortical neuron ions in human cortical neurons (HCN-1A) by BK (Ca) channel activity, inhibitory activity on acetylholinestrase, inhibition of thyrocyte proliferation [30], and treatment of Goiter formation [31], as a new therapeutic strategy and prevention of thyrocyte proliferation [31].

Costus pictus and *Costus igneus* are well known as "Insulin Plant", spiral ginger, fiery *Costus* or Spiral flag, step ladder belonging to family *Costaceae* (Zingiberaceae) rhizomatous, monostichous branched, leaves are produced on pseudostem, they possess valuable medicinal properties as anti-diabetic, anti-diuretic, anti-inflammatory, anti-helminthetic, anti-depressant, anti-oxidant, etc., [32.33]. *Costus speciosus* belongs to *Costaceae* an important medicinal plant used as indigenous medicine for treating diabetes, anti-helmintic, astringent, expectorant properties, as a tonic, relieving burning sensation, constipation, leprosy, asthma, bronchitis, anemia, and skin ailments [34].

MATERIALS AND METHODS:

Collection of Plant material:

The plant species were collected from different areas, *Costus pictus* were collected from Calicut University, Calicut, Kerala, India. *Costus igneus* from GKVK Bangalore and *Costus speciosus* collected from Bangalore University, Bangalore, Karnataka, India. Plants collected were identified, authenticated and maintained in the Department of Botany, voucher specimens are preserved Figure 1. The rhizome from these three species was freshly harvested, extracted, isolated, and utilized for further analysis.

Figure 1: a) *C pictus,* b) *C.igneus*, c) *C.speciosus*

Chemicals and standard reagents:

Diosgenin standard was purchased from Sigma Aldrich St Louis USA, other chemicals like Hydrochloric acid, Petroleum ether, n-hexane, Chloroform, Antimony tri chloride analytical grade (AR), were procured from Himedia, SDFC, SRL India and used for the extraction isolation, and TLC analysis mobile phase and spray reagent.

Extraction of Sapogenin in three species:

The isolation of crude sapogenin was obtained by hydrolysis process as described by Morris *et.al* [35] with slight modification. The freshly harvested rhizomes were washed thoroughly with liquid detergent and surface-sterilized under running tap water. The surface-sterilized rhizomes (5 g) were peeled, chopped, and refluxed with 3.5 M HCl (115 mL) for 3 hrs, hydrolyzed rhizomes were cooled to room temperature and filtered, and the residues were washed with water continuously till it reaches neutrality. The obtained residues were dried at 65^0C- 70^0C overnight. The dried residue was further extracted with petroleum ether by the hot maceration method for 6 hours. The extracts were concentrated and resulting solid particles were precipitated and dried to obtain crude sapogenin and re-suspended in chloroform. The extracts were allowed to evaporate at room temperature without disturbing overnight; a needle-shaped white crystal of diosgenin as pure sapogenin was obtained. The obtained crystals were separated and subjected to TLC profile and quantification by UPLC LC-SRM analysis.

TLC Profiling of the Diosgenin in Three species:

Preparation of the standard: The standard Diosgenin 10 mg/mL concentration was prepared in chloroform and the resulting stock solution was diluted to 1 mg/mL concentration as a working solution.

Preparation of sample for TLC: Diosgenin isolated from *Costus pictus*, *Costus igneus*, and *Costus speciosus* extracts were aliquoted as 10mg/ml concentration.

TLC (Thin Layer Chromatography) separations:

Diosgenin standard and isolated Diosgenin from *Costus pictus, Costus igneus, and Costus speciosus* were spotted

on activated TLC plates of silica gel 60 F254 aluminum sheets, Merck Darmstadt Germany. The TLC plates were activated at 110^{0}C for 10 mins before spotting, 10µg concentration of each sample and standard were applied on plates and dried. The spotted plates were activated for 10 min below 100^{0}C and developed for 20 mins, with mobile phase n-hexane: ethyl acetate (7:3); after development, the mobile solvent on the plate was dried in an oven below 100^{0}C. The developed plates were derivatized with 20% antimony chloride in chloroform by sprayer followed by drying in an oven at 105^{0}C for 10 mins. The Rf value was calculated with respect to standards and samples.

Extract preparations for LC-MS:

The three samples after hydrolysis and extracted with petroleum ether, a white crystalline product of Diosgenin were suspended in chloroform and dried under vacuum for complete dryness. The collected samples were re-suspended into 200 µL of methanol and vortexed well and sonicated for 1 min, centrifuged at 14,000 rpm for 5 mins at 10^{0}C. The supernatant was separated; 10 µL of each supernatant was diluted 100 times in methanol and centrifuged again at 14,000 rpm for 5 mins at 10^{0}C. The supernatant was transferred to fresh vials, and 10µl were injected for analysis.

UHPLC LC-MS SRM analysis:

An Agilent 1290 Infinity UHPLC system (Agilent Technologies, Pvt, Ltd India) coupled with a mass spectrometer (TSQ Vantage, San Jose CA the USA) for chromatographic separation. The temperature in the column oven was maintained at 40^{0}C and 10^{0}C was set up for the auto-sampler. The samples were injected into the system through needle injection mode at 10 µL injection volume, column Agilent Eclipse Plus, 1.8µ, C 18, 50 mm X 2.1 mm. The needle was washed with acetonitrile (0.1 % formic acid) before injection samples to avoid the carry-over problem. The mobile phase A 10 mM ammonium acetate in water (0.1% formic acid) and mobile phase B Acetonitrile (0.1% formic acid) solvents were used with a gradient mode of 0 to 2 min 50% B, 2-9 min 50-100% B, at 9-10 mins 100% B, at 10- 10.1 mins, 100 to 50% B, 10.1- 15 mins 50% B with a continuous run time of 15 mins. The flow rate was 0.3 mL/min. The MS system Thermo fisher TSQ Vantage operates both in positive and negative ionization mode using polarity. The operation conditions were as follows spray voltage of 4000+ve, 2800V; ion transfer, capillary temperature 270^{0}C, source temperature of 300C, vaporizer temperature of 100^{0}C; sheath gas flow rate of 15 Arb (arbitrary units) auxiliary gas flow rate of 10 Arb (arbitrary units) argon as the collision gas, S lens voltage, and the collision energy was regulated and optimized for respective metabolite with a scan time of (0.05 S) 50 millisec/ transition. The MS injector settings were as follows; 0 to 2 mins; waste. 2-9 mins; load, 9:15 mins, waste for the tandem mass spectrometry MS/MS scans, the pre-charged precursor ions for SRM were selected.

Preparation of Calibration curve for Diosgenin analysis:

A standard Diosgenin 10 mg/mL concentration was prepared in chloroform as a stock solution. An aliquot of serial dilution was made from lower concentration to higher concentration (1 ng to 5 ng) for the standard curve. Diosgenin was detected in ES +ve ionization mode; it is a relatively more hydrophobic compound. The linear regression of calibration curve with the mean (±SD) slopes, intercepts of R squared curves were obtained.

RESULTS:

Synthetic design:

The extraction of any metabolite being a very crucial and major parameter, in the process of phytochemical constituent analysis. The extraction of initially tried with maceration and Soxhlet apparatus there was not much difference being noticed. The use of Hydrochloric acid instead of Sulphuric acid, due to its low boiling point and good volatility, use of chloroform due to its good compatibility with Diosgenin. Thus, rhizome obtained from three species *Costus pictus, Costus igneus,* and *Costus speciosus* was hydrolyzed and the sapogenin was separated. The TLC analysis Figure 2 reveals the presence of Diosgenin Rf value 0.48, the UHPLC analysis RT value of 8.5, molecular weight 415.31, daughter ion 271.18.

Optimization of TLC analysis:

Thin layer chromatography is used widely in various areas like identification of sample purity, identification of compounds, biochemical analysis, used for the separation and identification of compounds. It is applied in qualitative testing of various medicines, food and cosmetic industries for estimation of impurities and separation of compounds. The chloroform extract obtained from the three species was applied on the silica per coated aluminum plates (E.Merck), n-hexane, ethyl acetate (7:3) were used as mobile phase as eluent's The Rf value of diosgenin (Rf-0.48) were obtained. The plates were derivatized by 20% Antimony chloride as a spray reagent. In the present study isolated diosgenin was detected on TLC plates as the preliminary analysis indicated the presence of Diosgenin. Later the samples were subjected to the UHPLC-LC-MS SRM method for quantification of diosgenin in all three species Figure 2. The results are summarized in Table No.1 the results obtained are based on the standard calibration curve of the Diosgenin standard.

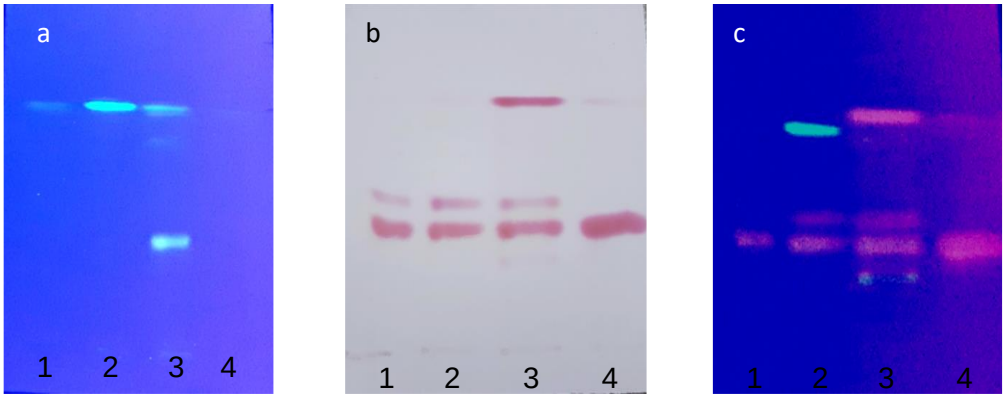


Figure 2: TLC Profile for Diosgenin

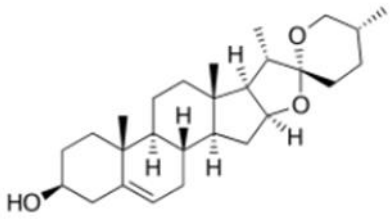
1) *C pictus*, 2) *C igneus*, 3) *C speciosus*; 4) Diosgenin standard,

2) a) Developed plates, b) &c) derivatized plates.

Rf = a/b

a- The distance from the starting point to the gravity center of the sample spot

b- The distance from the starting point to the front of the developing solvent



MW: 414.6

Diosgenin = 2.2/4.5 = 0.48 (Rf value of Diosgenin)

Optimization of UHPLC-SRM condition:

UPLC has a high resolution, speed, sensitivity, increase separation efficiency, a column with smaller particles (<1.7µm), faster results use less valuable solvents like acetonitrile increases the efficiency because of its high pH stability and peak shape and peak area stability. The mobile phase gradient conditions for UHPLC with acetonitrile and water with 0.1% formic acid solvents in LC-MS analysis represent the best gradient system (13). LC-MS analysis was performed by C18 column 1.8 µ, particle size 50 mm X 2.1 mm on Agilent 1290 Infinity UHPLC instruments, Agilent Eclipse plus column with a mixture of the solvent containing 10mM Ammonium acetate in water (0.1 % FA) and Acetonitrile (0.1% FA) as a mobile A and B as an ideal condition for estimation of Diosgenin by gradient elution. The mass spectro-photometric condition was optimized in both positive and negative ion modes, the positive ion mode was found to be more sensitive, the detection of the molecule forms as a quasi molecular ion (M+H)+ in MS analysis, 0.1% formic acid mobile phase facilitates separation. The mass spectrometric condition for Diosgenin Fig No. 3 estimation in both positive and negative mode, resulting in detection of Diosgenin obtained in the positive ion mode by Thermo fisher- TSQ Vantage voltage of 4,000 V ideal for the detection. The values demonstrated for *Costus pictus* Fig No. 4, *Costus igneus* Fig No. 5 and *Costus speciosus* Fig No. 6 contains diosgenin whereas a higher concentration of Diosgenin is reported in Costus speciosus 95.50 µg /g, *Costus igneus* 82.82 µg/g and *Costus pictus* 70.70 µg/g of the starting material (5g) fresh weight with Fig No: 7,8 R2= 0.9994 and Y=5159.76 +25326.3x.

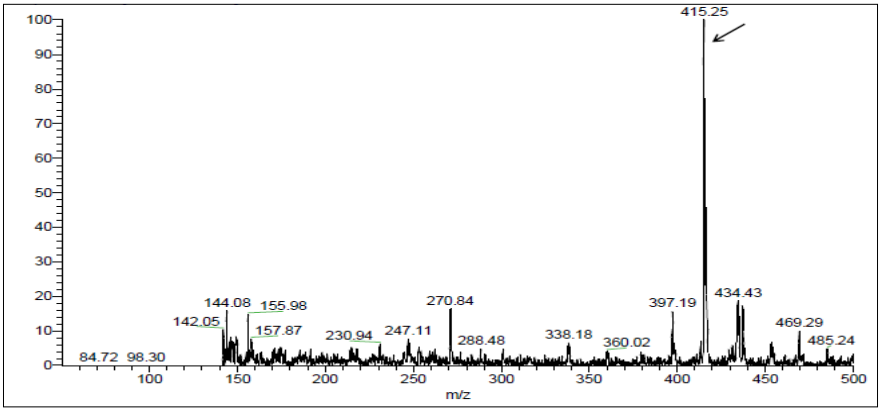


Figure 3: Diosgenin Standard

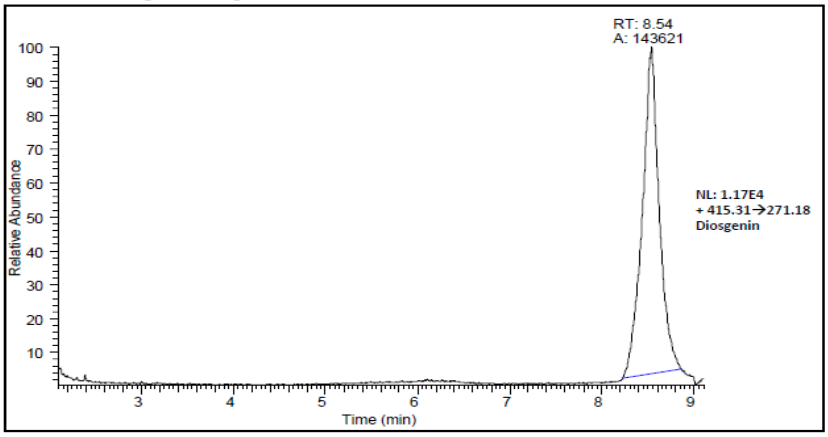


Figure 4: LC-SRM chromatogram of Diosgenin standard

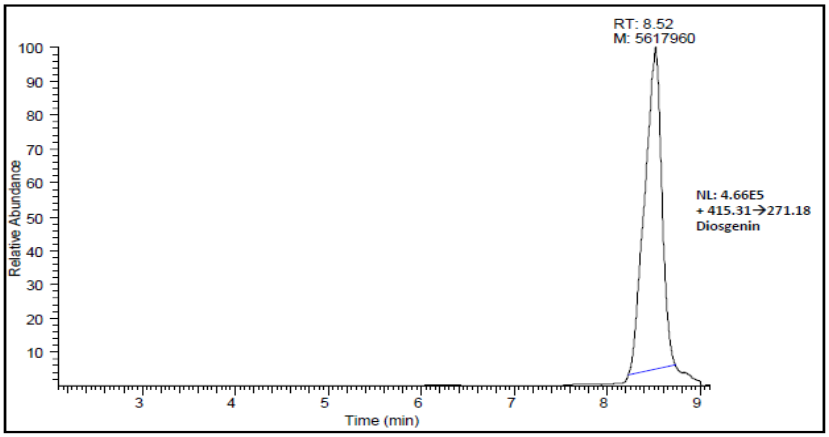


Figure 5: LC-SRM of *Costus pictus*

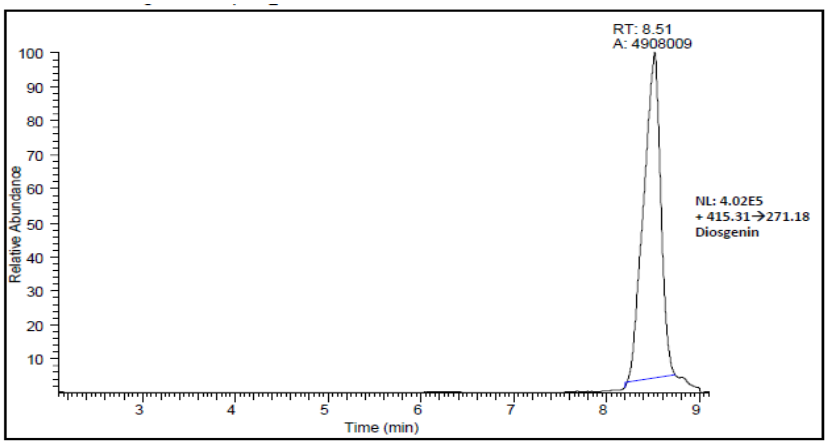


Figure 6: LC-SRM of *Costus igneus*

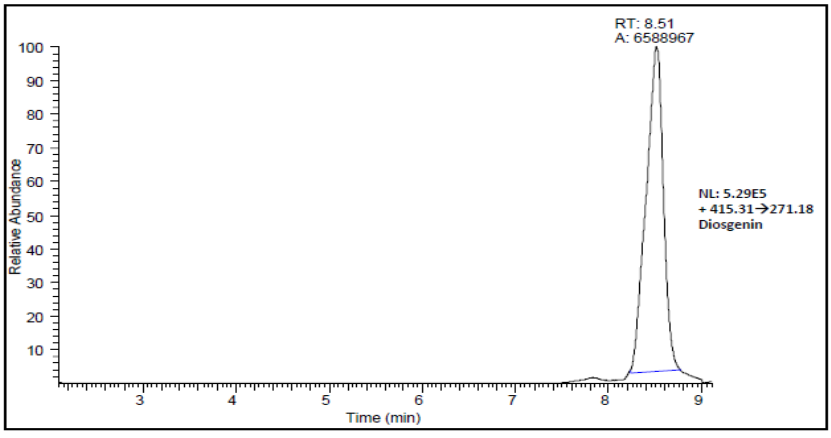


Figure 7: LC-SRM of *Costus speciosus*

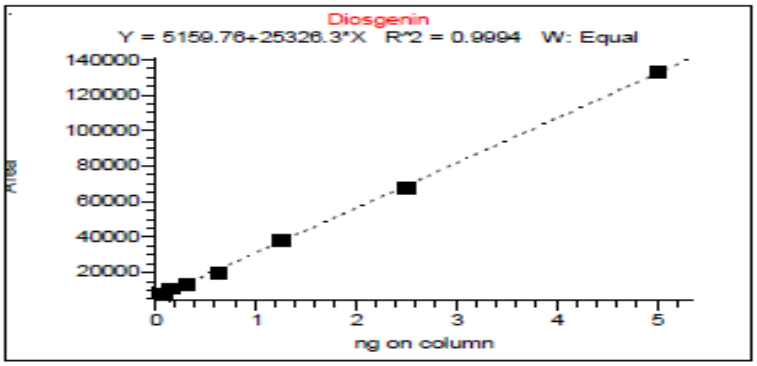


Figure 8: Calibration of standard curve

Table 1: Estimation of Diosgenin in each sample.

Amount of Metabolites in Samples (µg per g fresh weight)				
Sl.No	Metabolite	C.*pictus*	C.*igneus*	C.*speciosus*
1	Diosgenin	70.70	82.82	95.50

Calculations: [(ng on column (as given by std curve)X 2000) /(5gX1000)]

DISCUSSION:

The genus *Costus* belongs to *Costaceae* are perennial tropical herbaceous flowering plants, they are characterized and identified based on their spirally arranged leaves. They are used as a stimulant, diuretic, anti-diabetic, anti-inflammatory, anti-septic properties [36]. Diosgenin is a well-known steroidal sapogenin abundantly found in many medicinal herbs; it is mainly utilized as a major source of raw material which acts as a precursor molecule for the synthesis of steroidal drugs in pharmaceutical industries. It is having a wide array of pharmacological activities and possesses many medicinal properties manifested in treating many chronic diseases like cancer, neurological disorders like Parkinson's disease, Alzheimer's disease, brain injury, neuroinflammation, and ischemia [38].

The TLC is carried out for the identification of bioactive compounds, the Rf values of particular bio-active compounds provide an idea to determine their polarity and are useful for the selection of a particular solvent system for further isolation of phytoconstituents [38]. The ultra-high-performance liquid chromatography/Mass spectrometry/Selected Reaction Monitoring (UHPLC-MS/SRM) developed acts as an efficient tool for the quantification of metabolites from plant extracts [39] In the current study, Diosgenin was quantified from three *Costus* species and the UHPLC methodology helps to detect and identify nonamers in contrast with hexamers, with reduced run time, faster, more cost-effective for the analysis of the metabolites derived from plant extracts compared to HPLC analysis [40]. It also regulates Hypercholesterolemia, improves lipid profile [41]. Diosgenin can be utilized as a precursor for the synthesis of cortisone from Diosgenin [43].

CONCLUSION:

The extraction, isolation of Diosgenin in *Costus* species substantiate that medicinally important metabolites are found in all plants. Attempt for standardization of extraction, isolation detection of Diosgenin a steroidal compound was well demonstrated by TLC and UHPLC LC-MS analysis, which facilitates in the identification of the bioactive compound as an efficient tool. It is a sensitive and reproducible method for the detection and quantification of Diosgenin in various samples. LC-MS methods used to analyze thermolabile, polar molecules, and compounds of high molecular weight can be determined. There are 24 species identified in the *Costus* group with high therapeutic properties and are well known to possess many valuable bioactive compounds, hereby this present study supports further investigation of important metabolites in other *Costus* species of the same family and other species.

Acknowledgements: I would like to acknowledge the Metabolomics Facility at the Center for Cellular and Molecular Platforms (C-CAMP), GKVK Bengaluru for permitting me to carry out the UHPLC-SRM facility. I would like to acknowledge the technical services rendered by Ms. Padma Ramakrishnan Technology associate C-CAMP, GKVK, Bengaluru.

Conflict of Interest: All the authors mentioned in the manuscript do not have any conflict of interest.

Competing Interest declaration: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval: Not applicable

Source of Support: Nil

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Abbreviation: TLC (Thin Layer Chromatography), UHPLC (Ultra High-Performance Liquid Chromatography), LC (Liquid Chromatography), MS (Mass Spectrometry), SRM (Selective Reaction Monitoring).

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