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ISOLATION AND QUANTIFICATION OF STIGMASTEROL FROM METHANOLIC EXTRACT OF LEAVES OF *COSTUS SPECIOSUS* BY HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

The present study focusses on isolation and quantification of stigmasterol in methanolic extract *Costus speciosus* leaves. The method of isolation was simple involving extraction with methanol and further purification by column chromatography. The identity of stigmasterol was confirmed by infrared spectroscopy (IR) and nuclear magnetic resonance (NMR). A specific and rapid high performance thin layer chromatography (HPTLC) method was developed for analysis of stigmasterol for which separation was conducted on silica gel 60 F_{254} plates using n-Hexane: Ethyl acetate: Formic acid (8:2:0.1) as the solvent system. The method was observed to be linear in the range of 0.2-0.6 µg/ spot with correlation coefficient r^2 = 0.996. The LOD and LOQ obtained were 37.8 ng and 114.56 ng respectively. Thus, the proposed validated HPTLC method is new and was found to be accurate and convenient and could be used for rapid screening and quality control of herbal formulations consisting of *Costus speciosus.*

KEYWORDS: *Costus speciosus*, HPTLC, ICH guidelines, Stigmasterol, TLC densitometric.

INTRODUCTION

Costus speciosus is commonly known as 'crepe ginger belongs to the family Zingiberaceae (formerly known as Costaceae). Due to its medicinal and pharmacological attributes, it is extensively researched. *Costus speciosus* is widely grown in the deciduous forests of South India.[1] It is also called as 'insulin plant' by the virtue of its antidiabetic property. Its leaf extract is reported to have antioxidant and sudorific properties.[2] It has been valued commercially for industrially vital phytoconstituents such as, diosgenin, dioscin, costusosides[3] and tigogenin etc. Other important phytoconstituents isolated from *Costus speciosus* includes sitosterol, protodioscin, etc. The leaves of *Costus speciosus* is reported to possess anti-oxidant, anti-proliferative;[4] and anti-bacterial activities.[3] Extensive research studies have revealed that there is no isolation technique and HPTLC method for estimation of Stigmasterol from *Costus speciosus* plant. Hence attempt was made to isolate the same from the leaves of crepe ginger.

Due to its dependability and simplicity, chromatographic fingerprinting has become a crucial technology in recent years for determining the quality control of herbal medicines. High performance thin layer chromatography has become a standard and regular analytical procedure due to its high speed, low cost of operation and less analysis time. HPTLC is a sophisticated version of thin layer chromatography (TLC) where compounds having minimum or no ultraviolet (UV) absorption can also be detected. Stigmasterol was quantified by HPTLC from *Bryophyllum pinnatum.*[5] Using the same technique, stigmasterol and lupeol was simultaneously quantified from *Ficus religiosa.*[6] Along with lupeol, ursolic acid and betulinic acid, stigmasterol were simultaneously estimated from medicinal plants and marketed formulation.[7] Isolation and quantitative determination of stigmasterol by HPTLC was performed from *Tagetes erecta* and *Capsicum annum.*[8] An attempt was also made for the same from *Alpinia calcarata.*

A thorough literature survey showed no method has been reported for isolation and quantitative estimation of stigmasterol from *Costus speciosus* leaves, by HPTLC thus making this study ingenious. This method was found to be rapid and can be useful for quantitative estimation of stigmasterol.[9]

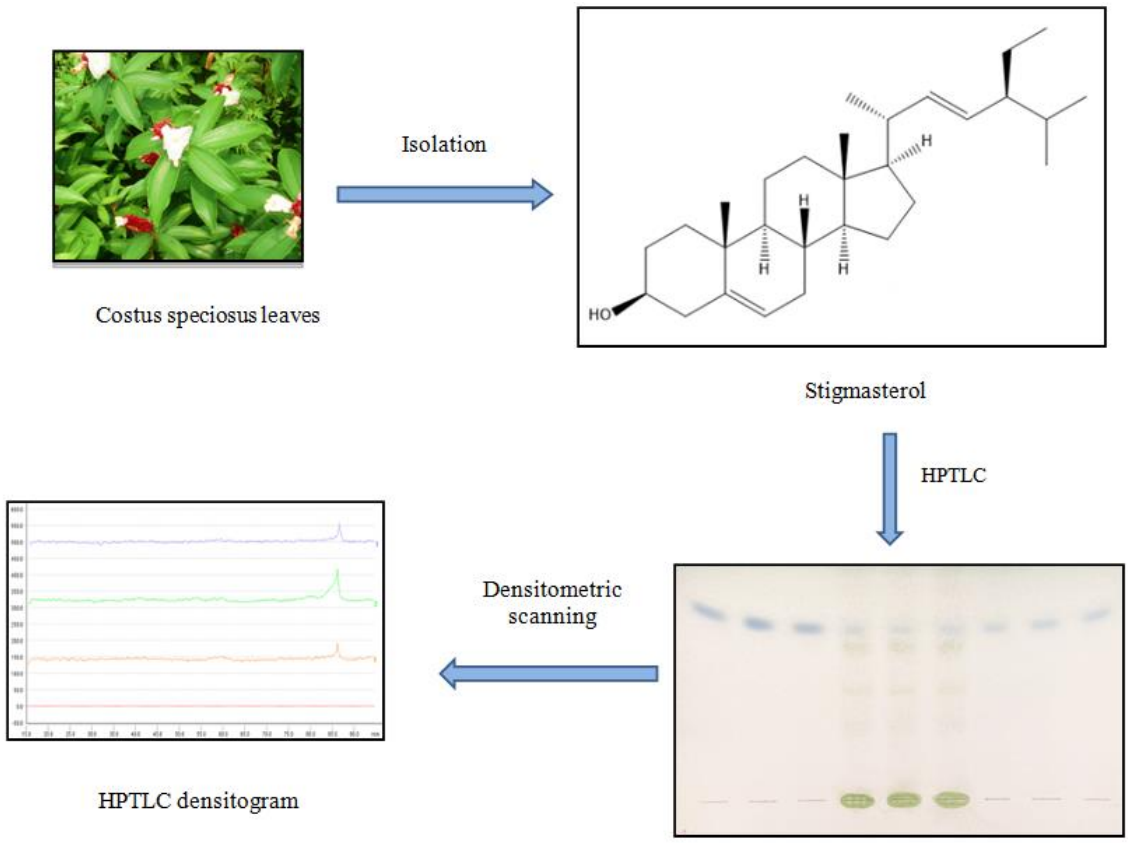

Figure 1: Graphical abstract.

2. MATERIAL AND METHODS

2.1. Chemicals

All laboratory grade chemicals were purchased from AMI chemicals, Mumbai, India. Standard stigmasterol (>95%) was procured from Yucca enterprises, Mumbai.

2.2. *Apparatus*

Glass wares such as beakers, pipettes and reflux assembly were purchased from Borosil, Mumbai, India.

2.3. *Plant material*

The leaves of C*ostus speciosus* were collected from local garden. A voucher specimen (ICT/MNPRL/CS/01) was submitted to Medicinal Natural Products Research Laboratory, Institute of Chemical Technology, Mumbai, India.

2.4. *Isolation of stigmasterol*

100 grams powdered leaf of *Costus speciosus* was subjected to defatting with 300mL of petroleum ether (60-80°) using reflux assembly for 1 hour. The powder was filtered, dried and was extracted with methanol (500mL) for 3 hours. The extract (50g) was separated, concentrated and was subjected to column chromatography for purification.

2.5. Purification of stigmasterol

The concentrated methanolic extract was adsorbed on silica gel (10g). The adsorbed extract was loaded in silica column (100-120 mesh) column. n-Hexane: Ethyl acetate: Formic acid (8:2:0.1) is was used as mobile phase for column chromatography. Stigmasterol was eluted in fractions 6-12 in. The fractions were combined and dried to obtain off white powder of stigmasterol (50mg).

2.6. Characterization of isolated stigmasterol

The isolated stigmasterol was characterized and its identity was ascertained with Fourier transform infra-red spectroscopy (FTIR) and proton NMR. FTIR was performed on Jasco FTIR 4600 series powered with spectra manager software. A small quantity of the isolated stigmasterol was placed on the sample holder after which the spectra was recorded. Proton nuclear magnetic resonance (NMR) was performed on Agilent series 101 and the results were interpreted using MestReNova x64 software. Small quantity of isolated stigmasterol was dissolved in $CDCl_3$ (deuterated chloroform) and placed in NMR tube. This was subjected to proton NMR and results were recorded and peaks were assigned. The spectra was found to be similar with the recorded and documented spectra.

2.7. Preparation of standard solution

25mg of standard stigmasterol was prepared in 25ml methanol (1000ppm) and different concentrations (200-1000 ng/spot) were loaded onto TLC plate to achieve calibration curve.

2.8. Chromatography conditions

Experiments were performed on a "10 x 10 silica gel 60 HPTLC F_{254} plates" (E. Merck). Prior to the sample

application, silica plates were activated at 100°C for 10 minutes. The applicator device was a CAMAG Linomat V automatic sample spotter (Camag Muttenz, Switzerland). The solutions were spotted in the form of band with the help of a 100 µL Hamilton syringe. The plate development was performed in a CAMAG glass twin trough chamber (20 x 10 cm). The mobile phase comprised of "n-Hexane: Ethyl acetate: Formic acid (8:2:0.1)" The developed plates were subjected to quantitative evaluation in a Biostep-DESAGA scanner unit equipped with ProQuant software. Densitometric scanning were performed at 257 nm. The identification of stigmasterol was confirmed by comparing the R_f value (0.7). The HPTLC run of standard stigmasterol, methanolic extract of Costus leaves and isolated stigmasterol is shown in Figure 4.

2.8.1. Validation of HPTLC method

For undertaking the validation of the method, International Conference on Harmonisation (ICH) guidelines were followed for precision, robustness, LOD and LOQ, specificity and accuracy. Each parameter study was undertaken in triplicates and the results were indicated in %RSD (relative standard deviation)

2.8.2. Linearity

A five point standard stigmasterol solutions with different concentrations were prepared and analysed ranging from 200-600 ng/spot (0.2-0.6µg/spot). The calibration curve of the same was determined.

2.8.3. Precision

Stock solution of stigmasterol was prepared in methanol and six 10µL (500ng/spot) bands were applied and analysed for determining the instrument precision. Six varied volumes of the same concentration were spotted on a plate and analysed to determine the variation arising from the method. Intra-day precision was evaluated by spotting six samples at three different concentrations (200, 300 and 400 ng/spot) on the same day. The inter-day precision studies were evaluated by comparing assays of three different days.

2.8.4. Limit of detection and limit of quantification

The limit of detection (LOD) of an analytical procedure is the least amount of analyte in the sample which can be detected but not necessarily quantified or quantitated to be an exact value. LOD was calculated using the following formula:

$$\text{LOD} = \frac{3.3 \times \text{standard deviation of the y-intercept}}{\text{slope of calibration curve}}$$

The LOQ or quantification limit of an individual analytical procedure is the lowest amount of analyte in the sample which can be quantitatively determined with suitable precision and accuracy. LOQ was calculated using the following formula:

$$\text{LOQ} = \frac{10 \times \text{standard deviation of the y-intercept}}{\text{slope of calibration curve}}$$

2.8.5. Specificity

The specificity of the method was confirmed by analysing standard stigmasterol and the one isolated from *Costus speciosus* leaves.

3. RESULTS

3.1. Isolation and characterization of stigmasterol

Stigmasterol was isolated from methanolic extract of leaves of *Costus speciosus* by simple solvent extraction followed by column chromatography. The isolated compound was characterized by UV, IR and NMR spectroscopy. UV absorption maxima was observed to be at 257 nm. IR spectrum showed peaks at 3305.39 cm^{-1} (hydroxyl), 1643.05 cm^{-1} (C=C stretching) and 1380.78 cm^{-1} (C-H bending). (Figure.2) The structure of the compound was confirmed by comparing it with the one present in the literature. NMR graph was also matched and was found to be the same as reported NMR of stigmasterol. (Figure.3.)

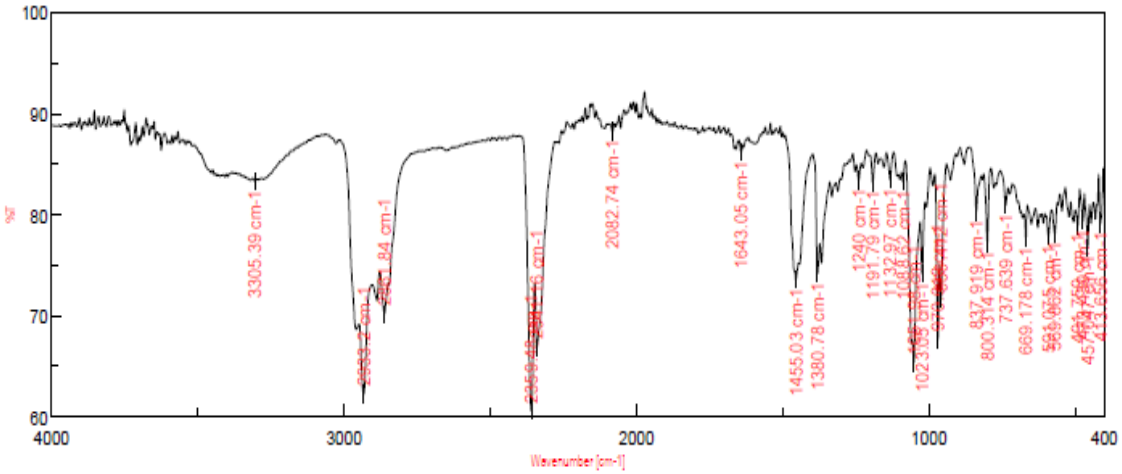

Fig. 2: IR spectra of isolated stigmasterol.

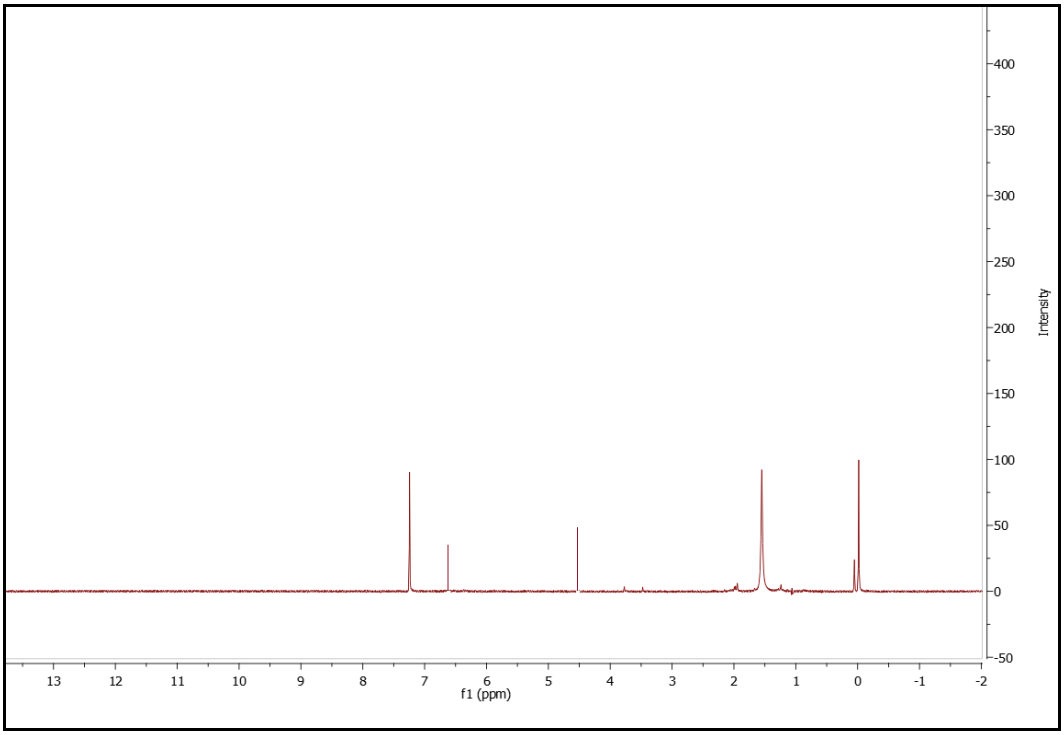

Fig. 3: NMR spectra of isolated stigmasterol.

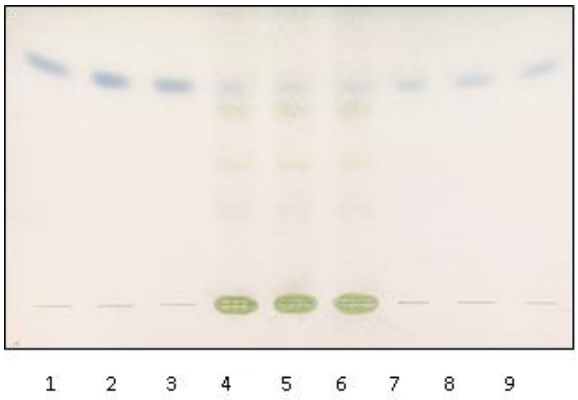

Figure 4: HPTLC chromatogram 1-3: standard stigmasterol, 4-6: Costus speciousus extract, 7-9: isolated stigmasterol.

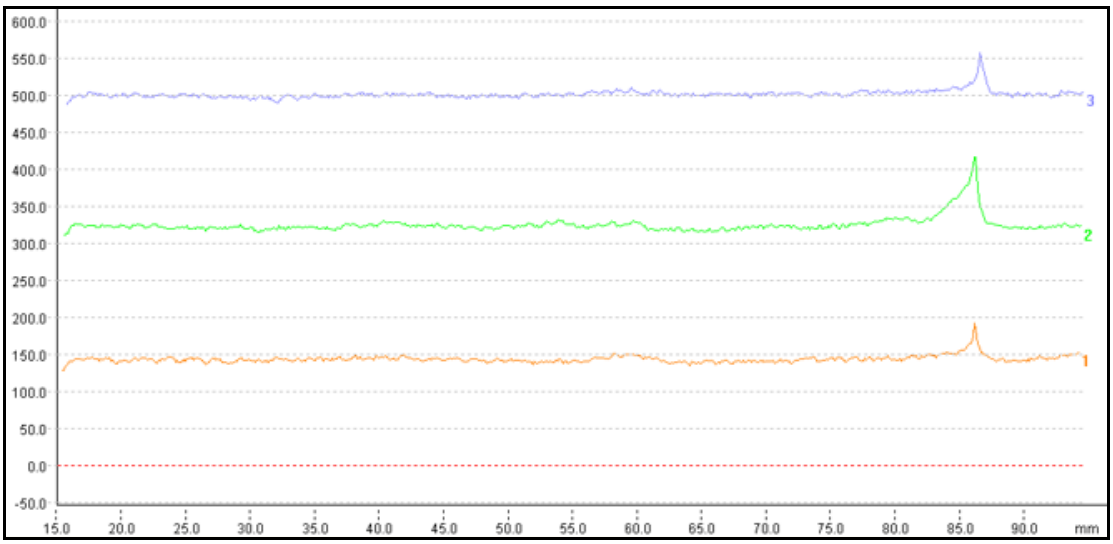

Figure 5: HPTLC chromatogram 1- standard stigmasterol, 2- Costus speciousus extract, 3- isolated stigmasterol.

3.2. HPTLC method validation

3.2.1. Linearity

The linear regression equation obtained was $y = 6.918x + 6.52$ ($r^2 = 0.996$). The result indicated a linear relationship between the concentrations and peak areas.

3.2.2. Precision

%RSD <3% was observed which denoted good precision, thus signofoying reproducibility of th method. Table 1.

Table 1: Intra-day and inter-day precision of stigmasterol.

Amount (ng/spot)	Intra-day precision		Inter-day precision	
	SD	%RSD	SD	%RSD
200	2.47	0.16	11.11	0.71
400	19.53	0.79	23.46	0.96
600	25.28	0.63	15.41	0.41
RSD = relative standard deviation				

3.2.3.LOD and LOQ

The LOD and LOQ were determined standard deviation and slope calculated from the calibration curve of stigmasterol. The limit of detection was found to be 37.8 ng/spot for stigmasterol while the limit of quantification was found to be 114.56 ng/spot.

3.2.4. Specificity

The specificity was evaluated by comparing the chromatogram of the sample with standard.

3.2.5. Robustness

The estimation was conducted by varying the mobile phase (solvent system) volume and composition and the results were indicated by %RSD values.

Table 2: Robustness.

Amount (ng/spot)	Mobile phase/ Solvent system	%RSD
200	n-Hexane: Ethyl acetate: Formic acid (8:2:0.1))	0.97
	n-Hexane: Ethyl acetate: Formic acid (7:3:0.1)	1.25

Table 3: Summary of validation parameters.

Parameters	Stigmasterol
Linearity	
i. Range	200-800 ng
ii. Correlation coefficient	0.996
iii. R_F value	0.53
Precision (%RSD)	
i. Inter-day precision	0.69
ii. Intra-day precision	0.52
LOD	37.8 ng
LOQ	114.56 ng
Specificity	Specific
Robustness	Robust

4. DİSCUSSİON

A number of medicinal properties have been linked to *Costus speciosus* and stigmasterol being major sterol present, it contributes in the synthesis of numerous hormones, including corticoids, androgens, oestrogens, and progesterone. The objective of the study was to establish a method of isolating stigmasterol from *Costus speciosus* and to quantify it by HPTLC as it was not attempted before. The powder was defatted initially with petroleum ether and then extracted with methanol and the crude extract was purified via column chromatography. Although use of column chromatography makes the process time consuming, but it is required to ascertain the purity of the compound. Further, stigmasterol was quantified by high performance thin layer chromatography and the method was validated for parameters such as linearity, robustness, precision, LOD and LOQ. The standard stigmasterol, Costus methanolic extract, and isolated substance have displayed strong peaks and good separation under the chromatographic conditions used. Thus, it can be ensured that this method can also be used for standardization of herbal formulations containing stigmasterol.

5. CONCLUSION

In conclusion, the HPTLC method employed in this study has proven to be efficient and reliable for the isolation and quantification of stigmasterol. Through careful sample preparation, chromatographic separation, and densitometric analysis, we were able to accurately determine the presence and concentration of stigmasterol in the tested samples. The isolation and quantification of stigmasterol hold significance in various fields such as pharmacology, food science, and nutrition due to its potential health benefits and biological activities. 50 mg of stigmasterol was obtained from 100g leaves of *Costus speciousus*. The developed HPTLC method provides a rapid and cost-effective means for analyzing stigmasterol content, which can aid in quality control, authentication, and standardization processes.

Conflict of Interest

The Authors declares that there are no conflicts of interest.

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