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IDENTIFICATION, QUANTIFICATION AND ANTIOXIDANT ACTIVITY OF SECONDARY METABOLITES IN LEAF AND CALLUS EXTRACTS OF *COLEUS FORSKOHLII*

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ABSTRACT

Though sixteen combinations of NAA + BAP, and NAA + KN, used in the study, induced callus from leaves of *Coleus forskohlii* with a variable response, 0.5mg / l NAA + 1.5mg / l BAP was the most efficient (98.8 ± 1.1). Evaluation of hydro-alcoholic, methanolic and aqueous extracts of leaves and call us for antioxidant activity by DPPH and TBARS methods proved that hydro-alcoholic extracts were more efficient compared to others. Extracts of leaves and callus respectively exhibited IC_{50} of 63.3µg/ml and 58.5µg/ml by DPPH; while only the callus extract exhibited 60µg/ml by TBARS. Proximity of callus IC_{50} of 58µg/ml to inhibition concentration indicates higher antioxidant activity compared to leaf extract. Quantification of polyphenols by HPLC exhibited three-fold increase in rosmarinic acid in callus (3.86mg/g), than leaves (1.25mg/g). Higher antioxidant activity of callus, due to rosmarinic acid, directs for further investigation into anti-inflammatory, analgesic and anti- depressant activity.

KEYWORDS: ***Coleus forskohlii,*** **Callus, HPLC, DPPH, TBARS, Rosmarinic acid**

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INTRODUCTION

Coleus forskohlii, an ancient medicinal plant of mint family, lamiaceae, variously called as Pashan bhedi, Pather chur, Makandi, Mayini, False boldo, Coleonol etc. In India, it is cultivated in Tamil Nadu, Gujarat and Karnataka for the purpose of drugs and in Thailand and parts of South-East Asia as a spice. It is mostly used in ayurvedic medicine in the treatment of heart ailments, asthma, bronchitis, insomnia, epilepsy, anaemia, inflammation and improvement of appetite facilitating digestion. Kotas, native tribals of Trichigadi in Nilgiris, treat their children suffering from constipation by feeding its root juice and the south Indians consider the decoction of tubers as tonic. Field reports from the Kumaon Himalayas suggested the use of fresh half cooked leaves as galactogogue. Leaves were found to be amoebicidal against trophozoites of *Entamoeba histolytica* and cures stomach problems[1]. Qualitative analysis of this plant showed the presence of terpenoids, tannins, flavonoids, phlobatannins, saponins and cardiac glycosides[2]. Rosmarinic acid, an ester of Caffeic acid and 3, 4-dihydroxyphenyllactic acid, a natural compound has been reported to have a multitude of biological activities, including anti-oxidative, anti-microbial and anti-inflammatory effects. The antioxidant property of rosmarinic acid protects against oxidants and free radical injuries in addition to its medicinal properties. Its activity, especially against rheumatic and inflammatory conditions makes it a sought-after substance for use in phytotherapy[3]. Rosmarinic acid was reported to have anti-HIV activities which make it a valuable product for the pharmaceutical and cosmetic industries[4].

The accumulation of rosmarinic acid was first reported in plant cell cultures of *Coleus blumei*[5] while forskolin was reported in MAFF 03-07124, a strain of *Agrobacterium rhigogenes,* in the hairy root cultures of *Coleus forskohlii*, while enhancement in the content of rosmarinic acid was seen in these cultures when elicitors like Yeast extract (YE), Salicylic acid (SA) and Methyl Jasmonate (MJA) were used[6]. Production and bio synthetic regulation of rosmarinic acid have been studied in root cultures of *Salvia officinalis*[7], suspension cells of *Lithospermum* erythrorrhizon[8], *Ocimum basilicum*[9], and *Heliotropium peruvianum*[10]. Presence of rosmarinic acid in medicinal plants, herbs and spices has beneficial and health promoting effects on human beings, readily accumulated in undifferentiated plant cell cultures and in some cases its concentrations are much higher than in the plant itself [11]. Although, antioxidant properties of *Coleus forskohlii* leaves have already been reported[12], studies on the phenolic compounds, including rosmarinic acid, have not been properly documented till now in the callus cultures. Therefore, an attempt has been made for the first time, to compare the antioxidant properties by DPPH and Lipid peroxidation between leaf and callus cultures, besides identification and quantification of phenolic compounds of *Coleus forskohlii* through HPLC.

MATERIALS AND METHODS

(i) Authentication of plant material

Coleus forskohlii plants were raised at the Plant Genetics Experimental Farm, Department of Genetics, Osmania University, from the cuttings obtained from ANGRAU Herbal Gardens, Hyderabad, A. P., India. Herbarium of this plant was authenticated by a Taxonomist, bearing Voucher specimen No. 0786 *C. forskohlii* (wild) briq, the Dept. of Botany, Osmania University, has been preserved. The dried leaf powder of *Coleus forskohlii* was qualitatively analyzed and standardized as per the guidelines contained in the WHO monographs (1998).

(ii) Induction of callus

Leaf segments (1.0-1.5cm) of *Coleus forskohlii* were inoculated onto M.S. basal medium[13], supplemented with different concentrations and combinations of auxin: 0.5 and 1.0 mg/l of α - Naphthalene acetic acid (NAA) with cytokinins: 1.0 and 1.5mg/l of Kinetin (KN) and also with

1.0, 1.5, 2.0 and 2.5mg/l of Benzyl amino purine (BAP), along with agar (w/v) before autoclaving at 15lbs at 121º C for 20 min and poured into the borosil culture tubes. The tubes were inoculated with leaf explants and incubated at 25 ± 1ºC under 16/8h photoperiod with cool white fluorescent lights for a period of 30-35 days for callusing. The induced callus was sub-cultured on the fresh callus medium after 35 days. The yield of callus was recorded after 30 days of subculture.

(iii) Chemicals

NAA, BAP and KN (Hi Media); H_2O_2, TCA, TBA, $FeSo_4$, HCl and NaCl of analytical grade; whereas 1, 1-Diphenyl -2-picrylhydrazyl (DPPH) and Ascorbic acid from Sigma Aldrich were used in the present study. The standards: Quercetin-3-O-glucoside, Chlorogenic acid, Caffeic acid, Coumaric acid and Myrecitin from Extra Synthase, while Rosmarinic acid procured from Cayman chemicals (U.S.A) were used in HPLC analysis of polyphenols. The solvents used for extraction and quantification of phenolics were of HPLC grade.

(iv) Extraction

(i) Aqueous extraction

Fresh leaves obtained from the field-grown plants and callus induced from these leaves were shade-dried for 7 days and powdered using a mechanical grinder[14]. Separately, 25grams of leaf powder and 2.5grams of callus powder were taken into conical flasks containing 200ml of Millipore water and kept in the dark for soaking for 72hrs. Every 24hrs, the soaked material was filtered through the muslin cloth to collect the extract. This procedure was repeated for three days by adding fresh water. The extracts collected every 24hrs, on three occasions, were pooled and subjected to lyophilization. Extract i.e. lyophilized powder was weighed to get the % of yield and stored at -20°C till it was subjected for analysis.

(ii) Methanolic extraction

Leaf and callus powders, weighing 25 and 2.5grams respectively, were taken separately into two conical flasks containing 200 ml of methanol (L R Grade), allowed soaking in dark for 72hrs. Every 24hrs, soaked material was filtered through the muslin cloth to collect the extract. This procedure was repeated for three days by adding fresh methanol. The extracts collected every 24hrs, on three occasions, were pooled and subjected to Rotavapor (Buchi). The extract was weighed to get the % of yield and stored at -20°C till subjected for analysis.

(iii) Hydroalcoholic extraction

Leaf and callus powders, weighing 25 and 2.5grams, respectively, were taken separately into two conical flasks containing 200 ml of Millipore water and Ethanol (L R Grade) in 1:1 ratio was allowed to soak in the dark for 72hrs. Every 24hrs, soaked material was filtered through muslin cloth to collect the extract. This procedure was repeated for three days by adding fresh water and ethanol. Extracts collected every 24hrs, on three occasions, were pooled and subjected to Rotavapor. The extract was weighed to get the % of yield and stored at -20°C till subjected for analysis.

(v) Anti-oxidant activity

Leaf and callus extracts were evaluated for their anti-oxidant activity by DPPH and TBARS methods.

(i) DPPH free radical scavenging assay

Antioxidant activity of leaf and callus extracts was studied by free radical scavenging assay (DPPH) method. Free radical scavenging activity of these extracts was assessed by the de-coloration of DPPH solution in ethanol[15]. Initially, 0.2ml of these extracts were mixed, separately, with Tris HCl buffer (pH 7.4) to make up to 1ml to which 1ml DPPH (500M in ethanol) was added. This was repeated by increasing 0.1ml of extracts while decreasing 0.1ml of buffer and making the volume up to 1ml to which 1ml of DPPH was added. These solutions in tubes were wrapped up with dark paper to avoid the effect of light and were allowed to incubate for 20min. in the dark. The absorbance of the resulting solutions was recorded at 517nm on a UV-visible spectrophotometer. The degree of de-coloration

of DPPH solution, which is the reflection of free radical scavenging detected at 517nm, indicates the efficiency of antioxidant activity of the extract. All the samples were tested in triplicates with ascorbic acid as a positive control. The potential antioxidant activity of extract was compared with standard, the ascorbic acid, by calculating 50% inhibitory concentration (IC_{50}) values.

$$\text{Percentage inhibition } (IC_{50}) = \left[\frac{\text{O.D. of Control} - \text{O.D. of Sample}}{\text{O.D. of Control}}\right] \times 100$$

Where O.D. of control is the absorbance of DPPH solution at 0 min and O.D. of sample is the absorbance of test samples, the leaf and callus extracts, after 20 min.

(ii) Lipid peroxidation assay

Lipid peroxidation inhibitory activity of leaf and callus extracts was studied on the lipoprotein of mitochondria isolated from the homogenized rat liver (from the Sprague Dawley female rat meant for culling at the NCLAS of NIN, CPCEA registered institute) and the protein component of isolated lipoprotein was estimated[16]. To this, an inhibitor containing a mixture of 200 mM $FeSO_4$, 100 nM H_2O_2 and 0.15 M NaCl (pH 7.0 with 0.1M HCl) was added. Later, different concentrations of an inducer, $Fe^{+2}H_2O_2$, was added to the sample, two min before incubation for 60 min at 37°C. Peroxidation was terminated by adding 20% trichloro acetic acid (0.5ml/ml) and thiobarbituric acid (1ml/ml) to the incubated mixture. Lipid peroxidation induced by the peroxidation activity of extracts on $Fe^{+2}H_2O_2$ was determined from the amount of malonaldehyde formation in the system by thiobarbituric acid reactive substances (TBARS) as per the method of Vitamin-E (α-Tocopherol) was used as standard and this experiment was repeated thrice.

(vi) Determination of total phenolic content (TPC)

Total poly-phenolic content of extracts, from each variation, was determined using Folin-Ciocalteu reagent and Gallic acid as standard. Aliquots of 20, 40, 60, 80, 100, and 120µl were taken out from a quantity of 1mg /10ml of leaf and callus extracts and each of the aliquot solution was made up to a volume of 500µl with distilled water. To each of these solutions, 3.5ml of 10% Folin-Ciocalteau reagent was added. After 2min, to each of this mixture, 2.5ml of 30% Na_2CO_3 (15g/200ml) was added. The extracts, after thorough mixing, were kept in water bath at 50°C for 15min, and later the absorbance was recorded at 760nm. Results were expressed as Gallic acid equivalents (mg GAE / L)[17].

(vii) Quantification of poly-phenols by HPLC

Quantification and identification of polyphenols viz., Rosmarinic acid, Quercetin-3-O-glucoside, Chlorogenic acid, Caffeic acid, Coumaric acid and Myrecitin, in the leaf and callus extracts was carried out by loading these extracts with 20µl loop injector into the stainless steel columns (25 cm X 4.6 mm) packed with Octadecylsilane bonded to porous silica (C18) (5µm) of HPLC by using their respective standards. Mobile phases A: Methanol (10%) and B: Methanol (70%) in Na_2HPO_4 buffers with pH 3.3 was used at a flow rate of 1ml/min.[18]. Quantification was made on the basis of corresponding peak area recorded by chromatopac c-R6A (Shimadzu). Reference standards were used for the preparation of standard curves.

RESULTS AND DISCUSSION

(i) Induction of callus

Callus is an unorganized mass of tissue, grown on the solid substrate medium, from different explants of plants. The results indicated that combination of 0.5mg /l NAA + l.5mg/l BAP was found to be the most effective and efficient (98.8 ± 1.1%) in inducing callus (Table 1). It was reported in some medicinal plants that the hormonal combinations of NAA with BAP;

0.5mg/l NAA with 3.0 mg/l BAP in *Solanum nigrum*[18] and 3.0 mg/l NAA with 1.0mg/l BAP in *Artemesia vulgaris*[19] were found to be more efficient in inducing callus.

(ii) Antioxidant activity

(i) DPPH free radical scavenging activity

The DPPH method has been widely used to evaluate the free radical scavenging ability of antioxidants. During DPPH test, the change of color of reaction mixture from purple to yellow with decreased absorbance at a wavelength of 517nm determines the antiradical potential of antioxidants. The IC_{50} is the concentration of extract required to quench 50% DPPH under the chosen experimental conditions. Antioxidant activity of leaf extracts of some medicinal plants was reported earlier[20]. The IC_{50} values of hydroalcoholic extracts of leaves (63.33µg) and callus (58.53µg) were found to be more potent compared to the IC_{50} values of methanolic extracts of leaf (81.25µg) and callus (73.29µg), while the IC_{50} value of Vitamin-C, the standard Ascorbic acid, was found to be 5.54 µg. However, aqueous extracts of either the leaf or callus did not exhibit any activity (Table 2). The antioxidant activity of callus extract was found to be more potent than that of the leaf extract, probably due to higher concentration of rosmarinic acid in callus extract. The hyper antioxidant activity observed in hydroalcoholic extract of callus (58.53%) could be due to more accumulation of active principles, the phenolic compounds, with one or more hydroxyl groups, like rosmarinic acid in the callus extracts. The antioxidative property of the phenolics has been predicted mainly due to their redox potential[21] and might be the major contributors to the antioxidant activities of these extracts[22]. Almost similar IC_{50} values of both the hydroalcoholic extract of callus indicates that they are potent antioxidants which may be due to the presence of higher content of rosmarinic acid and other phenolic compounds in callus extracts. These antioxidative constituents present in *Coleus forskohlii* may be responsible for free radical scavenging activity. The data revealed that the leaf and callus extracts of *Coleus forskohlii* act as free radical inhibitors and thus as primary antioxidants that react with free radicals[23] may be due to the addition of sugars, phosphates, nitrates and calcium[24]. The presence of sucrose concentration in the M.S. medium mainly influences the production of secondary metabolites in phenylpropanoid pathway. The antioxidant activity of leaf and callus extracts as expressed by DPPH was due to their H^+ atom donating ability to peroxy radicals, thus inhibiting the oxidation of fatty acid chain termination[25].

(ii) Lipid per-oxidation (TBARS) method

Neither leaf nor the callus extracts of all the three solvents, could inhibit formation of 50% TBARS. To substantiate this point, a quantity of 60µg of hydroalcoholic extract of callus could only inhibit ~48% of TBARS formation. In order to determine the capability of extracts in reducing oxidative stress, production of TBARS in hydroalcoholic extracts showed minimum activity on lipid peroxidation. The quantity of malonaldehyde produced, as a byproduct, by $Fe^{+2}H_2O_2$ during peroxidation was measured by the levels of TBARS produced. The IC_{50} value of α-tocopherol, the standard, was found to be 76.03 µg. (Figure 1).

(iii) Quantification of total phenolic content

Quantification of total phenolics of leaf and callus extracts of *Coleus forskohlii* was found to contain almost similar contents of 4.83mg/ml and 4.90mg/ml respectively (Table – 3). However, it was found that *Stevia rebaudiana* callus contains significantly higher total phenolic compounds (35.86mg/g) than its leaves (25.18mg/g) on dry weight basis[26]. As callus growing in a nutrient-rich culture medium is exposed to more carbon influx than the field-grown plant parts, it may influence the metabolic flux for the biosynthesis of elevated levels of phenolics [27]. Similar reports with highest antioxidant activity and high phenolic content was reported in the extracts of medicinal plants [28].

(iv) Separation and quantification of polyphenols by HPLC

HPLC analysis of leaf powder revealed the presence of rosmarinic acid, quercetin-3-O-glucoside, chlorogenic acid, caffeic acid, coumaric acid and myrecitin, while the callus exhibited the presence of only the former four components. The presence of phenolic compounds; chlorogenic acid, caffeic acid, syrengic acid, coumaric acid, ferulic acid, myrecitin and quercetin in the leaves of *Coleus forskohlii* has been documented previously[29] but the presence of these compounds in callus has not yet been reported. Hence, for the first time in the present investigation, the rosmarinic acid along with quercetin-3-O-glucoside, chlorogenic acid and caffeic acid were identified and quantified. The accumulation of rosmarinic acid in callus extracts accounts for >60% of the total phenolic content (Table 3). In this study, the quantification of individual polyphenols in the leaf and callus extracts in comparison with the retention time, and the UV spectra of authentic standards were calculated on the basis of peak area of each compound, through HPLC (Figure 2 - 4). Further, the yields, nature of compounds and materials from extracts are strongly dependent on the solvents used due to the presence of different concentrations of bioactive compounds with different polarities. The concentration of each polyphenol was found to be higher in the leaves except the rosmarinic acid and chlorogenic acid confirming that the biosynthesis of polyphenols is accelerated by light exposure and serves as a filtration mechanism against UV-B radiation[30.] The concentration of rosmarinic acid in callus (3.86mg/g) was found to be 3-fold higher than that of the leaves (1.25mg/g). Similar results of higher accumulation of rosmarinic acid were reported earlier in callus cultures of *Ocimum basillicum*[31]. The combination of NAA with BAP used as growth regulators in combination with nutrients may influence the rosmarinic acid synthesis in callus cultures of *Coleus forskohlii*. Thus it appears that *Coleus forskohlii* contains powerful antioxidants both in leaves and callus.

Table 1
Induction of callus in Coleus forskohlii

NAA+BAP (mg/l)	% of callus response
0.5 + 1.0	83.3± 2.3
0.5 + 1.5	98.8 ± 1.1*
0.5 + 2.0	82.1 ± 0.9
0.5 + 2.5	79.1± 1.9
1.0 + 1.0	42.3± 2.1
1.0 + 1.5	64.5 ± 3.2
1.0 + 2.0	44.0 ± 3.5
1.0 + 2.5	48.6 ± 2.5
NAA + KN (mg/l)	
0.5 + 1.0	23.0 ± 2.0
0.5 + 1.5	33.6 ± 2.7
0.5 + 2.0	36.5 ± 1.8
0.5 + 2.5	42.5 ± 2.0
1.0 + 1.0	52.6 ± 1.7
1.0 + 1.5	45.0 ± 1.2
1.0 + 2.0	74.1 ± 1.3
1.0 + 2.5	87.5 ± 1.8

Values are given as mean ± SD (n=6)

Table - 2
Scavenging of free radicals by leaf and callus extracts of Coleus forskohlii

S.No.	Extract Conc. (µg)	% inhibition by methanolic extract		% inhibition by Hydroalcoholic extract	
		Leaf	Callus	Leaf	Callus
1	10	7.24 ± 0.00	2.34 ± 0.55	8.85±1.00	9.80 ± 1.44
2	20	14.73 ± 0.00	6.86 ± 1.00	13.03±0.80	19.73±0.99
3	30	23.32 ±1.02	15.48 ± 0.66	20.27±1.06	30.81±1.90
4	40	25.72 ± 1.72	23.76 ± 0.59	28.12±2.27	35.71±0.99
5	**50**	33.62 ± 0.84	32.27 ± 2.25	38.37±0.96	**51.58 ±1.00***
6	60	39.50 ± 1.11	41.13 ± 1.19	51.53±2.43	53.94 ±0.12
7	70	43.70 ± 0.99	48.37 ±1.56	58.29±2.03	57.64 ±1.23
8	80	49.88 ± 1.41	54.13 ± 0.63	62.02±1.07	65.20 ± 0.46
9	90	54.00 ± 1.30	63.79 ± 2.13	67.28±1.45	75.40 ±1.78
10	100	57.74 ± 1.36	65.10 ±1.38	81.46±3.62	77.66 ± 0.63

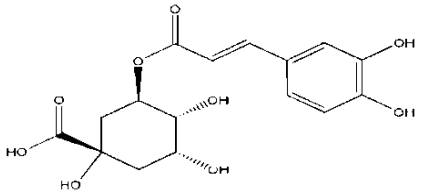
Values are expressed in mean ± SD, (n=5)

Table - 3
Content of polyphenols in the extracts of Coleus forskohlii

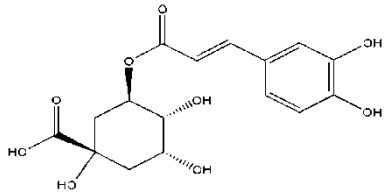
Extract	Total Polyphenols	Rosmarinic acid	Chlorogenic acid	Caffeic acid	Coumaric acid	Myricetin	Quercetin-3-O-glucoside
Leaf	4.83 ± 0.01	1.25 ± 0.08	0.15 ± 0.005	0.16± 0.02	0.03±0.01	0.04±0.01	0.24 ± 0.02
Callus	4.90 ± 0.02	3.86 ± 1.19	0.17 ± 0.015	0.11± 0.01	0.29 ± 0.02	----	----

Data represented as mean ± SD of each of the three replicates (n = 3) at a concentration of mg/g.

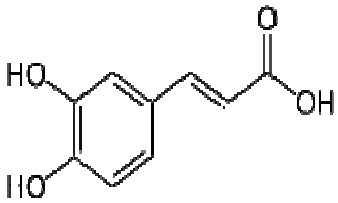
Structure of polyphenols



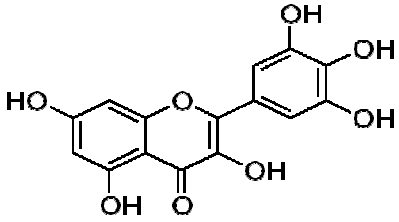
Rosmarinic acid



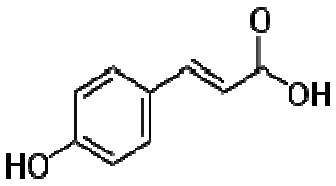
Chlorogenic acid

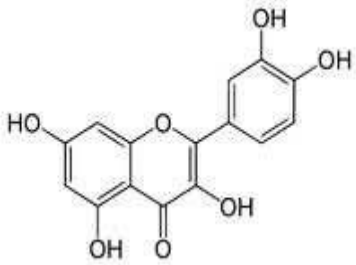


Caffeic acid

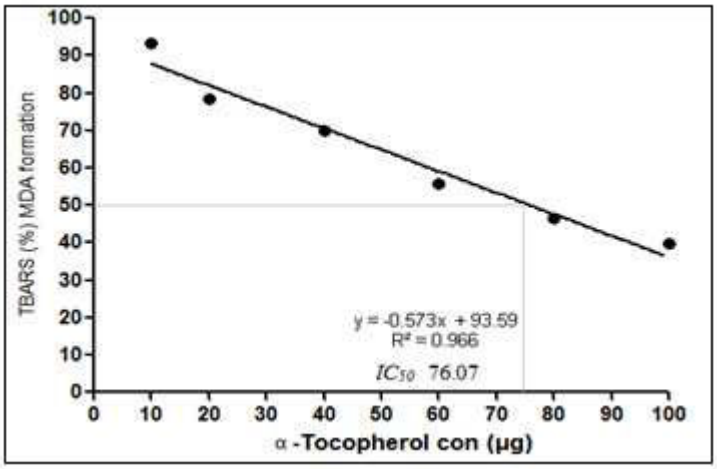


Coumaric acid





Myrecetin ***Quercetin – 3- O- glucoside***



Values are expressed as mean ± SD, n=5

Figure 1

Linearity expression using % inhibition and concentration of Antioxidant activity of α–Tocopherol as exhibited by lipid peroxidation method

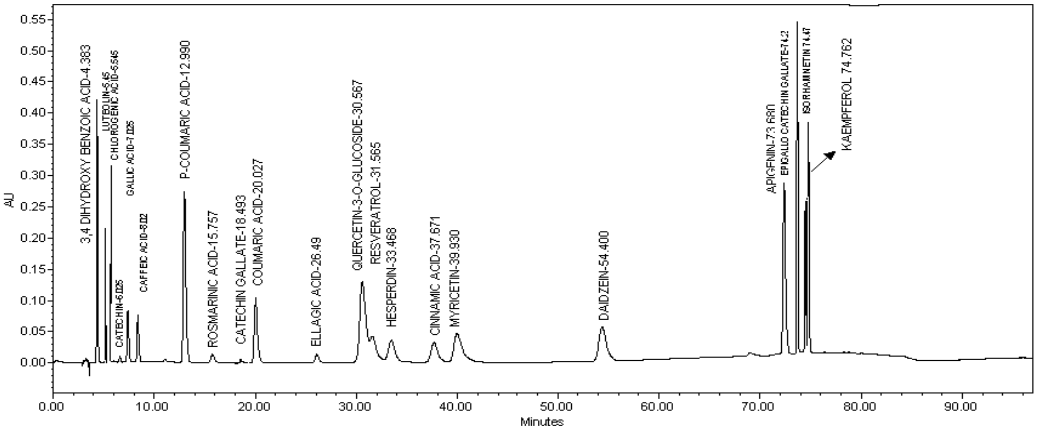


Figure 2

RP –HPLC profiles of retention times of standard polyphenols using PDA detector

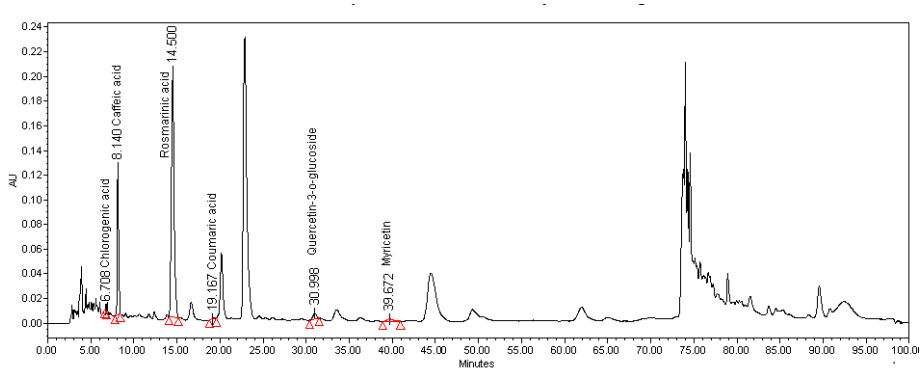


Figure 3
RP –HPLC identification and quantification of polyphenols in leaf extracts of Coleus forskohlii

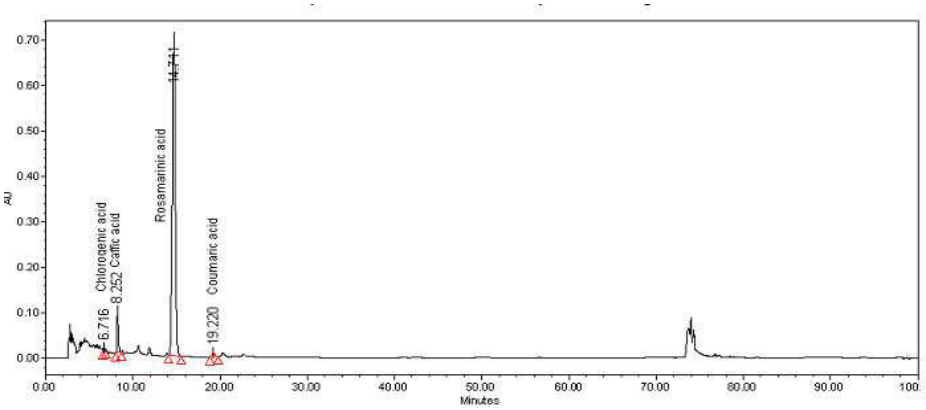


Figure 4
RP –HPLC identification and quantification of polyphenols in callus extracts of Coleus forskohlii

CONCLUSION

The wide medicinal properties of *Coleus forskohlii* may be due to the presence of different polyphenols, particularly the higher content of rosmarinic acid, and their antioxidant properties. In the present investigation, identification and quantification, through HPLC, the higher content of rosmarinic acid to the extent of more than 3-fold was achieved in the callus extracts. The study also exhibited antioxidant activity of extracts of *Coleus forskohlii* by both the DPPH and lipid peroxidation methods. The data obtained in the present investigation clearly demonstrates that *Coleus forskohlii* can be exploited in future for medicinal purposes by manipulating an increase in the content of various polyphenols, especially the rosmarinic acid through *in vitro* callus cultures. As callus can be grown indefinitely *in vitro* at different concentrations, the conditions for its growth can be easily regulated and hence, the method developed in this study has greater potential for producing rosmarinic acid in larger quantities on an industrial scale.

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