Simultaneous Quantification of Forskolin and Iso-Forskolin in *Coleus forskohlii* (Wild.) Briq. and Identification of Elite Chemotype, Collected from Eastern Ghats (India)

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**ABSTRACT**

**Background:** *Coleus forskohlii* is a well-known industrially important medicinal plant, for its high forskolin content. **Objective:** A simple, selective, and sensitive high-performance thin layer chromatography (HPTLC) method was developed and validated for simultaneous quantification of forskolin and iso-forskolin in *C. forskohlii* germplasm collected from the Eastern Ghats, India. **Materials and Methods:** Chromatographic separation of the targeted marker(s) was obtained on precoated silica plates using toluene: ethyl acetate: methanol (90:30:0.5, v/v/v) as the mobile phase. **Results:** Densitometric quantification of forskolin and iso-forskolin was carried out at 545 nm. Forskolin and iso-forskolin were identified by comparing the ultraviolet spectra of standard and sample track at R<sub>r</sub> of 0.64 ± 0.02 and 0.36 ± 0.01, after derivatization with anisaldehyde sulfuric acid reagent. The linearity of both the analytes was obtained in the range of 300–1200 ng/spot with the regression coefficient (R) of 0.991 and 0.986. Recovery of analyte(s) at three levels, namely, 100, 150, and 200 ng/spot was found to be 100.46% ± 0.29%, 99.84% ± 0.33%, 100.02% ± 0.78%, and 99.76% ± 0.62%, 99.56% ± 0.35%, 100.02% ± 0.22%, respectively, for forskolin and iso-forskolin. The content of forskolin and iso-forskolin varies from 0.046% to 0.187% and 0.002% to 0.077%, respectively (dry weight basis), the maximum content of both the markers was found in NBC-31, from Thakurwada, Maharashtra. **Conclusion:** The developed HPTLC method was linear, accurate, and reliable as per the International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use guidelines. The study aids in the identification of elite chemotype for commercial prospection of industrially viable medicinal crop. **Key words:** Chemotype, *Coleus forskohlii*, forskolin, high-performance thin layer chromatography, iso-forskolin

**SUMMARY**

- 12 Samples are collected from different locations of the eastern ghat regions
- Quantification of two major marker forskolin and iso-forskolin
- The maximum content of both the markers was found in NBC-31, from Thakurwada, Maharashtra

**INTRODUCTION**

*Coleus forskohlii* (Wild.) Briq. (family: Lamiaceae) is perennial species available in tropical and subtropical parts of India, Pakistan, Sri Lanka, etc. The roots have been used in various Ayurvedic formulations and cosmetic products with huge industrial potential in the near future. The roots have been used as condiments, for the preparation of pickles, and juice is used in constipation. Tribes of Trichigadi in Nilgiri, South India, consider the decoction of tuberous roots as tonic. The species is industrially demanded for its forskolin contents, which is used to treat glaucoma, cardiac problems, and also used in the treatment of certain types of cancers.

Forskolin is a diterpene which interacts with various membrane proteins including adenyl cyclase, the glucose transporter, the voltage-gated potassium channel, and ligand-gated ion channel. The major chemical constituents of *C. forskohlii* are diterpenoids, namely, dactyl forskolin, 9-deoxyforskolin, 1, 9-deoxy forskolin, 1...
9-dideoxy-7-deacetyl forskolin in addition to forskolin (7-acetoxy-8, 13-epoxy-1, 6, 9-trihydroxy labd-14-en-11-one),[6,8] alpha-amyrin, betulolin acid, alpha-cedrol, and beta-sitosterol. [9] Recently, two more new labdane diterpene glycosides, forskoditerpenosides A and B, were also isolated from the ethanolic extract of the whole plant. [10] Iso-forskolin is also known as 6-acetyl-7-deacetyl forskolin, an analog of diterpene forskolin and also present in C. forskohlii. [11,12] Iso-forskolin increased cyclic adenosine monophosphate (cAMP) level in rat liver homogenate and relaxed the histamine-induced contraction of isolated guinea pig lungs and trachea smooth muscle. [13,14] Recently, iso-forskolin was reported to activate adenylyl cyclase isoforms. [15] The variation, as well as maximum forskolin content (1.024%) among the natural germplasm in C. forskohlii, was previously reported by our group from Nilgiri Hills and Western Himalayan regions of India. [16,17] Simultaneous densitometric quantification of forskolin and iso-forskolin in the germplasm collected from the natural population (Eastern Ghat regions) of C. forskohlii is still not available to the best of our knowledge and literature survey. Thus, the major objective of the present study is to develop and validate a simple, precise, selective, and reproducible high-performance thin layer chromatography (HPTLC) method for separation and simultaneous quantification of forskolin and iso-forskolin in C. forskohlii. Further, this is essential to record the variation in the natural population of targeted species for identification of elite chemotype of C. forskohlii to promote the commercial cultivation and industrial prospection of this species.

**EXPERIMENTAL**

**Plant materials and extraction**

C. forskohlii (roots) samples were collected in the month August 2014 from different locations of Eastern Ghats, India. Totally 72 plants from 12 different populations (each 5–7 plants) were collected and a specimen is deposited in the herbarium repository of CSIR-NBRI [Table 1]. Root sample(s) were washed, chopped, shade dried, and coarsely powdered. Accurately about 2 g of powdered sample was soaked in 100% methanol (100 ml × 3 times) at room temperature (27°C ± 2°C) for 24 h. The sample was filtered and the residue was again soaked with fresh methanol. The procedure was repeated thrice. The pooled filtrate was concentrated using rotator vacuum distillation at 40°C for further use. Working solution of plant samples (1.0 mg/ml) and standard compounds, i.e., forskolin and iso-forskolin (0.1 mg/ml) were freshly prepared from stock sample on the same day for analytical work. Sample and standard compound dilution were filtered through 0.45 μm filter paper (Millipore, USA) before HPTLC injection.

**Reagents**

Toluene, ethyl acetate, methanol, and HPTLC-precoated silica gel 60 GF254 (20 × 10 cm) plate (Merck, India) were used. Forskolin (Sigma-Aldrich, France; purity 98%) and iso-forskolin (Natural Remedies, Bangalore; purity 95%) were used as standard for quantification studies. Before use, solvents were filtered and sonicated for 15 min. All other chemicals and reagents used were of analytical grade purity.

**Stock solution of standard compounds and samples**

A stock solution of samples and the marker compounds of strength 10 mg/ml and 1 mg/ml, respectively, were prepared in methanol and stored at 40°C for further use. Working solution of plant samples (1.0 mg/ml and standard compounds, i.e., forskolin and iso-forskolin (0.1 mg/ml) were freshly prepared from stock on the same day for analytical work. Sample and standard compound dilution were filtered through 0.45 μm filter paper (Millipore, USA) before HPTLC injection.

**High-performance thin layer chromatography method**

**Apparatus**

A CAMAG Linomat V automated TLC applicator, CAMAG twin trough glass chamber, and a CAMAG TLC scanner model 3 equipped with CAMAG Wincats IV software were used during the study at a temperature of (27°C ± 2°C) and at relative humidity.

**Chromatographic experiments**

Sample and standard compounds were applied on precoated silica gel 60F254 HPTLC plate with 6 mm-wide bands positioned 15 mm from the bottom and 8 mm from the side of the plate, using a CAMAG 100 μl sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (CAMAG, Switzerland) under the flow of N2 gas. These conditions were kept constant throughout the analysis of samples. The linear ascending development was carried out in the CAMAG glass twin trough chamber (20 cm × 10 cm) after saturation with the mobile phase. The mobile phase was selected using a Vario system, wherein varying ratio and polarity were tried. The mobile phase consisting of toluene: ethyl acetate: methanol (09:03:0.05 v/v/v) was optimized for quantitative study. The saturation time of the TLC chamber in the mobile phase was optimized to 10 min for a good resolution of the tested markers and the total run time was ~25 min at room temperature (27°C ± 2°C). TLC plate was developed up to a distance of 80 mm from the point of application, derivatized with anisaldehyde sulfuric acid, and dried in hot air oven at 105°C. Scanning of the TLC plate was performed using the CAMAG TLC Scanner 3 at single wavelength λmax × 545 nm in ultraviolet absorbance mode for all tracks; slit dimension was 4 mm × 0.45 mm.[18]

**Validation of the developed method**

Validation of the developed quantitative HPTLC method includes the evaluation of parameters such as specificity, linearity, sensitivity, accuracy, recovery, precision, and robustness as per the ICH guidelines (2005). [19]

Table 1: Passport data of Coleus forskohlii collected from different locations of Eastern Ghats

| Collection number | Location/district/state | Soil type | Altitude (m) | Latitude | Longitude |
|-------------------|------------------------|-----------|--------------|---------|-----------|
| NBC-26            | Parsoni/Nagpur/Maharashtra | Black soil | 310         | 21°22'32.51''N | 79°09'07.31''E |
| NBC-27            | Nanhavadi/Maharashtra   | Black soil | 268.59      | 21°04'57.64''N | 79°21'39.56''E |
| NBC-28            | Bhawer/Maharashtra      | Black soil | 267.68      | 20°18'41.47''N | 78°34'48.00''E |
| NBC-29            | Narkhed/Maharashtra     | Black soil | 416.76      | 20°18'41.47''N | 78°34'48.00''E |
| NBC-30            | Pandurana/Maharashtra   | Black soil | 417.98      | 21°16'12.00''N | 78°34'48.00''E |
| NBC-31            | Thakurwa/Katol/Maharashtra | Black soil | 635         | 18°45'21.57''N | 73°20'31.67''E |
| NBC-32            | Kurnool/Andhra Pradesh  | Red soil   | 4023        | 101°20'31.67''N| 76°40'12.53''E |
| NBC-33            | CIMAP/Bengaluru/Karnataka | Red gravel soil | 909.75    | 12°16'30.92''N | 77°35'40.43''E |
| NBC-45            | Chamundhi hills/Karnataka | Stony soil | 1024.69     | 21°07'15.74''N | 79°22'58.74''E |
| NBC-54            | Panchgani Hills/Maharashtra | Stony soil | 310         | 21°07'15.74''N | 79°22'58.74''E |
| NBC-55            | Khadak/Pune/Maharashtra | Stony soil | 1450        | 21°07'15.74''N | 79°22'58.74''E |
| NBC-56            | Khambhat Ki Ghati/Maharashtra | Stony soil | 2186        | 21°07'15.74''N | 79°22'58.74''E |
RESULTS

Calibration and quantification

Linearity was achieved with a concentration range of 300–1200 ng/spot for both standard compounds, forskolin and iso-forskolin [Figure 1], with regression coefficients ($R^2$) 0.982 and 0.973 and correlation coefficients ($r$) 0.991 and 0.986, respectively [Table 2, Supplementary Figures 1 and 2]. The result of quantitative determination of forskolin and iso-forskolin in *C. forskohlii* is presented in Figure 2. Forskolin and iso-forskolin content in germplasm varied from 0.046% to 0.187% and 0.002% to 0.077%, respectively (dry weight basis). NBC-31 from Thakurwada, Maharashtra, was found to be rich (elite) chemotype in both the markers [Figure 2].

Specificity

The specificity of the methods was determined by analyzing the bands of standard compound and samples. The bands for forskolin and iso-forskolin in sample solutions were confirmed by comparing the $R_f$ and ultraviolet spectra with the reference standards [Supplementary Figures 3 and 4]. The peak purity of these compounds was also assessed by comparing the spectra at three different levels, i.e., peak start, peak apex, and peak end positions, respectively [Figures 3 and 4].

Precision

Instrumental precision was checked by repeated scanning of the spot of forskolin and iso-forskolin each six times. The repeatability of the sample application and measurements of peak area was expressed in terms of percent relative standard deviation (%RSD). The intraday precision study was achieved at the different concentration levels of 300, 600, 900, 1200 ng/spot. Forskolin and iso-forskolin were spotted three times within 24 h and expressed in terms of %RSD. For the interday precision study, the same concentrations of 300, 600, 900, and 1200 ng/spot of forskolin and iso-forskolin were used over a period of 5 days and expressed as %RSD. To estimate the limit of detection (LOD) and the limit of quantification (LOQ), the signal-to-noise ratio was determined. The LOD was considered as 3:1 and the LOQ as 10:1. In the present study, the LODs for forskolin and iso-forskolin estimation in the samples were 1289.87 and 1296.29 (ng) and the LOQs were 3908 (ng) and 7524.45 (ng), respectively [Table 3].

Accuracy

The accuracy of the methods was determined by analyzing the percentage recoveries of forskolin and iso-forskolin in samples. To obtain it, three sets were prepared from *Coleus* species. The samples were spiked with three different concentrations: 100, 150, and 200 ng/spot [Table 4]. The spiked samples were recovered in triplicate and then analyzed by the proposed HPTLC method [Table 4].

DISCUSSION

The HPTLC method was optimized with a view to developing a precise, sensitive, and selective method for simultaneous quantification of forskolin and iso-forskolin in *C. forskohlii*. Solvent systems toluene: ethyl acetate: methanol (9:3:0, v/v/v) gave the best resolution of forskolin ($R_f$ ± standard deviation [SD]: 0.61 ± 0.02) and iso-forskolin ($R_f$ ± SD: 0.39 ± 0.01) in the presence of other compounds in the sample extract and enabled the quantification of the focused standard compounds [Figures 3 and 4]. The purity of the bands in the samples was confirmed by comparing band spectra of samples with the corresponding band spectra of standard compounds at the start, middle, and end positions of the bands [Supplementary Figures 5 and 6]. Quantification of forskolin and iso-forskolin in the extracts was performed at the maximum absorption spectrum $\lambda_{max}$ = 545 nm. The linear range for forskolin and iso-forskolin was 300–1200 ng/spot, with respective correlation coefficients of $r$ = 0.991 and 0.986, respectively. In the proposed method, the LODs and the LOQs were found to be 1289.87, 1296.29 and 3908.71, 3928.16 ng/spot for the compounds forskolin and iso-forskolin, respectively [Table 2]. This indicated that the proposed HPTLC method for simultaneous quantification of forskolin and iso-forskolin exhibits a good sensitivity.

Accuracy and intra- and inter-day precision were chosen to determine the precision of the developed HPTLC method. The accuracy of the proposed method was tested by analyzing the percentage recoveries

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**Table 2:** Statistical analyses of calibration curves in the high-performance thin layer chromatography determination of forskolin and iso-forskolin

| Parameter                  | Forskolin* | Iso-forskolin* |
|----------------------------|------------|----------------|
| Linearity range (ng/spot)  | 300-1200 ng/ml | 300-1200 ng/ml |
| $R_f$                      | 0.64       | 0.36           |
| Regression equation        | $y=11.144x+4013.9$ | $y=9.4243x+7524.5$ |
| Correlation coefficient ($R^2$) | 0.991     | 0.986          |
| Regression coefficient ($r$) | 0.9825   | 0.973          |
| Average                    | 12372      | 14592.675      |
| Slope                      | 11.1442    | 9.4243         |
| SD                         | 4354.306579 | 3700.334744  |
| SE                         | 704.6347288 | 744.8449258  |
| LOD (µg/spot)              | 1289.87    | 1296.29        |
| LOQ (µg/spot)              | 3908.71    | 3928.16        |
| Intercept                  | 4013.85    | 7524.45        |

* n=3; mean±SD. LOQ: Limit of quantification; LOD: Limit of detection; SD: Standard deviation; SE: Standard error

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**Figure 2:** Quantification of forskolin and iso-forskolin in collected 12 germplasms of *Coleus forskohlii*
of forskolin and iso-forskolin in samples. To obtain it, three sets of *C. forskohlii* samples were prepared. The samples were spiked with three different concentrations and spiked samples were recovered in triplicate and then analyzed by the proposed HPTLC method.

Quantification of forskolin and iso-forskolin in the roots of *C. forskohlii* revealed that there is a significant difference in the content of metabolite among the populations. The highest content of forskolin and iso-forskolin was found in the Thakurwada, Maharashtra, population of 0.18% and 0.077%, respectively. Hence, the study suggests the variation in the content of forskolin and iso-forskolin among the populations which can be due to the change in the phytogeographical factors, soil nature, and microflora of the regions. Our results have shown that the proposed HPTLC method for simultaneous quantification of forskolin and iso-forskolin in *C. forskohlii* is simple, reproducible, specific, precise, and accurate. In addition, the developed HPTLC method was validated for linearity, LOD and LOQ, and accuracy, and was applied to the analysis of both forskolin and iso-forskolin in respective samples. The developed HPTLC method can be applied for simultaneous quantification of forskolin and iso-forskolin.

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**Table 3:** Data of intra- and inter-day precision study

| Concentration (ng/spot) | Amount of standard | Forskolin |  |  |  | Iso-forskolin |  |  |
|-------------------------|-------------------|----------|---|---|---|----------------|---|---|
|                         |                   | Intra-day|  |  |  | Inter-day |  |  |
|                         |                   | %RSD     | Mean RSD | %RSD | Mean RSD | %RSD | Mean RSD | %RSD | Mean RSD |
| 300                     |                   | 1.033    | 0.738 | 0.782 | 0.599 | 0.131 | 0.427 | 0.489 | 0.510 |
| 600                     |                   | 0.529    | 0.457 | 0.641 | 0.387 | 0.692 | 0.574 | 0.624 | 0.592 |
| 900                     |                   | 0.525    | 0.516 | 0.285 | 0.574 | 0.692 | 0.574 | 0.624 | 0.592 |
| 1200                    |                   | 0.942    | 0.641 | 0.489 | 0.510 | 0.692 | 0.574 | 0.624 | 0.592 |

RSD: Relative standard deviation (*n=3*)

**Table 4:** Recovery studies of forskolin and iso-forskolin at 100, 150, and 200 ng addition by the proposed thin layer chromatography densitometric method

| Sample | Amount of forskolin present in sample (ng) | Amount of forskolin added (ng) | Theoretical added value (µg) | Amount of forskolin analyzed (µg) | Recovery (%) | Average recovery | Amount of iso-forskolin present in sample (µg) | Amount of iso-forskolin added (µg) | Theoretical added value (µg) | Amount of iso-forskolin analyzed (µg) | Recovery (%) | Average recovery |
|--------|--------------------------------------------|-------------------------------|-----------------------------|----------------------------------|--------------|-----------------|-----------------------------------------------|-----------------------------------|-----------------------------|----------------------------------|--------------|-----------------|
| NBC-26 | 55.78                                      | 100                           | 155.78                      | 159.15                           | 102.16       | 100.46          | 112.66                                        | 111.89                            | 99.31                       | 99.76                             | 100.25       |                  |
|        | 55.46                                      | 150                           | 205.46                      | 204.22                           | 99.39        | 13.02           | 163.02                                        | 163.44                            | 100.25                     |                              |                          |                  |
|        | 55.52                                      | 200                           | 255.52                      | 255.05                           | 99.83        | 12.82           | 212.82                                        | 212.26                            | 99.73                       |                              |                          |                  |
| NBC-27 | 46.37                                      | 100                           | 146.37                      | 145.18                           | 99.73        | 11.40           | 111.40                                        | 100.76                            | 99.73                       |                              |                          |                  |
|        | 45.84                                      | 150                           | 195.84                      | 195.33                           | 99.73        | 11.89           | 161.89                                        | 160.66                            | 99.24                      |                              |                          |                  |
|        | 46.36                                      | 200                           | 245.36                      | 245.45                           | 100.03       | 12.01           | 212.01                                        | 212.28                            | 100.12                     |                              |                          |                  |
| NBC-28 | 130.78                                     | 100                           | 230.78                      | 231.23                           | 100.19       | 100.02          | 159.38                                        | 159.66                            | 100.17                     |                              |                          |                  |
|        | 130.03                                     | 150                           | 280.03                      | 279.26                           | 99.72        | 60.22           | 210.22                                        | 209.88                            | 99.83                      |                              |                          |                  |
|        | 130.34                                     | 200                           | 330.34                      | 330.85                           | 100.15       | 59.55           | 259.55                                        | 259.78                            | 100.08                     |                              |                          |                  |
CONCLUSION

C. forskohlii is an industrially important medicinal plant and is the only natural source of forskolin and its derivative diterpenoids (s). A linear, accurate, reproducible, and precise method was developed for simultaneous quantification of forskolin and iso-forskolin markers in this species for intraspecies variation in phytogeographical region of Eastern Ghats (India). Germplasm of Thakurwada, Maharashtra, was identified as elite germplasm, having the highest content of both the targeted marker(s). This study is relevant for the identification of elite chemotype and authentication of quality raw materials; in addition, this also promotes the cultivation of identified elite chemotype(s) of C. forskohlii to fulfill the commercial demand of these bioactive diterpenoids (forskolin and iso-forskolin) in the pharmaceutical industry.

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Conflicts of interest

There are no conflicts of interest.

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