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Development of *In-vitro* root and micro tuber for the production of forskolin in *Coleus forskohlii*

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Abstract
Roots, tuberous roots and micro-tubers were successfully produced from tissue culture derived explants of *Coleus forskohlii* for forskolin production in *in vitro* condition. MS medium (Murashige and Skoog 1962) supplemented with IAA, NAA and different concentrations of sucrose were used. Effects of IAA, NAA derivatives were compared for growth and forskolin production in cultured roots on different explants with MS Media under different culture condition. IAA, NAA derivatives induced synthesis of forskolin shows the probability for scaling up of the production of this compound in untransformed root culture. The maximum forskolin content (0.014%) was observed in IAA 1.0+NAA 1.0 mg/l combination after 45 days of *in vitro* root culture. Micro- tuber and tuberous roots were formed after 60 days at the base of the shoots on the solid MS Medium with different Sucrose concentration. The number of tuberous root was varied from 12 ± 0.20 to 16 ± 0.32/shoots and the number of micro-tubers was varied from 04 ± 0.24 to 11 ± 0.26/shoots. The weight of the tuberous roots and micro-tuber varied from 3.2 ± 0.22 to 4.5 ± 0.34 g/shoots and 1.2 ± 0.22 to 5.1 ± 0.28 g/shoots respectively. Optimum concentrations of Sucrose were most effective in tuberous roots and micro-tuber formation. The maximum micro-tubers (11 ± 0.26/shoots) and forskolin content (0.06%) was observed in IAA 1mg with 70% sucrose concentration. This amount of forskolin content was nearly equal to that of wild plant.

Keywords: *Coleus forskohlii*, tuberous root, micro-tubers, forskolin

Introduction
Coleus forskohlii Briq., belongs to the family Lamiaceae is an ancient, perennial aromatic herb with fasciculate tubers. Numerous ethno medicinal uses of the tuberous roots and leaves of *C.forskohlii* for human as well as veterinary diseases are noticed in the ancient literature. (Bhat *et al*., 1977) [3] and Paulus (1979) isolated a pharmacologically active compound of labdane diterpene from this plant named as forskolin.The tuberous roots are found to be a rich source of forskolin, is a diterpenoid that activates of Cyclic Adenosine Monophosphate (CMP) in the cells. The tuberous root-derived drug is conventionally used in the treatment of heart diseases, abdominal colic, respiratory disorders, insomnia, convulsions, asthma, bronchitis, intestinal disorders, burning sensation, constipation, epilepsy, and angina (Ammon and Müller 1985) [2]. Forskolin not only enhances burning of fat but also inhibits accumulation and storage of fats (Okuda *et al.,* 1992) [11]. Forskolin may also be able to regulate insulin secretion (Yajima *et al*., 1999). Current findings on the pharmacological activity of forskolin have been widely reviewed (Alasbahi and Melzig 2010; Kavitha *et al.,* 2010) [1, 6], because *C.forskohlii* is the only known source of forskolin, it has been seriously collected from its natural habitation and it is now listed as endangered (Sharma *et al*., 1991). While the present requirements for forskolin are being met through large-scale cultivation, the herbal industry is still facing difficulties because cultivated material shows deviations in forskolin content, which ranges from 0.1% to 0.44% (Vishwakarma *et al.,* 1988) [15]. The emerging demand for forskolin as an effective therapeutic agent and the difficulties related with finding reliable yields has led to the assessment of various biotechnological tools for sustainable production of forskolin. In several cases, biosynthesis of secondary metabolites is differentiation dependent (Yeoman and Yeoman 1996) [18] optimization of the culture medium and growth conditions is important for sustainable production of secondary metabolites (Chan *et al.,* 2010) [5]. In this present study, the effect of plant growth regulator and sucrose concentration on plant morphogenesis, synthesis and quantification of forskolin content in *in vitro* roots and micro-tuber production of *C.forskohlii*.

Materials and methods
Explant, media and culture condition
Explants, culture conditions, *in vitro* root and micro-tuber induction young leaves, node and inter nodal explants were collected from the grown field of *C.forskohlii.* The explant sterilization procedure already described in Chapter 4.2.2..All the explant inoculated on MS Medium supplemented with growth regulators in individually and combination of Auxins and Cytokines (0.1-3.0 mg/l IAA, NAA and kinetin) with 3% sucrose concentrations. Cultured explants were incubated at 25 ± 2°C under 8-16 h dark photoperiods and sub-cultured intervals 2-4 weeks. After two weeks, the developed shoots were transferred to the different sucrose concentrations containing medium (3-8%) for micro-tuber induction. The numbers of *In vitro* roots, micro-tubers and tuberous roots are developed on the shoots; their fresh and dry weight of the *In vitro* roots and micro-tubers were calculated.

Extraction, Identification and estimation of Forskolin
1g dried powder was obtained from *in vitro* culture of roots and micro-tubers and stirred with 10ml Acetonitrile for 2hrs. After, the supernatant was pipetted out into volumetric flask. This procedure was repeated twice (total 3 extractions per sample), and the samples were then diluted to the final volume with acetonitrile, finally the sample was filtered

through 0.45 µm nylon membrane filter. (Brian T *et al*, 2003) and identified the forskolin compound by GC-MS analysis and estimation of forskolin by HPLC method.

Identification of forskolin by LC-MS analysis

Apparatus

LC/mass spectrometry (MS) system.—(Waters Symmetry) mass spectrometer and Applied Bio System LI 3200Q PRAP LC with AS3000 autos ampler, P4000 pump, and UV6000LP detector, using an XTerra RP18 column, 75x 4.6 mm, 5 µm particle size (Waters) at ambient temperature. The mobile phase consisted of water+0.1% formic acid (A) and acetonitrile +0.1% formic acid (B). At a flow rate of 0.5 ml/min, the gradient elution was 0 min 50A/50B, 4min 0A/100B, 7min 0A/100B, 7.5min 50A/50B to 10 min. The injection volume was 5µL. Best results were obtained in positive electro spray ionization (ESI) mode, with ionization voltage set to 25V, source voltage to 5.5 kV, and probe temperature to 450ºC. (Brian T *et al*., 2003) [4]. The LC/MS experiment was performed to confirm the identity of the peak of interest. The flow rate was modified to 0.5 ml/min to allow better detection of the compound.

Estimation of Forskolin by HPLC analysis

Apparatus

The HPLC- 2998 Photodiode Array (PDA) Detector (Waters system) with C-18 column has been used for the experiment. The mobile phase consisted of water and acetonitrile (50/50) and a flow rate of 0.5 ml/min with gradient elution was 0min to 20 min and absorbance is 210nm.

Results and Discussion

***In-vitro* root culture**

The individual and combination effects of IAA and NAA supplements in MS medium was studied on of excised roots of *C.forskohlii* for its growth. The roots and shoots were developed on MS medium with combination of Auxin and Cytokines (IAA 1.0 and KIN 0.1 mg/l) (Fig 1a). The results were observed after two subcultures. The numerous roots were developed from leaf and inter nodal explants in Auxins (IAA and NAA) combination. The maximum numbers of roots were observed in IAA 1mg/l and NAA 0.5mg/l in the nodal explants (Fig 1b) and IAA 1.0mg/l in the leaf explants (Fig 1e). At equal concentrations of Auxin combination (IAA 1mg/l and NAA 1mg/l) growth of the roots was vigorously developed after 30 days of culture and later formed knotted, mass of primary roots as well as secondary roots with laterals (Fig 1f).

IAA is found to be an important and strong hormonal activity of root induction. The fresh and dry weight of mass root culture was calculated and estimate the forskolin compound.

Table 1: Effect of sucrose concentration in *in vitro* tuberous root and micro-tubers formation on MS Medium supplemented with IAA – 1.0 mg/l

Sucrose Concentration (%)	Morphogenesis of roots	No. of Tuberous roots / Micro-tubers/Shoots	Total Fresh weight of tuberous root and tubers (g)/shoots	Total Dry weight of tuberous root and tubers (g)/shoots)
3.0	NR	-	-	-
4.0	NR	-	-	-
5.0	TR	12 ± 0.20^{b}	3.2 ± 0.22^{b}	0.8 ± 0.22^{b}
6.0	TR	16 ± 0.32^{a}	4.5 ± 0.34^{a}	1.1 ± 0.30^{a}
7.0	MT	11 ± 0.26^{a}	5.1 ± 0.28^{a}	1.2 ± 0.28^{a}
8.0	MT	08 ± 0.28^{b}	2.8 ± 0.20^{b}	0.7 ± 0.20^{b}
9.0	MT	04 ± 0.24^{c}	1.2 ± 0.22^{c}	0.3 ± 0.32^{c}

Means within a column followed by the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5 % level. NR- Normal roots, TR-tuberous roots, MT-Micro-tubers

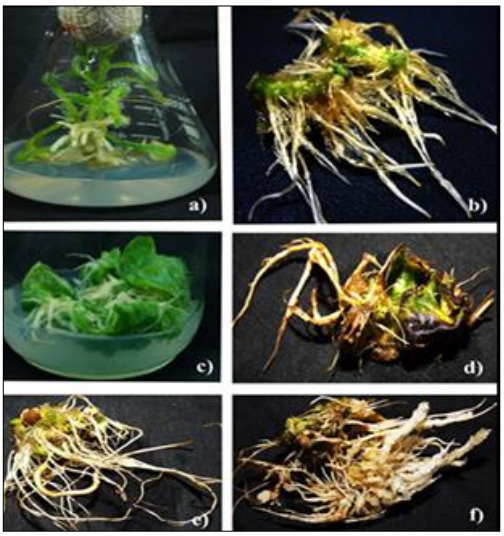

Fig 1: Effect of Auxin and sucrose concentrations in *in vitro* root formation of *C.forskohlii* a, Nodal explants grown in IAA 1.0+Kin 0.1mg/l showing shoots and root development. b ,Inter nodal explants grown in IAA 1.0 + NAA 0.5 mg/l showing root development. c,d ,The leaf explant sub cultured in IAA 1.0 mg/l showing root development. e, The leaf explant grown in IAA 1.0mg/l showing root development in mass. f ,The inter nodal explant in IAA 1.0 +NAA 1.0mg/l Mass of root not development

Effect of MS Media, Sucrose for *In vitro* micro-tuber production

The present study clearly has demonstrated the use of Auxins alone or in combination with MS media for rooting of *C.forskohlii*. The effects of Auxins with different concentration sucrose were studied for *in vitro* tuberous root and micro-tuber production in *C.forskohlii.* IAA 1.0 mg/l supplemented with six various concentrations of sucrose 3-8% was attempted. Among the tried concentrations, sucrose 5 to 6% induced tuberous root on the similar periods 7 to 8% sucrose concentration developed micro-tubers in the shoots after 60 days. Microtuberization was shown only in shoot cultures of *C.forskohlii* in 5 to 8% sucrose containing medium. The maximum number of tuberous roots (16 ± 0.32) formation in 6% of sucrose (Fig 2-c,d) and micro-tubers (11 ± 0.26) developed in 7% sucrose concentration (Fig 2 e,f). Based on the average fresh weight and dry weight of tuberous roots and micro-tubers the yield were calculated (Table 1). The increase the concentration of sucrose above 8% the number of tuberous root and tubers formation got decreased. Comparing the sucrose was higher than others, which that sucrose was beneficial to the formation of tuberous root and tuber formation *In vitro* culture condition. It influences of forskolin accumulation and increased levels of forskolin. Tuberous roots and micro-tubers weight yield was higher than other normal roots and thus could yield increased forskolin and can be used its production. This is the first report to producing tuberous roots and micro-tuber formation of *C.forskohlii*. The effect of sucrose and hormone major component of MS media on the induction of tuberous root and tubers in *in vitro* and the actual role of major element needs further research. In a similar manner to the phyto-hormones, sucrose has been shown to play a key role in the *in vitro* tuberous root and tuber formation of *C.forskohli*

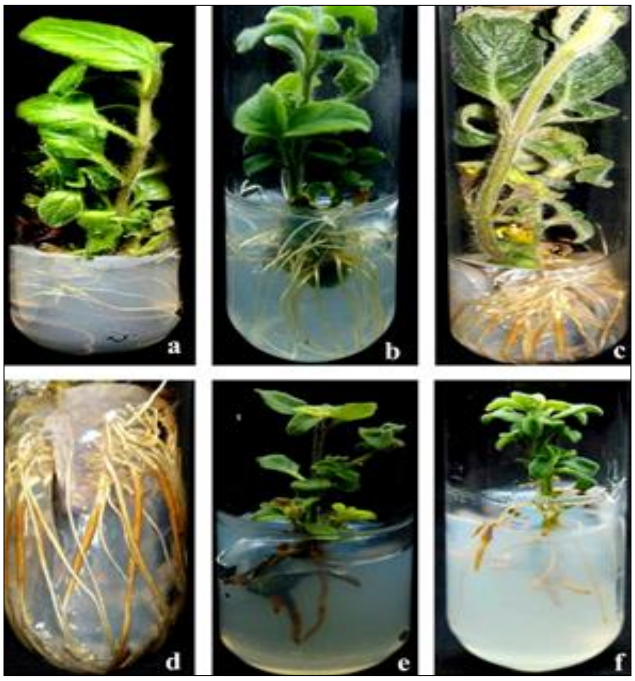

Fig 2: Effect of Auxins and sucrose concentration in *in vitro* micro-tuber and tuberous root Formation a, b- Normal root formation from the shoot in 3-4% sucrose with IAA 1.0 mg/l after 15-20 days c, d- Tuberous roots formation in 5-6% sucrose with IAA 1.0mg/l after 45-60 days e, f–Micro tubers formation in 6-8% sucrose with IAA 1.0mg/l after 45-60 days

As a carbon source (sucrose), could not only provide for synthesis of new cell compounds, but also played a significant role in regulating the water absorption of plantlets in tissue culture (Du *et al.,* 2009). Definitely, higher sucrose concentration in growth media was shown to stimulate bulb induction (Zel *et al*., 1997) or potato like tuberization (Prat, 2010) and development of tubers was delayed on media containing lower sucrose concentration in dissimilarity to media with 3% sucrose as already observed by Lawrence and Barker (1963) on potato. Results of this experiment revealed that formation of tuberous root and tuber in medium containing 5-8% sucrose developed well but, tuberous roots and tubers could not develop well in the medium either with low and high concentration of sucrose. Possibly, low concentration of sucrose could not provide sufficient carbon and osmotic pressure for tuberous roots and tuber induction, and while the concentration sucrose should be increased; it was difficult for plantlet to absorb water due to the decrease of medium water potential or the available water in the medium, which was wouldn't' conduct the water and growth in turn photosynthesis. Therefore consequently, the formation of tuberous root and tuber affected by both lower and higher concentrations of sucrose concentrations in the medium.

Forskolin confirmation by LC-MS Analysis

The LC/MS experiment was performed to confirm the identity of the peak of interest. The flow rate was modified to 0.5 ml/min to allow better resolution of the compound. Although the retention time was 4.7 min, the UV spectra matched with the standard peak of interest in the LC and LC/MS methods. In positive ESI mode, the spectrum of forskolin showed signals at m/z 428.2 $[M+NH_4]^+$ and 411.1$[M+H]^+$. Forskolin compound confirmation was done by LC-MS method compare to standard forskolin peak. All the samples exhibited same Retention time (RT) indicating the presence of compound in all the samples. (Retention Time 4.7 min - Fig. 3 - 5.)

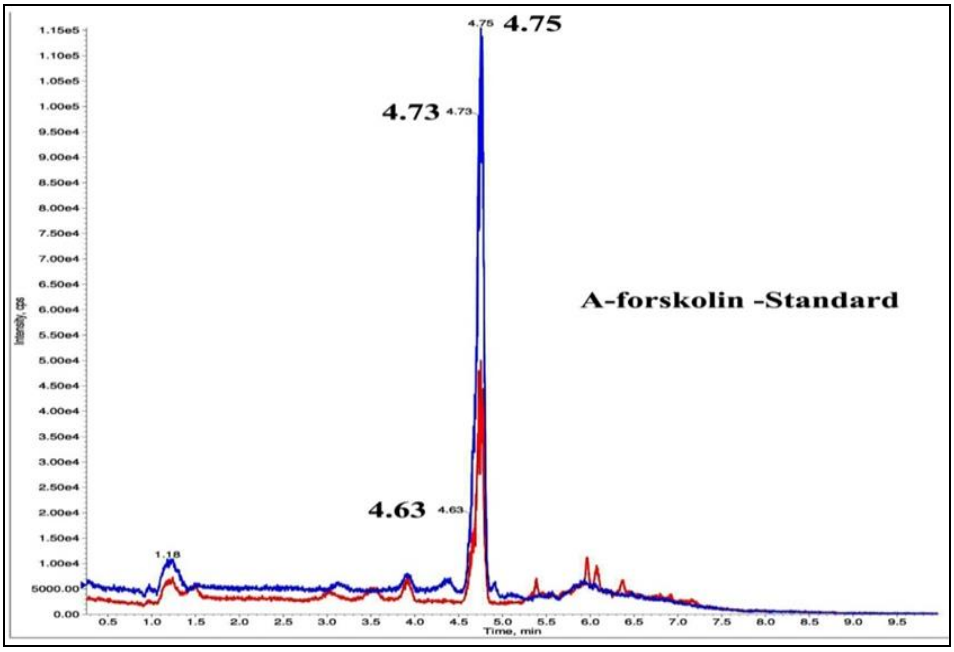

Fig 3: LC-MS analysis of Standard Forskolin

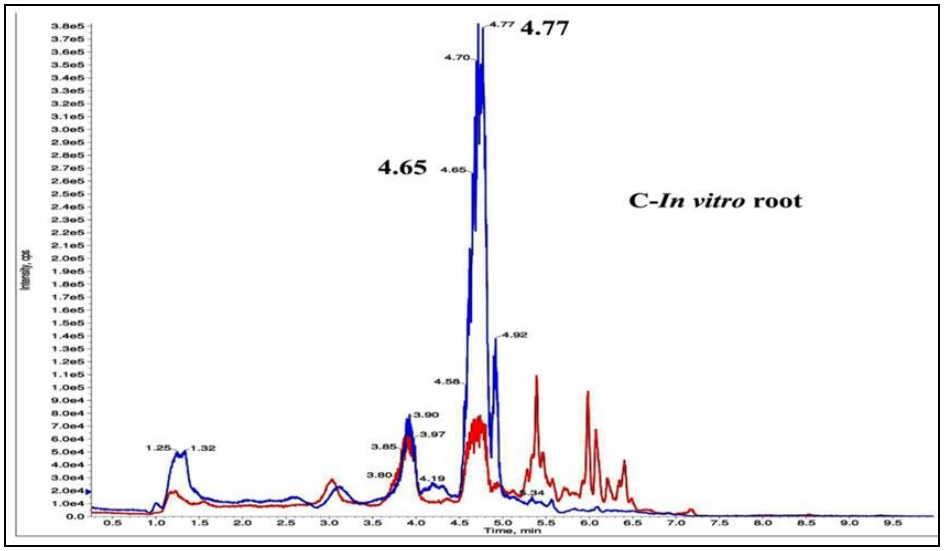

Fig 4: LC-MS analysis of Forskolin in *in vitro* root of *C.forskohlii*

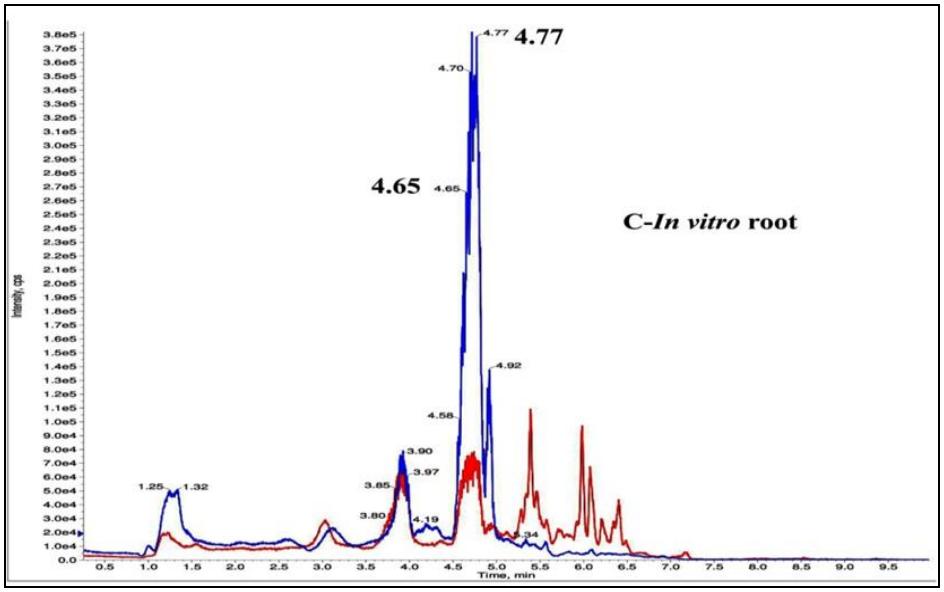

Fig 5: LC-MS analysis of Forskolin in *in vitro* micro tuber of *C.forskohlii*

Estimation of forskolin in *in vitro* root and micro-tuber by HPLC Analysis

The HPLC analysis was carried out to compare forskolin level in *in vitro* raised root and micro-tubers of *C.forskohlii*. Forskolin was quantified using HPLC when compared with standard (98%- Fig. 6) the elution of extracts of *in vitro* micro-tubers and root presented the amount of forskolin, with a retention time of 3.3 min in standard and 3.2, 3.4 in *in vitro* samples (Fig 7-8). The maximum forskolin production in *in vitro* culture of root and micro-tuber induction was 0.14 mg/g (0.014%) and 0.61mg/g (0.06%) correspondingly. In compare, the forskolin content in the root culture slightly higher than previous report (Mukherjee *et al.*, 2000) [10] and the first report forskolin production *in vitro* micro-tuber induction of *C.forskohlii*. Forskolin production in *in vitro* cultures of *C.forskohlii* has been described by Mersinger *et al.*, (1988) [9], Krombholz *et al.*, (1992) [7] and Sen *et al.*, (1992) [13], the forskolin content greater than has been reported earlier and was comparable to the forskolin content in normal tuberous roots of plants cultivated in the field for a year (Yanagihara *et al.*, 1995) [17].

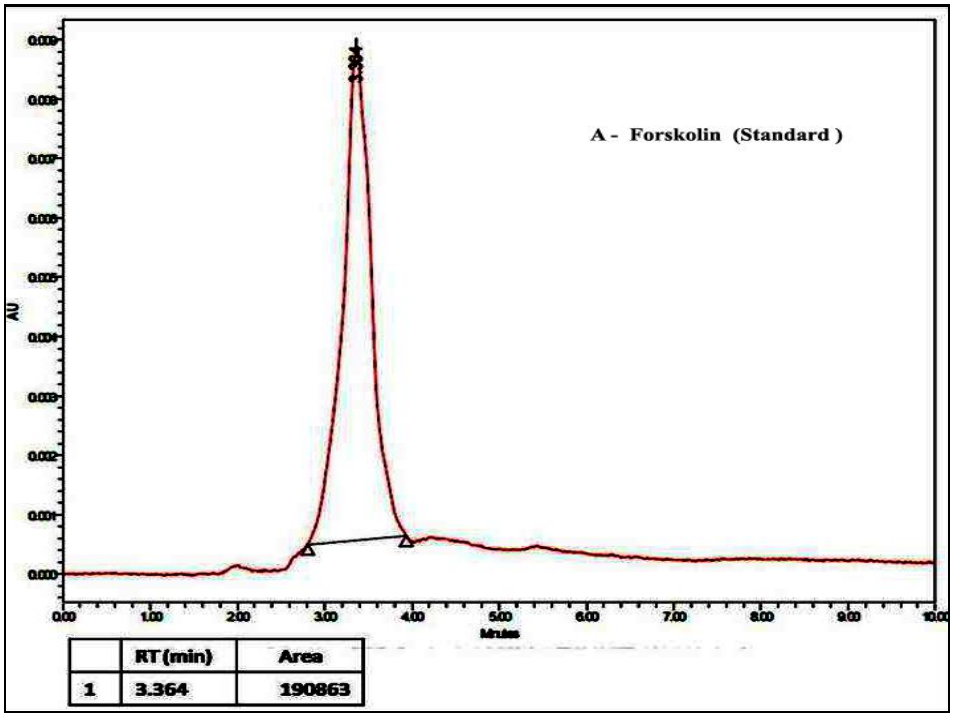

Fig 6: HPLC analysis of standard Forskolin

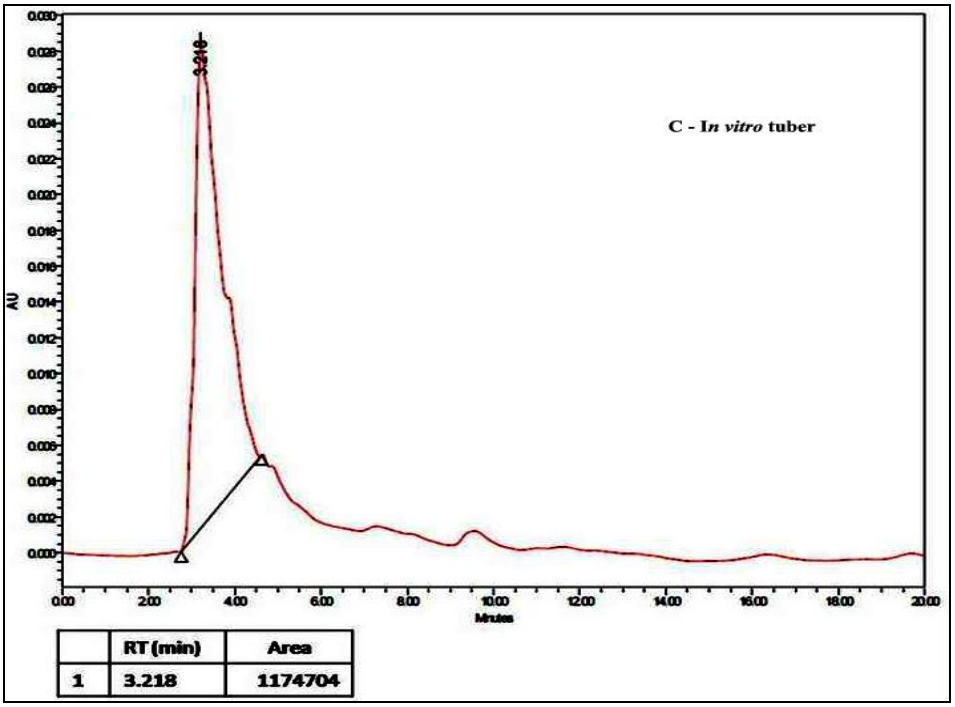

Fig 7: HPLC analysis of Forskolin in *in vitro* tuber of *C.forskohlii*

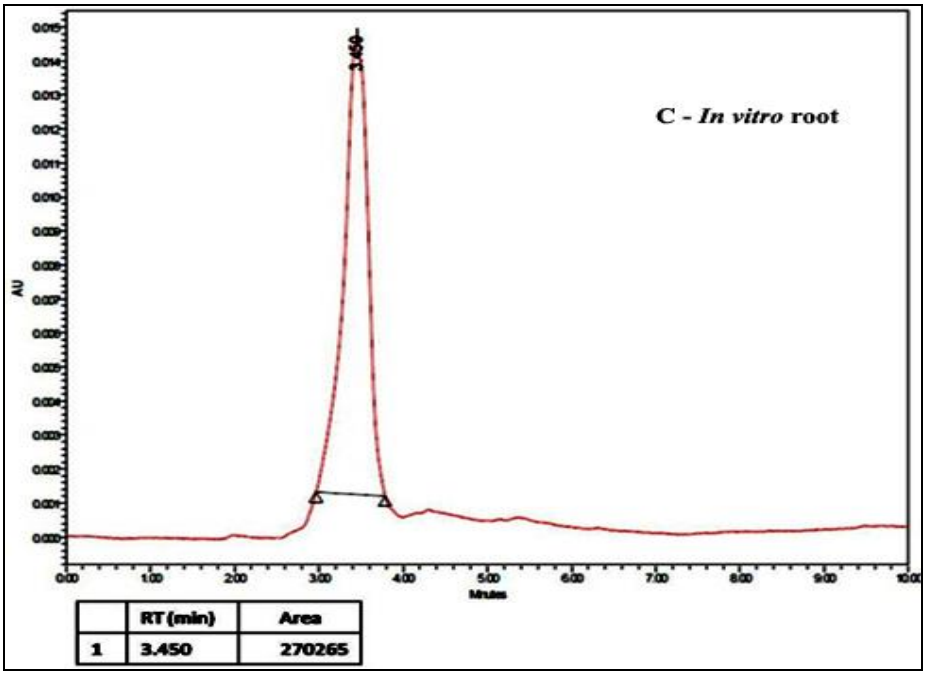

Fig 8: HPLC analysis of Forskolin in *in vitro* root of *C.forskohlii*

Conclusion

The effects of Auxins, Cytokinins and different concentrations of sucrose were studied for *in vitro* tuberous root and micro-tuber production of *C.forskohlii*. IAA 1.0 mg/l supplemented with six various concentrations (3-8%) of sucrose was attempted. Among the tried concentrations, sucrose 5 to 6% induced tuberous root in the similar periods 7 to 8% sucrose concentration developed the micro-tubers in the shoots after 60 days. Microtuberization was shown only in 5 to 8% sucrose containing medium. The maximum number of tuberous root (16 ± 0.32) and micro tuber micro-tubers (11 ± 0.26) was achieved in 6% and 7% sucrose concentration respectively. Then the increase concentration of sucrose above 8% resulted in the decreased number of tuberous root and micro tubers. Forskolin content was confirmed and compared in *in vitro* root and micro tuber of *C.forskohlii* by using LC-MS Analysis and HPLC analysis. The higher amount forskolin production in *in vitro* culture of root and micro-tuber induction was 0.14 mg/g (0.014%) and 0.61mg/g (0.06%) correspondingly. The forskolin content was found in higher amount in micro-tuber then normal *in vitro* root. The results suggested that sucrose play a key role in the in vitro tuberous root and micro tuber formation of *C.forskohlii*. MS media contain major components play a vital role in the induction of tuberous root and micro tubers in *in vitro* condition. In my knowledge this is the first report to producing tuberous roots and micro-tuber formation of *C. forskohlii* in *in vitro* condition

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