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Original Article

# IDENTIFICATION, QUANTIFICATION AND VALIDATION OF STIGMASTEROL FROM ALPINIA CALCARATA USING HIGH-PERFORMANCE THIN LAYER CHROMATOGRAPHY METHOD

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**ABSTRACT**

**Objective:** The present study is designed to develop a new simple, precise, rapid and selective high-performance thin-layer chromatographic (HPTLC) method for the determination of stigmasterol in methanolic rhizomes extract of *Alpinia calcarata.*

**Methods:** As per International Conference on Harmonization (ICH) guidelines we have applied different concentrations of stigmasterol as standard on HPTLC plates for the quantification of stigmasterol from the *Alpinia calcarata* rhizomes. The concentration of standard stigmasterol is 1 mg/ml.

**Results:** The retention factor of stigmasterol was 0.58. Linearity was obtained in the range of 50 ng-250 ng for stigmasterol. The developed and validated HPTLC method was employed for stigmasterol in methanolic rhizomes extract of *Alpinia calcarata* for standardization of the content of the marker. The linear regression data for the calibration plots showed a good linear relationship with r=0.99977 for stigmasterol, respectively Satisfactory recoveries of 99.77 % were obtained for stigmasterol.

**Conclusion:** The results obtained in validation assays indicate the accuracy and reliability of the developed HPTLC method for the quantification of stigmasterol in methanolic rhizomes extract of *Alpinia calcarata*

**Keywords:** *Alpinia calcarata* Roscoe, Stigmasterol, HPTLC


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## INTRODUCTION

*Alpinia calcarata* Roscoe belonging to the family Zingiberaceae is a rhizomatous perennial herb, which is commonly used in the traditional medicinal systems in Bangladesh, India, Indonesia, Thailand, Malaysia, SriLanka, Taiwan, Cambodia, and Vietnam [1]. *Alpinia calcarata* is used in the oriental part of the world as a food additive, spice and in indigenous system of medicine. It grows in dense forests at high altitudes and is considered as a native of India [2]. *Alpinia calcarata* rhizomes are branched and dense with a light to dark brown colour and known to possess a broad spectrum of medicinal properties. This is a very good source of pinocembrin 6 which induces mitochondrial apotopsis in colon cancer cells [3].

Experimentally, rhizomes are shown to possess antibacterial [4], antifungal [5], anthelmintic, antinociceptive [6], antioxidant [7], aphrodisiac [8], antidiabetic activities [9], rheumatism, fever and anticancer activity. It is also widely used to relieve colds and reducing swellings [10, 11]. High blood pressure, diuretic, stomach problems, analgesic [12], anticandidal, antiplatelet, antispasmodic [13] antiulcerous hypotensive [14, 15] insecticidal, muscle relaxant and Uterine stimulant.

Nowadays, HPTLC has become a routine analytical technique due to its advantages of reliability in quantitation of analytes at the micro and even in nanogram levels and cost effectiveness [16]. It has proved a very useful technique because of its low operating cost, high sample throughput and the need for minimum sample clean-up. The major advantage of HPTLC is in reducing analysis time and cost per analysis [17]. However, no single method was found in the literature to our knowledge to detect stigmasterol in methanolic extract of rhizomes of *Alpinia calcarata*. Therefore, the present study aimed to develop an HPTLC method for analysis of stigmasterol in *Alpinia calcarata* rhizomes which have not yet been reported.

The developed method was optimized and validated in accordance with International Conference on Harmonization (ICH) guidelines. Here, we developed a simple high-performance thin-layer chromatographic method for the rapid analysis of stigmasterol compounds in *Alpinia calcarata* rhizomes. The method was found suitable for rapid screening of plant materials for their genotypic assessment and can be performed without any special sample pretreatment.

## MATERIALS AND METHODS

### Materials

### Standard and chemicals

Stigmasterol (purity 99%), was purchased from Sigma-Aldrich, New Delhi. Ethyl acetate, toluene, glacial acetic acid and methanol used in the present research work were of HPLC grade and were procured from E. Merck Mumbai, India.

### Collection of plant material

The Rhizomes of *Alpinia calcarata* were collected in the Month of July from the Kolli Hill-Chinna Kovilur, Tamil Nadu, India. The plant was identified and rhizomes of *Alpinia calcarata* were authenticated and confirmed from Dr. S. John Britto, Director, Rapinat herbarium, St. Joseph College, Tiruchirapalli, and Tamil Nadu for identifying the plants. The voucher specimen number PP001 (14.07.2016).

### Preparation of methanol extracts

The rhizomes of *Alpinia Calcarata* were washed in running water, cut into small pieces and then shade dried for a week at 35-40 °C, after which it was grinded to a uniform powder of 40 mesh size. The methanol extracts were prepared by soaking 100 g each of the dried powder plant materials in 1 L of methanol using a soxhlet extractor continuously for 10 hr. The extracts were filtered through whatmann filter paper No. 42 (125 mm) to remove all unextractable matter, including cellular materials and other constitutions that are insoluble in the extraction solvent. The entire extracts were concentrated to dryness using a rotary evaporator under reduced pressure. The final dried samples were stored in labeled sterile bottles and kept at-20 °C. The filtrate obtained was used as sample solution for the further HPTLC analysis [17].

**Identification and quantification of stigmasterol from *Alpinia Calcarata* by HPTLC**

**Sample preparation**

All the chemicals, including solvents, were of analytical grade from E. Merck, India. The HPTLC plates Si $60F_{254}$ (20 cmX10 cm) were purchased from E. Merck (India). Standards of stigmasterol (99% purity) were purchased from Sigma (New Delhi, India). 100 mg/ml of methanolic extracts of rhizomes of *Alpinia calcarata* was taken for analysis. The extracts were filtered and vacuum dried at 45 °C. The dried extracts were separately redissolved in 1 ml of methanol and sample of varying concentration (5-30 µl) for stigmasterol were spotted for quantification. 1 mg of standard stigmasterol were prepared in 1 ml of chloroform, and different amounts of (50-250 ng) stigmasterol were loaded onto a TLC plate to get the calibration curve [18-21].

**Thin layer chromatography**

A Camag HPTLC system equipped with an automatic TLC sampler $ATS_4$, TLC scanner 3 and integrated software Win CATS version 3 was used for the analysis. Samples were washed on a precoated silica gel HPTLC plates Si $60F_{254}$ (20 cm x 10 cm) plate of 200 µm-layer thickness, for quantification of stigmasterol in rhizomes of *Alpinia calcarata*. The samples and standards were applied on the plate as 8 mm wide bands with a constant application rate of 150Nl $s^{-1}$, with an automatic TLC sampler ($ATS_4$) under a flow of $N_2$ gas, 15 mm from the bottom, 15 mm from the side, and the space between two spots was 6 mm in the plate.

**Detection and estimation of stigmasterol**

The linear ascending development was carried out in a camag twin through chamber (20 cm x 10 cm), which was pre-saturated with a 25 ml mobile phase, toluene: ethyl acetate (8.0: 2.0 v/v) for stigmasterol for 30 min, at room temperature (25 °C±2 °C) and 50±5% relative humidity. The length of the chromatogram run was up to 90 mm. Subsequent to the development; the TLC plate was dried in a current of air, with the help of air dryer, in a wooden chamber with adequate ventilation.

The dried plate was dipped into freshly prepared libermann burchard reagent. Quantitative estimation of the plate was performed in the absorption-reflection mode at 538 nm, using a slit width 6.00 x 0.45 mm, with data resolution 100 µm/step and scanning speed 20 mm/sec. The source of radiation utilized was a tungsten lamp emitting continuous visible spectra of 366 nm. Determination of stigmasterol in methanolic extracts of rhizomes of *Alpinia calcarata* was performed by the external standard method, using pure standards. Each was carried out in triplicate [22, 23].

**Method validation**

This method was validated as per the ICH guidelines (International Conference on Harmonization in 1994, 1996 and 2005), the method validation parameters checked were linearity, precision, accuracy and recovery, limit of detection, limit of quantification, specificity, robustness and ruggedness. All measurements were performed in triplicates [24-26].

**Calibration curve and linearity**

The calibration was performed by analysis of working standard solutions of stigmasterol (50 to 250 ng for *Alpinia calcarata*) were spotted on precoated TLC plate, using semi automatic spotter under a nitrogen stream. The TLC plates were developed, dried by hot air and photometrically analyzed as described earlier. The calibration curves were prepared by plotting peak area versus concentration (ng/spot) corresponding to each spot.

**Recovery**

To determine the recovery, known concentrations of standards were added to a pre-analyzed sample of *Alpinia calcarata* rhizomes. The spiked samples were then analyzed by the proposed HPTLC method and the analysis was carried out in triplicate.

**Precision**

A stock solution containing stigmasterol compound was prepared in chloroform and six 10 µl (1000 ng/spot) bands were applied and analyzed by the developed method to determine instrument precision. Six different volumes of same concentration were spotted on a plate and analyzed by the developed method to determine variation arising from the method itself. To evaluate intra-day precision, six samples at three different concentrations (1000, 2000 and 3000 ng/spot) for stigmasterol were analyzed on the same day. The inter-day precision was studied by comparing assays performed on three different days.

**Limit of detection and limit of quantification**

The detection limit (LOD) of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. LOD was calculated using the following formula

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

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

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

**Specificity**

The specificity of the method was ascertained by analyzing standard compound stigmasterol and the compound stigmasterol is present in the rhizomes of *Alpinia calcarata*.

**Method specifications**

Silica gel 60 F254 precoated plates (20x 10 cm) were used with toluene: ethyl acetate (8.0:2.0 v/v) for stigmasterol as solvent system. The 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 80 mm and scanned in fluorescence mode at 366 nm. The contents of stigmasterol in the *Alpinia calcarata* were determined by comparing the area of the chromatogram of standard stigmasterol with a calibration curve of the marker compound of *Alpinia calcarata*, considering the isolated compound to be 100% pure.

**RESULTS**

**Optimization of HPTLC chromatographic conditions**

HPTLC fingerprint patterns have been therefore evolved for methanolic extracts of rhizomes of *Alpinia calcarata*. Stigmasterol standard was quantitated accurately using silica gel $F_{254}$ HPTLC pre-coated plates with the mobile phase toluene: ethyl acetate (8.0: 2:0 v/v), the Rf value was about 0.58. The chromatographs of stigmasterol and methanolic extract of rhizomes of *Alpinia calcarata* are shown in (fig. 1). The Rf value of stigmasterol was matched with the Rf value of extract was about 0.58 was shown in peak (fig. 2 (a) and (b)). A steroid compound stigmasterol was identified and isolated by HPTLC techniques.

**Validation of HPTLC method**

**Calibration curve and linearity**

The calibration curve was prepared by plotting peak area versus concentration (ng/spot) corresponding to each spot (fig. 3). The regression equation and correlation curves for stigmasterol in *Alpinia calcarata* were regression via height y= 6.234+0.101X and r= 0.99315 sdv= 6.52. Fig. 3 (a) and regression via area y=132.296+2.085 X and r=0.99977 sdv=1.17. fig. 3(b).

**Accuracy and recovery**

The results showed that the percentage recoveries after sample processing and application were in the range of 99.77 % to 100.11 % (stigmasterol) (table 1). The percentage of stigmasterol in *Alpinia calcarata* rhizome (table 2).

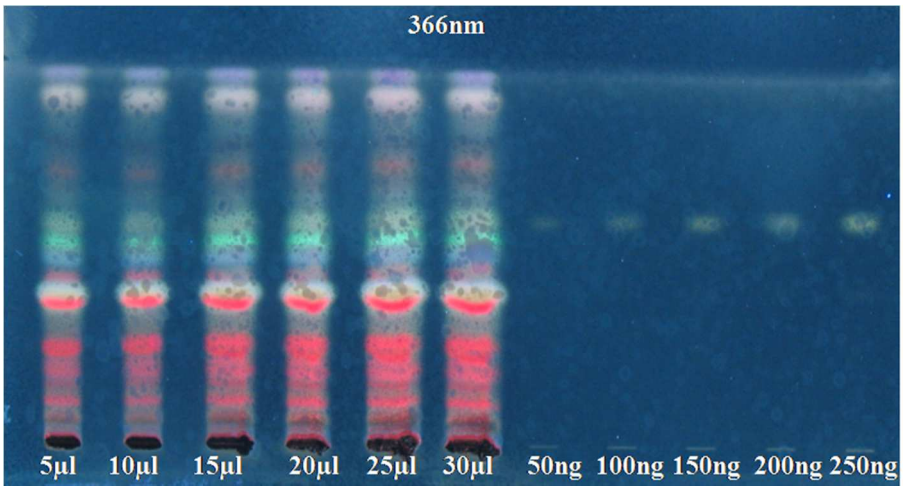


**Fig. 1: Quantitative estimation of stigmasterol in *Alpinia calcarata* rhizomes**

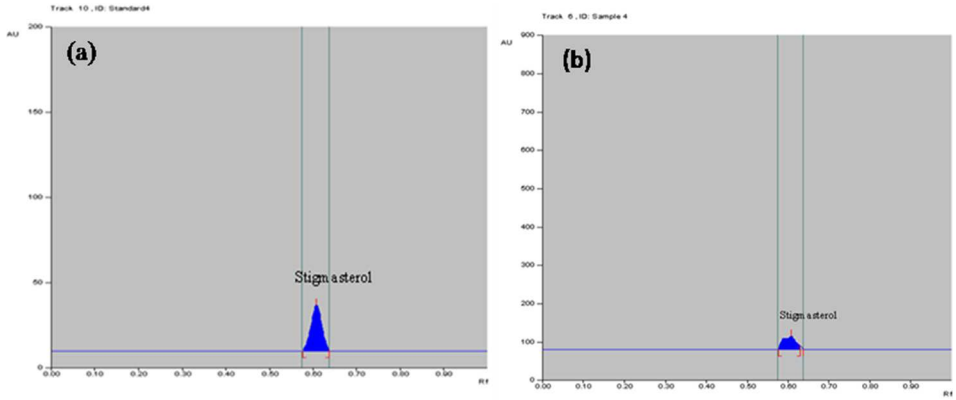


**Fig. 2: (a) HPTLC chromatogram of standard stigmasterol; (b) HPTLC chromatogram of stigmasterol in *Alpinia calcarata* rhizomes**

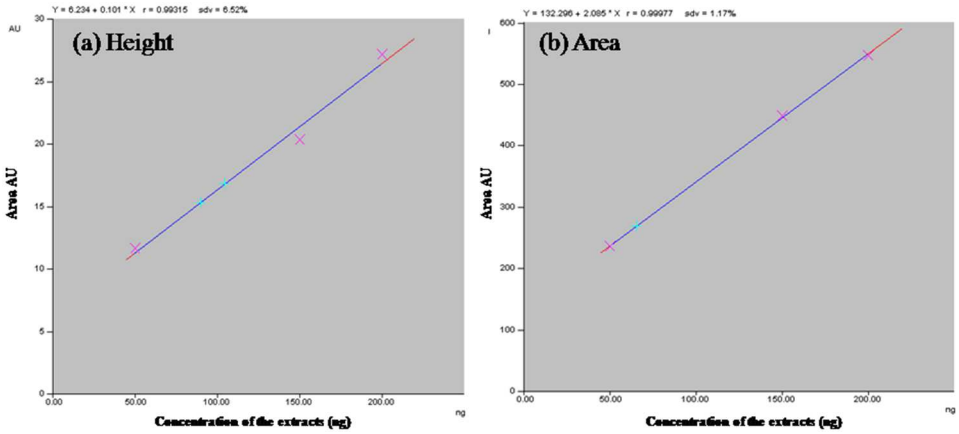


**Fig. 3 Linear graph for Stigmasterol in *Alpinia calcarata* in all tracks (concentration vs. area)**

**Precision**

The developed method was found to be precise as indicated by percent RSD (Relative Standard Deviation) not more than 1.5 (tables 3 and 4).

**Specificity**

It was observed that the other herbal constituents present in the formulations did not interfere with the peak of stigmasterol. Therefore the method was specific. The spectrum of standard compound stigmasterol and the corresponding spot present in *Alpinia calcarata* matched exactly, indicating no interference by the other plant constituents and excipients. The peak purity of stigmasterol was assessed by comparing the spectra at three different levels like peak start (S), peak apex (M) and peak end (E) positions of the spot. Good correlation r = 0.99977 and SD = 1.17 for stigmasterol were obtained between the standard and sample overlain spectra of stigmasterol (fig. 4).

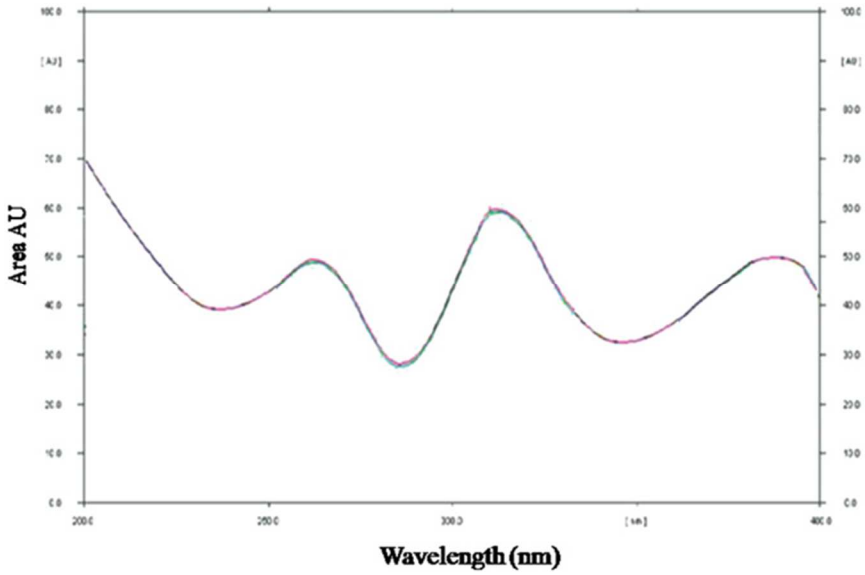


**Fig. 4: Spectral comparison of standard Stigmasterol (green colour) and Stigmasterol quantified from *Alpinia calcarata* rhizomes (pink colour)**

**Table 1: Recovery study of stigmasterol by HPTLC (*n*=3)**

| Compound | Amount of compound present in the plant material (mean, mg/100 mg) | Amount of standard added (mg) | Amount of standard found in the mixture (mg) | Recovery (%) |
|---|---|---|---|---|
| Stigmasterol | 1912 | 1912 | 3830.31 | 100.11±1.14 |
| | | 3824 | 5725.33 | 99.77±0.93 |

n is number of determination

**Table 2: Amount of Stigmasterol in *Alpinia calcarata* rhizomes (n=3)**

| Compound | Quantity (mean) (mg/100 mg) | mean±SE | CV (%) |
|---|---|---|---|
| Stigmasterol | 1.912 | 1.912±0.005 | 0.26 |

n is number of determination, SE is standard error, CV is cumulative value

**Table 3: Intra-day and inter-day precision of the method (n = 6)**

| Compound | Amount (ng/spot) | Intra-day precision | | | Inter-day precision | | |
|---|---|---|---|---|---|---|---|
| | | Mean area | SD | %RSD | Mean area | SD | % RSD |
| Stigmasterol | 1000 | 1580.81 | 1.53 | 0.096 | 1540.50 | 1.67 | 0.108 |
| | 2000 | 3165.32 | 1.71 | 0.054 | 3215.35 | 1.98 | 0.061 |
| | 3000 | 4790.20 | 1.48 | 0.030 | 4681.80 | 1.84 | 0.039 |

n is number of determination, SD is standard deviation, RSD is relative standard deviation

**Table 4: Summary of validation parameter**

| Parameters | Stigmasterol |
|---|---|
| Linearity | |
| (i) Range | 50-250 ng |
| (ii) Correlation coefficient | |
| (a) Height | 0.99315 |
| (b) Area | 0.99977 |
| (iii) Rf value | 0.60 |
| Precision (%RSD) | |
| (i) Instrument precision (CV%, n=6) | 1.62 |
| (ii) Method precision (CV%, n=6) | 2.91 |
| LOD (ng/spot) | 50 |
| LOQ (ng/spot) | 214 |
| Specificity | Specific |
| Robustness | Robust |
| Ruggedness (%RSD) | 0.8110 |

n is a number of determination, RSD is a relative standard deviation, CV is cumulative value, LOD is Limit of detection, LOQ is Limit of quantification, Rf is retention factor.

**Limit of detection and limit of quantification**

The limit of detection was found to be 80 ng/spot for stigmasterol while the limit of quantification was found to be 212 ng/spot for stigmasterol (table 4).

**Robustness**

Robustness tests examine the effect of the operational parameters on the analysis results. By introducing small changes in mobile phase composition, the results indicated that the method was robust (table 5).

**Table 5: Robustness of the method (n=6)**

| Compound | Amount (ng/spot) | Mobile phase | %RSD |
|---|---|---|---|
| Stigmasterol | 1000 | Toluene: Ethyl acetate (7: 3v/v) | 0.96 |
| | | Toluene: Ethyl acetate (8:2 v/v) | 1.45 |

n is number of determination, RSD is relative standard deviation

**Ruggedness of the method**

It expresses the precision within laboratories variations like different days, different analyst, and different equipment. The ruggedness of the method was assessed by spiking the standard 6 times in two different days with the different analyst (table 4).

**DISCUSSION**

HPTLC is a simple, rapid and accurate method for analyzing plant material. HPTLC fingerprint has better resolution and estimation of active constituents is done with reasonable accuracy in a shorter time. The HPTLC method can be used for phytochemical profiling of plants and quantification of compounds present in plants. With increasing demand for herbal products as medicines and cosmetics, there is an urgent need for standardization of plant products [16]. HPTLC finger print analysis has become the most of its simplicity and reliability. It can serve as a tool for identification, authentication, qualitative, quantitative analysis and quality control of herbal drug [27]. Thin-layer chromatography (TLC) and HPTLC methods are commonly applied for the identification, assay and testing for purity, stability, dissolution or content uniformity of raw materials (herbal and animal extracts, fermentation mixtures, drugs and excipients) and formulated products (pharmaceuticals, cosmetics, nutriments) [28]. Stigmasterol is reported to show antioxidant [29], anti-inflammatory [30], analgesic and anthelmintic [30], hepatoprotective and anticancer activity [31-35].

Previous study has reported that quantitative analysis of stigmasterol and *dl*-α-tocopherol acetate, two marker compounds in *Leptadenia reticulate* by high-performance thin-layer chromatographic methods [20]. Quantitative determination of stigmasterol and lupeol from *Ficus religiosa* by HPTLC [17]. HPTLC method which has been developed and validated for quantitation of stigmasterol from petroleum ether extract of dried leaf, stem and flower of *Ageratum conyzoides*[36]. Qualitative and quantitative estimation of galangin from *Alpinia galangal* and *Alpinia officinarum* by HPTLC method [37].

Determination of stigmasterol in dietary supplements by gas chromatography was performed [38], and HPTLC determination of stigmasterol was done [39]. Four new labdane-type diterpenoids, calcaratarins A-D (1-4), along with six known labdane-type diterpenoids, a known element-type sesquiterpenoid, and a known coumarin were isolated from the rhizomes of *Alpinia calcarata* [40]. The major components of essential oils isolated from *Alpinia calcarata* rhizomes were camphene (3.86%), beta-myrcene (4.39%), eucalyptol (14.05%), linalol (2.48%), pyrazine (1.72%), L-camphor (7.90%) and berneol (5.67%) might possess significant anti-inflammatory activity and analgesic effect and could be a potential source for treatment of different inflammatory diseases [41]. Phytochemical analysis of the *Alpinia calcarata* rhizomes extracted using soxhlet apparatus with different solvents revealed the presence of polyphenols, tannins, flavonoids, steroid, glycosides and alkaloids [1]. Beta alanine, isovaleric acid, catechol, citral protocatechuic acid, iso menthone, nerol umbelliferone, chrysin, apigenindi-methyl ether, vanillic acid, myristic acid, acetoxycha-vicol acetate, nerolidol, himachalol, dihydro resveratrol, confertin, valerenic acid, alpha–linolenic acid isolated from rhizomes of methanolic extract of *Alpinia calcarata* by LCMS/MS analysis [2]. Isolation of stigmasterol and β-sitosterol from the Rhizomes of *Alpinia allughas* Ros and revealed to the presence of steroids, terpenoids, alkaloids and phenolics [42].

The presented study clearly gave evidence of the bioactive quantitative of stigmasterol in methanolic extracts of *Alpinia calcarata* rhizomes. The developed HPTLC method for the quantification of above stigmasterol compounds is simple, precise, specific, sensitive, and accurate. Further, this method can be effectively used for routine quality control of herbal materials as well as formulations containing any or both of these compounds.

**CONCLUSION**

In conclusion, an HPTLC method has been developed with some modifications and it can be used for the quantitative determination of stigmasterol in methanolic extract of rhizomes of *Alpinia calcarata*; its main advantages are its simplicity, accuracy and selectivity. The average recovery values of stigmasterol were found to be about 99.94%, which showed the reliability and suitability of the method. This method could also be used for the estimation of these compounds in other herbal preparations and might be useful for standardization purposes.

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**AUTHOR CONTRIBUTION**

All authors contributed equally to this manuscript.

**CONFLICTS OF INTERESTS**

The authors declare that they have no conflict of interest. It has not been published elsewhere. That it has not been simultaneously submitted for publication elsewhere. All authors agree to the submission to the journal.

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