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Assay of Steroidal Sapogenin in *Costus Speciosus* Rhizomes

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Diosgenin, a steroidal sapogenin, was isolated from rhizhomes of *Costus speciosus* by reported method. It was characterized by TLC, UV and FT IR spectroscopy and purity was determined by HPLC. Amount of diosgenin in the free and glycosidic form was determined colorimetrically, after subjecting the purified sample to preparative TLC. Result shows that the majority of diosgenin is in the glycosidic form.

Key Words: *C. speciosus*, Rhizomes, Steroide sapogenin.

INTRODUCTION

Costus speciosus (Koen.ex Retz.)Sm. (Family Zingiberacea) is a succulent herb with tuberous rhizomes, stems spirally twisted leaves spiral, oblong to oblanceolate, acute or acuminate, often cuspidate, glabrous above, silky-pubescent beneath, flowers white in dense spikes, bracts bright-red, fruit a 3-valved capsule, found plentifully in Bengal, Kashmir, Himachal Pradesh, Uttar Pradesh and Kerala[1].

The rhizomes are feebly astringent and considered as tonic, antihelmintic, abortifacient, depurative and aphrodisiac. The juice of fresh rhizome is purgative. The decoction of rhizomes and those of *Cyperus rotundus* Linn. and the bark of *Azadirachta indica* A. Juss. is used for the treatment of jaundice, in Meghalaya. A mixture of saponins from the rhizome shows significant antiinflammatory, antiarthritic and antifertility activities. It shows pronounced inhibitory effects on germination of the smut spores. Total saponin mixture, showed antiinflammatory and oestrogen activity in sprayed rats, significantly increased uterine weight and uterine glycogen concentration and produced proliferate changes in uterus and vagina. It also showed spasmodic effect of varying degree on isolated ileum and isolated tracheal preparations[2].

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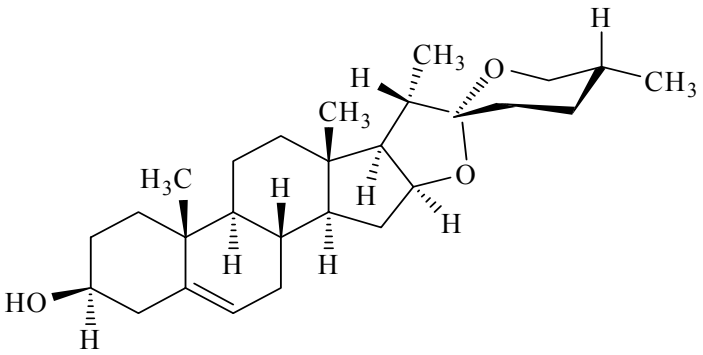
A steroidal sapogenin isolated from the plant is diosgenin[3,4]. The plant has become all the more important because of the presence of diosgenin in its rhizome[5]. The plant can be easily propagated through rhizome cuttings and has been a good source of steroid industry in India[6]. Akhila *et al*.[7] gave the evidence of site of biosynthesis of diosgenin and its sponins. Radioactively labeled free and conjugated diosgenis were isolated from different parts such as stem, leaf, flower, seed and rhizome of *C. speciosus*. Their studies revealed that diosgenin was biosynthesized in leaves and then translocated to all the parts of the plant. Glycosidation of diosgenin takes place in all the parts of the plant and diosgenin glycosides are stored in rhizomes, seeds and flowers. Deglycosidation of saponin was observed only in the rhizome indicating that some enzyme is present in the rhizome, which is responsible for the hydrolysis. Diosgenin occurs both in free and glycosidic form, dioscin, the prominent steroidal saponin isolated, alongwith β-sitosterol-β-D-glucopyranoside, prosapogenin-B of dioscin, prosapogenin-A of dioscin, gracillin, 3-O-(α-L-rhamnopyranosyl (1 to 2)-β-D-glucopyranosyl)-26-O-(β-D-glucopyranosyl)-22-α-methoxy-(25R)-furost-5-en-3-β-26-diol,methyl protodioscin and protodioscin in *C. speciosus*. They were isolated and their structures elucidated by Singh and Thakur[8]. The present paper deals with the estimation of diosgenin content in the free and glycosidic form in the rhizome of *C. speciosus*.



Diosgenin

EXPERIMENTAL

The rhizhomes of *C. speciosus* were collected from Malappuram district, Kerala and authenticated by Prof. T. Ahammedkutty, Department of Botany, Farook College, where a voucher specimen is deposited.

Liebermann-Burchardt test: To 1 mL of methanolic extract, 1 mL of chloroform, 2-3 mL of acetic anhydride and 2 drops of concentrated sulphuric acid were added. Dark green colour of the solution indicated the presence of steroids.

HPLC was performed using Shimadzu Model HPLC system (Shimadzu, Tokyo, Japan) consisting of LC-10ATVP pump, SPD M10AVP photodiode array detector, CLASS-VP 6.12 SP5 integration software and a rheodyne injection valve fitted with a 20 µL injection loop. Baseline resolution of diosgenin was obtained at 25 ± 2 ºC using a Phenomenex Luna C_{18} column (250 × 4.6 mm. i.d 5 µm) and an isocratic solvent system consisting of methanol:water (90:10 v/v). The mobile phase was passed through 0.45 µ PVDF filter and degassed before use.

UV spectrum was taken using a Shimadzu UV-1700 Model (Shimadzu, Tokyo, Japan) with a double beam spectrophotometer. Quartz cuvettes were used for UV sampling.

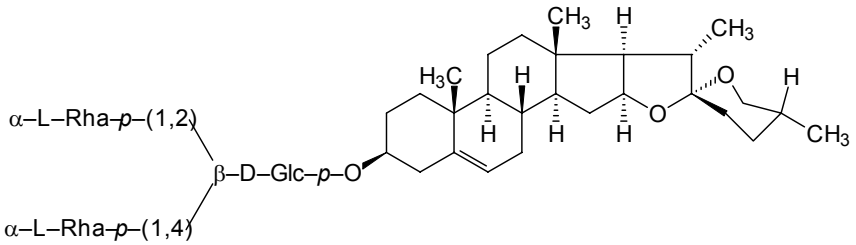
IR spectrum was taken on Shimadzu-8400S Model (Shimadzu, Tokyo, Japan).

Preparative thin layer chromatography was done using pre-coated silica-gel plates 60F254 with layer thickness 0.25 mm (E. Merck), micro syringe (25 µL, Hamilton, #802) and a UV chamber with λ_{254} nm and λ_{365} nm.

All chemicals used were GR grade-E.Merck, India.

RESULTS AND DISCUSSION

Isolation and purification: Diosgenin was isolated from the rhizome of *C. speciosus* using a previously reported method[6]. The shade-dried rhizome of *C. speciosus* was powdered; 100 g of the powder was completely defatted with 300 mL petroleum ether (b.p 60-80 ºC) using a Soxhlet apparatus. The dried defatted material was hydrolyzed with HCl (3 N) for 4 h on water bath using a reflux water condenser. The hydrolyzed material was freed from chloride ions by washing several times with distilled water. It was then dried completely first in air and then in oven between 100-105 ºC. The material was then extracted exhaustively in a Soxhlet apparatus using *n*-hexane for 6 h. The extract, thus obtained was reduced to 20 mL by rotary flash evaporator. Remaining solvent was removed using water bath.



Dioscin

The dried extract containing diosgenin was purified by dissolving in a minimum amount of hot methanol, which on cooling yielded crystalline diosgenin. Further purification of diosgenin was achieved by repeated

crystallization in cold methanol followed by washing with methanol, which resulted in needle shaped white crystals. The crystals gave positive colour reaction with Libermann-Burchard reagent and was soluble in chloroform, methanol, acetone and *n*-hexane[9,10].

Determination of purity using HPLC: Methanol:water in the proportion 85:15 (v/v) was used as the solvent system. The flow rate was kept constant at 1.0 mL/min and the chromatogram was integrated at 210 nm. The standard solution of diosgenin was injected in duplicate and the average detector response was measured. The percentage purity of isolated compound was detected by area normalization method. The HPLC chromatogram showed the presence of an additional compound at an retention time of 9.67 minutes indicating that further purification was needed.

TABLE-1
RETENTION TIME AND PEAK AREA IN HPLC

Detector A-210 nm peak number	Retention time	Area	Area (%)
1	3.26	2129071	74.57
2	9.67	725889	25.40

Characterization of diosgenin: UV spectroscopic characterization-standard solution of concentration 50 µg/mL in hexane was prepared and used for analysis. The baseline correction was done using hexane and UV spectrum of isolated diosgenin was recorded. The λ_{max} of isolated diosgenin was 216 nm similar to standard diosgenin.

Fourier transform infrared spectroscopic characterization - 500 µg of isolated compound was dissolved in 5 mL chloroform. The background spectrum of chloroform was recorded using a fixed volume NaCl window. Then the FTIR spectrum of the diosgenin was recorded by the same method. Scan number was set as 20. Integration was done using Kubelka munk conversion. FTIR spectrum showed characteristic absorbance at specific wave number, which matches the spectrum of standard diosgenin in library. The spectrum of dosgenin was found to contain characteristic absorptions at 3453 cm^{-1} (-OH), 2853 cm^{-1} (C-H stretching), 1458 cm^{-1} (C-H bending), 980 cm^{-1} (vinyl double bond), 875 cm^{-1} (C-O-C stretching), *etc*.

Preparation of hydrolyzed extract: 1 g of the shade-dried and powdered rhizome of the *C. speciosus* was refluxed with 10 mL of 2.5 N HCl for 4 h to hydrolyze the material. Cooled, filtered and washed until the residue was free from chloride. Dried the material at 100 ºC and further extracted with a mixture of petroleum ether (b.p 60-80 ºC) and isopropanol (12: 1). The solution was filtered and the solvent was removed by distilla-

tion under reduced pressure. The residue was then dissolved in 10 mL of a mixture of petroleum ether (b.p 60-80 ºC) and isopropanol (12: 1) and the solution was used for preparative TLC.

Preparation of unhydrolyzed extract: 1 g of the shade-dried and powdered rhizome of the *C. speciosus* was extracted with a mixture of petroleum ether (b.p 60-80 ºC) and isopropanol (12: 1). The solution was filtered and the solvent was removed by distillation under reduced pressure. The residue was then dissolved in 10 mL of a mixture of petroleum ether (b.p 60-80 ºC) and isopropanol (12: 1) and the solution was used for preparative TLC.

Estimation of diosgenin: Assay of diosgenin in hydrolyzed and unhydrolyzed extract was done by spectrophotometric method. Standard diosgenin with different concentrations were spotted on a pre-coated TLC plate and developed using petroleum ether:isopropanol (12.0:0.5) solvent system. The developed plate was spotted using anisaldehyde-sulphuric acid spray reagent. The band corresponding to diosgenin was scrapped and dissolved in methanol. The absorbance of each solution was recorded at 429 nm. A calibration curve was plotted, with absorbance against concentration, by repeating the procedure with different concentrations of standard diosgenin. The procedure was repeated with both the samples and from the calibration curve, the amount of diosgenin present in each sample was estimated. The amount of diosgenin in glycosidic and free form was estimated to be 1.76 and 0.22 %, respectively. The result shows that majority of diosgenin in the rhizome is present in the glycosidic form.

Steroidal sapogenins are regarded as the most suitable raw material for steroid drug production. Two third of the global demand for steroid precursors is being fulfill from diosgenin and there is an increase in the demand for it[11]. *C. speciosus* is one of the important raw materials being used for the commercial production of steroids in India.

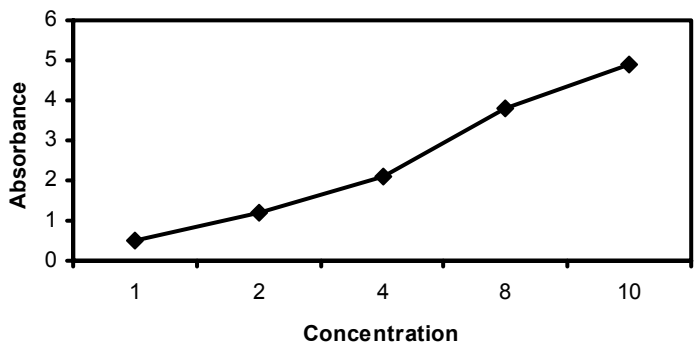
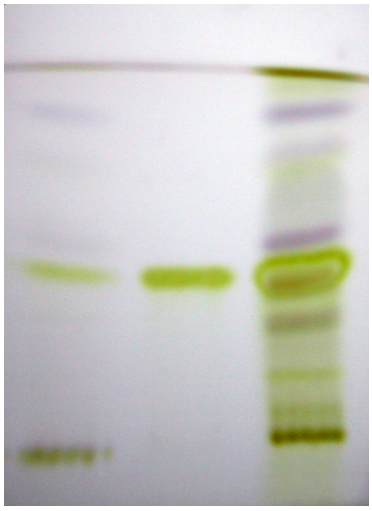


Fig. Calibration curve (estimation of diosgenin)

TLC profile of extracts (hydrolyzed and unhydrolyzed) of *C. speciosus*



1 2 3

1. Extract of *C. speciosus* (unhydrolyzed) 2. Standard diosgenin
3. Extract of C. speciosus (hydrolyzed)

TABLE-2
ESTIMATION OF DIOSGENIN IN HYDROLYZED AND UNHYDROLYZED SAMPLE

Sample	Absorbance	Concentration (µg/mL)
Hydrolyzed sample (Total diosgenin content)	4.7	9.9
Unhydrolyzed sample (Diosgenin in free state)	0.7	1.1

TABLE-3
ASSAY OF DIOSGENIN IN THE RHIZOME OF *C. speciosus*

Amount of the dried rhizome	1 g
Volume of the extract applied	5 µL
Absorbance of hydrolyzed sample	4.7
Concentration of diosgenin (from the graph)	9.9 µg
Amount of diosgenin present in 1g of rhizome	1.98×10^{-2} g
% of diosgenin in hydrolyzed sample (Total diosgenin content)	1.98
Absorbance of unhydrolyzed sample	0.7
Concentration of diosgenin (from the graph)	1.1 µg
Amount of diosgenin present in 1g of rhizome	0.22×10^{-2} g
% of diosgenin in unhydrolyzed sample (free state)	0.22
Amount of diosgenin in glycosidic form	1.76 %

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