SUMMARY AND CONCLUSION

Every plant produces a vast number of metabolites; which are classified accordingly as primary and secondary metabolites. Primary metabolites are the essential part of the plant’s biochemical system, which gives rise to secondary metabolites as an end product of metabolism and helps in defence system. The bioactive nature of secondary metabolites attracts the scientist’s attention to define their medicinal value. So, study of the metabolite profile of plants bearing medicinal value is gaining interest.

*Coleus forskohlii* Briq., (Family- Lamiaceae) is also an important medicinal plant of Ayurvedic, Unani and other traditional systems of medicine. The plant is distributed in India, Pakistan, Sri Lanka, Brazil and tropical East Asia. Medicinal preparations of *Coleus species* have long been used in Hindu and Ayurvedic traditional systems of medicine particularly in the treatment of epilepsy, conjunctivitis, abdominal colic etc. (*Evans 2009*).

*C. forskohlii* contains forskolin, which has been demonstrated to possess hypotensive, spasmolytic, cardiotonic and antiplatelet activity. Because of its unique ability to stimulate adenylate cyclase, it has been considered as a promising drug for the treatment of glaucoma, congestive cardiomyopathy and asthma (*Vishwakarma et al., 1988*). Other diterpene and terpenoids like deacetylforskolin, 1,9-deoxyforskolin, 9-deoxyforskolin and 1,9-dideoxy-7-deacetylforskolin have been reported in the roots of coleus. The secondary metabolites have a great structural diversity and classified occurrence. Each plant species has its unique group of secondary metabolites, which generally reveal high intra-specific variation (*Hartmann 1996*). Thus, studies related to metabolite and molecular profiles of coleus, with \textit{in vitro} production of forskolin were designed.

For present investigation, coleus samples were collected from different geographical regions of Indian subcontinent (*Table 15*). Total ten samples were collected, authenticated and voucher specimens were deposited (voucher specimen number JH/BNPL/CF1-CF10/2010), in BNPL.

Coleus samples were standardized according to Pharmacopoeial guidelines, various physicochemical parameters determined and results are presented in *Table 17*. Value of FOM is found in between 0.13% to 0.42%. Extractive values were determined for
all the samples using solvent alcohol and water, which ranges 19.24% to 23.96% for alcohol soluble extractives and 19.75% to 23.86% for water soluble extractives. The total ash value for coleus samples was found in the range of 4.83% to 8.43% while, acid-insoluble ash for coleus ranges 1.48% to 2.8%. All samples pass the limits as per the Indian pharmacopoeia. Powder microscopic evaluation was carried out for all coleus samples. The prototype of microscopic characters of powdered drug are summarized and presented as Fig.9-12. Powder microscopic characteristics mainly embrace starch grains, periderm cells, fibres, vessels and vessel elements.

For phytochemical evaluation of herbal drugs, TLC is being employed extensively as it enables rapid analysis of herbal extracts with minimum sample clean-up requirement and it provides qualitative and semi-quantitative information of the resolved compounds. Hence, fingerprint profiles of various extracts of C. forskohlii roots were developed. In order to develop fingerprints roots of coleus were thoroughly extracted using methanol; to protect the integrity of phytochemicals, extracts were concentrated under reduced pressure using rota vapour and stored in refrigerator (4°C) in well closed container.

The solvent system was optimized for HPTLC procedure keeping an aim of qualitative (fingerprinting) and quantitative estimation of marker constituents present in herbal extracts and formulations. Mobile phase composed of toluene : methanol, 18:1.5 (v/v) leads to separation of more than 12 substances with good resolution (Fig.13) for fingerprinting which have compact and well resolved spot and of active marker forskolin at Rf 0.21 ± 0.02. Good resolved spots were obtained when the chamber was saturated with mobile phase for 15 min at 24°C and 50% relative humidity. The TLC chromatograms of different samples (Fig.15 to 24) and peak data (Table 18) obtained after the scanning of developed plate confirms separation of forskolin along with 15 more substances. The samples collected from Salem, Bengaluru, Coimbatore, Ahmedabad and Sangli were found metabolically similar and containing all the 16 substances. Similarly, very little variations were observed in the content of each substance (in relation to area percentage) among different samples as obtained by HPTLC fingerprinting. These detailed fingerprint profiles generated with chemical markers can be used for the quality control (Xie et al., 2006).

Multiple components in herbal medicines can be simultaneously separated by using various chromatographic techniques like high performance thin layer chromatography
(HPTLC) (Chen et al., 2006), high performance liquid chromatography (HPLC) (Yao et al., 2011), gas chromatography (GC) (Lu et al., 2006), and capillary electrophoresis (CE) (Sun et al., 2003). However, use of chromatographic fingerprint in analysis of metabolite diversity, similar to RAPD in molecular markers is uncommon and can be easily done by developing multiple fingerprints and analyzing them for determination of metabolic similarity in between species or among samples of same species collected from different regions. Hence, TLC fingerprints of chloroform extract of coleus samples which were collected from different places were developed using various solvent systems to access their similarity/diversity. For proposed analysis; other than toluene: methanol (18:1.5, v/v) two more solvent system were developed for separation of different components of chloroform extract with greater resolution and compact spots. Chloroform: methanol (9:0.2, v/v) and toluene: ethyl acetate: formic acid (9:2:0.2, v/v/v) were other two solvent system in which fingerprints were developed.

Total 15 substances were detected (Table 19) in chloroform extract by using toluene: methanol (18:1.5, v/v), 13 substances (Table 20) in chloroform: methanol (9:0.2, v/v) and 17 substances (Table 21) were separated using toluene: ethyl acetate: formic acid (9:2:0.2, v/v/v) as solvent system.

From the dendrogram (Fig. 65) and similarity matrix (Table 24) constructed from chromatographic fingerprinting data, it is clear that all sample divided in two main cluster each with five sample. Sample C1, C8, C2, C9 and C10 stands in one cluster while; sample apart from these holds another cluster. Since, division of samples in two main cluster and further divided in 3 subclusters each. Samples collected from Belgum (C4) and Satara (C7) recorded highest similarity co-efficient 0.939 in similarity matrix for their metabolites whereas, sample C3 and C10 implies 0.683 in similarity matrix which is minimum among any two sample.

Chromatographic fingerprinting of petroleum ether extract leads to detection of 16 substances along with forskolin (Fig. 66). Data obtained after scanning the derivatized plate is summarized in Table 25. Substance with reproducible mark and detection are documented along with their particular Rf.

Extraction of essential oil using hydro distillation method may leads to either loss of certain volatile components or alteration of heat labile constituents. Earlier analysis
were based on extraction of essential oil by hydro distillation and further chemo-profiling using GC-MS but, in present investigation GC-MS analysis using head space auto sampler for profiling of highly volatile and heat labile constituents was carried out for the first time. Head space assisted GC-MS analysis of volatile constituents present in *C. forskohlii* samples belonging to ten different locations of Indian subcontinent led to the identification of more than 35 constituents. The identified constituents with their respective percentages, *R*<sub>t</sub> and CAS number are summarised in Table 26. The major constituents present were α-pinene (5.4 - 33.9%), camphene (8.0 - 26.7%), β-pinene (3.5 - 21.8%), bornyl acetate (3.4 - 17.6%), decanal (2.1 - 20.6%), isobornyl acetate (0.5 - 55.9%), *l*-phellandrene (0.3 - 5.2%) and benzoic acid, methyl ester (0.1 - 6.8) in all samples.

No chromatographic method has been reported so far to study the effect of geographical conditions on the quality of *C. forskohlii*. Hence in present study, accordingly repeatable methods has been developed for the chromatographic fingerprinting (*Wagner et al., 2009*) and the quantitative estimation of forskolin as well. The methods for quantitative estimation were validated according to the ICH guidelines and made functional for the estimation of forskolin content in roots of *C. forskohlii* using HPTLC and HPLC.

The regression data obtained from HPTLC analysis calibration curves (n=3) were found to be linear over a wide concentration range of 50–500 ng/spot with respect to the peak area (Table 27). Similarly, in HPLC analysis calibration was found to be linear from 50–1000 µg/mL with respect to peak area.

The HPTLC method was found specific on comparison of the spectra of forskolin at *R*<sub>f</sub> 0.21 ± 0.02 in standard and samples (super-imposed, Fig.92). While HPLC method was also found specific on comparison of the *R*<sub>t</sub> of the peak and peak shape at peak start, peak apex and, at peak end. The proposed methods were used for extraction and subsequent estimation of forskolin from pre-analysed samples after spiking with 50, 100 and 150% of standard forskolin, which demonstrated good recovery (Table 28 and 34). The repeatability of sample application and measurement of peak area were expressed in terms of % RSD at three different concentration levels for HPTLC 100, 200 and 300 ng/spot were selected, whereas 100, 250 and 500 µg/mL for HPLC method. The low % RSD values (≤ 2%) obtained indicated precision of the method. Similarly, the low values of % RSD were obtained after introducing small changes in
temperature, relative humidity and mobile phase composition for HPTLC, whereas column temperature, mobile phase composition and detection wavelength for HPLC method indicated robustness of the method (Table 31 and 37). The LOD of proposed HPTLC method (S/N 3:1) was found 13.01 ng/spot, whereas LOQ (S/N 10:1) 39.03 ng/spot. Similarly, LOD and LOQ for HPLC method were found 12.2 μg/mL and 36.6 μg/mL, respectively.

Forskolin content was found highest in sample collected from Bengaluru which recorded 0.22% and 0.227% w/w of forskolin by HPTLC and HPLC, respectively. Forskolin content in other samples were also determined by both techniques however, there was no considerable difference among the results obtained by these two techniques. Comparison of forskolin content determined by two different methods has been summarized and presented graphically in Fig.105.

There is much variation in forskolin content among the collected coleus samples; as samples were obtained from different provinces which may be a major reason behind this variation. Other than geographical and climatic conditions there are several factors which govern the accumulation of secondary metabolites in medicinal plants. Hence, it is necessary to evaluate the crude drug sample for its key metabolites content.

In context with quality control attribute of herbal drugs, chromatographic fingerprint is one of the method of choice; besides chromatographic fingerprints, molecular fingerprints has been developed by various scientists which can act as supporting tool in authentication and to find out adulteration in various herbs. Application of molecular techniques would increase quality control attributes, can facilitate plant conservation (Pirttilä et al., 2001) as well as prevent biological piracy or adulteration/substitution with lower grade herbs. Furthermore, techniques such as amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) are useful in studying plant diversity, genetic transformation and clonal fidelity determination of micropropagated plants (Moyo et al., 2008).

High medicinal value of C. forskohlii and its increasing demand directs studies regarding variability in genetic profile among the samples collected from different places in India, which may lead to an important database for authentication as well as determination of polymorphism.
Modification of a DNA isolation protocol or blend of two or more different procedures to obtain DNA of the desired quality is quite often (Varma et al., 2007). Most of the protocols suggest use of fresh and highly growing plant part usually young leaves, which are often devoid of secondary metabolites and contain high amount of nucleic acid. Present investigation was carried out on secondary metabolite rich coleus roots (dried) hence; isolation of DNA from such metabolite loaded sample was very complicated. Modified CTAB buffer (Table 6 and Method 4.7.2) was used in current isolation of genomic DNA. Developed protocol yields DNA of high purity and free from polyphenols and polysaccharides from dry mature roots. The level of DNA purity was determined by the ratio of absorbance obtained at 260/280nm. The ratio of OD of 260/280 in the range of 1.7-1.9, indicates high purity DNA.

PCR conditions were optimized for proper amplification of DNA. Several factors were taken into consideration like Mg$^{++}$ concentration, dNTPs concentration in reaction mixture, primer amount and genomic DNA for reaction mixture. Concentration of Mg$^{++}$ was finalised to 2.5 mM, dNTPs was 250 µM in total reaction mixture of 15 µL and concentration of primer was optimized to minimum 10 pmol as starting material for reaction. However, 30 ng of genomic DNA was found appropriate for PCR amplification and 1 µL Taq DNA polymerase was found suitable for 15 µL of total master mix for PCR.

Among 20 primers, 5 generated reproducible, unique, monomorphic and polymorphic fragments (Fig.107-111; Table 41) in PCR amplification using the genomic DNA extracted from roots of coleus. Results obtained from RAPD analysis of ten samples of the genus Coleus, revealed that the number of fragments amplified for each primer varied between 7 and 11 fragments and their size ranged from 290 bps - 2800 bps. The total number of band for all five primers was 48, the total number of monomorphic bands was 5, the total number of polymorphic bands was 43, and the polymorphism percentage was 89.58%. The characterization of the fragments generated by the array of the five primers is surveyed and summarized in Table 41 which revealed that the most effective primer was OPA-14 which produced 11 bands. The primers gave the following ratios of polymorphic bands 80% for OPA-A, 90% for OPA-2, 85.17% for OPA-4, 90% for OPA-8 and 100 % for OPA-14. The highest ratio of polymorphism was 100% (OPA-14) while the lowest ratio was 80% (OPA-1). All the five primers used in the present study produced high degree of polymorphism.
Results for similarity in terms of genetic nature lies in between 0.588-0.783 but lower limit of similarity in metabolic profile was 0.683, this confirms unity in the chemical nature of collected samples of coleus. So there was a disparity among the molecular fingerprints and chromatographic fingerprints. Thus it can be assumed that, climatic condition of different regions affected the molecular make-up but plant species tries to produce similar kind of metabolites instead of different conditions.

Since, coleus is an important medicinal plant which is gaining peoples interest because of forskolin, it was necessary to carry out studies on its alternative source remedy for *in vitro* production. Present report for *in vitro* production of forskolin has been summarized in three main topics as development of callus culture effect of elicitors/precursors and suspension culture.

Initiation of callus was found in cultures supplemented with NAA + 6-BA (1.0 ppm each), IBA + 6-BA (2.0 ppm each). During the maintenance callus culture supplemented with NAA + 6-BA (1.0 ppm each) showed best growth compared to culture grown on IBA + 6-BA (2.0 ppm each). Although previous reports on coleus callus culture by Sharma et al 1991 suggested White media for callus growth and maintenance but in present study MS media found to be significant in maintaining the growing ability of callus.

It was found that hormonal combination IAA + Kinetin + 6-BA (1.0 ppm each), IAA + IBA (1.0 ppm each) + 6-BA (2.0 ppm) and IAA + NAA (1.0 ppm each) + Kinetin (0.5 ppm) were found best for shooting. Present study also reported induction of roots using IAA (1.0 ppm). It was further observed that growth of shoots was poor in case of hormonal combination IAA + Kinetin + 6-BA (1.0 ppm each), while cultures supplemented with IAA + IBA (1.0 ppm each) + 6-BA (2.0 ppm) and IAA + NAA (1.0 ppm each) + Kinetin (0.5 ppm) were growing vigorously, while later hormonal combination was also prone to induce rooting in shoots. Culture supplemented with IAA + NAA (1.0 ppm each) + Kinetin (0.5 ppm) were vigorously rooted in further transfers and developed into plantlet (*Fig.122*), successfully. Hence, present study suggested a new, rapid, and economic protocol can be used for micropropagation of coleus.

Number of shoots was found maximum in case of IAA + IBA (1.0 ppm each) + 6-BA (2.0 ppm), around 17 shoots were formed per culture during the maintenance.
Maximum shoot length was observed as 7.07 cm in culture nourished on MS media supplemented with IAA + NAA (1.0 ppm each) + Kinetin (0.5 ppm) which records highest number of leaves as well. However, shoots initiated on MS + IAA + NAA (1.0 ppm each) + Kinetin (0.5 ppm) were self rooted in same media during further maintenance on same media.

The presence of forskolin in in vitro cultures was confirmed by TLC and hence quantitative estimation in calli obtained from leaves was done using CAMAG system consisting of Linomat 5 spotting device and Scanner 3. Results of assay of forskolin (Table 54) revealed that callus obtained from MS media supplemented with NAA + 6-BA (1.0 ppm each) produce higher amount of forskolin compared to callus developed and maintained of MS + IBA + 6-BA (2.0 ppm each) hence, it was selected for further studies in relation to elicitation/precursor.

Elicitation is one of the best approaches for enhancing the production of secondary metabolites from medicinal plants and it has been shown that elicitors affect plant secondary metabolism by modulating the rates of biosynthesis or accumulation of constituents.

Studies regarding alteration in media component (sucrose) levels reveal higher concentration of sucrose favours growth of calli. From kinetic study it is certain that mass of cells also increases with increase in sucrose concentration. Growth rate of cultures supplemented with 1 and 2% sucrose were found to be minimal, although the higher amount of sucrose favours growth but cultures supplemented with 4 and 5% sucrose were having similar growth rate. Higher concentration of sucrose also increases the mass of calli. It might be due to rich carbon supply in the exponential growth phase of calli.

Inference drawn from the observations regarding effect of MJ on callus culture can be summarized as at lower concentration MJ do not affect growth rate or morphology of callus however at higher concentration (100 µM) it stimulates growth rate as well as greenish brown calli turned yellowish brown in colour. TDZ is having property to mimic both auxin and cytokinin like effects on growth and differentiation of cultured explants. In present study, lower concentration TDZ did not showed any significant effect on callus morphology or growth rate however, at higher concentration (2.27 and 4.45 µM), organogenesis like response was observed in fewer cultures. Chitosan at
lower concentration showed positive effect on callus growth. The cultures treated with chitosan adversely get affected as concentrations rose from 10 to 100 mg/L this may be due to increased membrane permeability of cell leads to cell death and phenolic oxidation.

Forskolin content of various samples (Table 63) was analyzed by previous HPTLC method, which showed sucrose concentration treatment was found beneficial for production of biomass as well as forskolin as 5 % sucrose supplementation leads to production of 670 mg/Kg forskolin. However, callus maintained on 3% and 4% sucrose yields 593.33 and 650 mg/Kg forskolin, respectively similar to the report of Tripathi et al 1995 regarding highest growth rate and forskolin production in callus culture supplemented with higher percentage of sucrose. Maximum forskolin yield was obtained in callus maintained on MS + NAA + 6-BA (1.0 ppm each) + 50 µM MJ which contain 826.67 mg/Kg forskolin at 84 days. Treatment with TDZ leads to production 680 mg/Kg forskolin, which was higher than the yield of callus supplemented with only NAA and 6-BA (Control); while, CHT treatment leads to formation 573.33 mg/Kg forskolin.

Hence, it can be concluded that in callus grown on MS + NAA + 6-BA (1 ppm each) supplemented with 5 % sucrose, 2.27 µM TDZ, 50 mg/L CHT and 50 µM MJ may produce good amount of forskolin.

Callus supplemented with NAA+ 6-BA (1 ppm each) was used for the development of suspension cultures. The suspension culture was maintained on MS + NAA+ 6-BA (1.0 ppm each) with optimal treatment of 5 % sucrose, 2.27 µM TDZ, 50 mg/L CHT, and 50 µM MJ as well as on MS + NAA+ 6-BA (1.0 ppm each) which served as control. The quantitative estimation of forskolin in suspension culture of C. forskohlii revealed that treatment of cultures favours the production of forskolin. Suspension culture gives rise to significant results as suspension without elicitation treatment produces 626.67 mg/Kg forskolin, while suspension with treatment managed to produce forskolin up to 740.00 mg/Kg calculated on dry weight basis.

The present study was especially designed for in vitro production of forskolin but we observed presence of significant amount of rosmarinic acid in same cultures hence, the samples were also analysed for the content of rosmarinic acid. As sucrose concentration was key factor for production of biomass and metabolites, rosmarinic acid content was also found increased in cultures supplemented with higher
concentration of sucrose which was in the range of 10.17 to 14.97 % w/w. However, results obtained on assay of rosmarinic acid after CHT treatment of \textit{in vitro} cultures were showed increase in concentration as compared to control but slightly lesser effect in comparison to sucrose. Forskolin content in cultures supplemented with MJ were highest, but rosmarinic acid content was highest in cultures supplemented with TDZ. The content of rosmarinic acid was in the range of 16.83 to 20.81 % w/w in cultures supplemented with different concentration of MJ.

Maximum rosmarinic acid yield was obtained in callus maintained on MS + NAA +6-BA (1 ppm each) + TDZ 4.45 µM which contain 23.58% w/w of rosmarinic acid. Suspension culture control and with combined elicitation produces 12.7 %w/w and 19.68 %w/w of rosamarinic acid, respectively. Nevertheless, the rosmarinic acid content obtained was approximately 8 - 10 folds higher than the natural leaves of \textit{C. forskohlii}. Hence, it can be concluded that the present protocol could act as pioneer in simultaneous \textit{in vitro} production of forskolin as well as rosmarinic acid.
CONCLUSION

- Present study on *C. forskohlii* is the first report regarding development of chromatographic and molecular fingerprints of the 10 different samples of coleus obtained from different provinces of India.
- A new, fast, accurate, simple, economic, and validated HPTLC and HPLC methods for quantitative estimation of forskolin add credits to this report.
- GC-MS fingerprints of volatile constituents using headspace were also developed and reported for the first time in present study.
- Fast extraction procedure for the qualitative estimation (chromatographic fingerprints) was also suggested as a part of Q.C. analysis.
- Genomic DNA isolation from the dried and mature roots of coleus has been reported for the first time.
- Comparative assessment of polymorphism based on RAPD data and chromatographic fingerprints data was carried out for the first time, which revealed genetic diversity among coleus is much higher as compared to metabolic diversity.
- The present study also report successful establishment of in vitro cultures for production of forskolin.
- Callus cultures developed on MS media supplemented with NAA + 6-BA (1.0 ppm each) were found best for in vitro production of forskolin.
- Present study also suggest protocol for successful micro-propagation of coleus plants based on MS + IAA + IBA (1.0 ppm each) + 6-BA (2.0 ppm) and MS + IAA + NAA (1.0 ppm each) + Kinetin (0.5 ppm).
- Further studies regarding alteration in media, elicitation, and precursor feeding reveal that in callus grown on MS + NAA +6-BA (1 ppm each) supplemented with 5 % sucrose, 2.27 μM TDZ, 50 mg/L CHT and 50 μM MJ may produce good amount of forskolin, have been reported for first time, in respect to the secondary metabolite production.
- The suspension culture were maintained on MS + NAA+ 6-BA (1.0 ppm each) with optimal treatment of sucrose, TDZ, CHT, and MJ revealed that treatment of cultures favours the production of forskolin.
- Simultaneous presence of rosmarinic acid along with forskolin was reported in all developed cultures for first time and quantitative estimation results indicates
cultures produces approximately 8 - 10 folds higher rosmarinic acid than the natural leaves of *C. forskohlii*.

- However, present investigation is preliminary study and it requires extensive research work to be carried out in order to increase the biomass in cultured condition and also an increase in the production forskolin. Thus a more elaborate work is required based metabolomics in respect to proteomics to understand mechanism involved in forskolin production. However, metabolomics in respect to genomics for better correlation between different accessions for the production of secondary metabolites.