

Original article

Effect of plant hormones and zinc sulphate on rooting and callus induction in *in vitro* propagated *Coscinium fenestratum* (Gaertn.) Colebr stem and their role in estimation of secondary metabolites

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Abstract

Coscinium fenestratum (CF) (Gaertn.) Colebr. is a hard woody climber medicinal plant belongs to the family, Menispermaceae. The plant is commonly known as tree turmeric because the stem content yellow berberine, the major active constituent. The plant is widely available in Western Ghats region but became endangered due to over exploitation and very slow germination rate. Hence, the alternate *in vitro* tissue culture has established for reduced germination rate and to extract more amount of plant constituents from the stem callus. Full strength MS medium supplemented with 2, 4-D at 0.1 mg/l and kinetin at 2 mg/l gave callus growth in 46 days and the growth mechanism is observed first time through SEM study of callus. Thereafter, rooting of callus occurred in IBA and kinetin in combinations in half strength MS medium whereas direct rooting of stem occurred with IBA and zinc sulphate (IBA at 2 mg/l and ZnSO₄ at 3 mg/l) supplemented in half strength MS media along with coconut water, within 18 days. Thereafter, extracted callus is identified with TLC, followed by estimated with HPLC and HPTLC and resulted methanol extract of CF showed higher content of berberine than aqueous extract.

Key words: Berberine, callus, chromatographic method, estimation, *in vitro* culture, organogenesis, plant hormones, ZnSO₄

1. Introduction

Plant tissue culture is an old practice for the *in vitro* development of the desired plant parts from which increased number of plant secondary metabolites is isolated very easily. The main objective of plant tissue culture is to develop plantlets or callus for such plant species which are endangered or threatened due to over exploitation or the rate of seed germination is very longer time and to enhanced the active constituents by sub-cultured as per required times. This method helps to grow the plants aseptically or repeated number of multiplication of young parenchymatous cells that accumulate impurity free major plant constituents and isolation of same becomes very easy.

Looking at the above objectives, the present study was selected a plant species which is endangered but economically important, *i.e.*,

Maramanjil. Scientifically, the plant is known as *Coscinium fenestratum* (CF) (Gaertn.) Colebr. (Family: Menispermaceae) is a large dioecious woody climber tree. The plant is indigenous to the Indo-Malayan region, distributed in Sri Lanka, India, Malaysia, Vietnam, Myanmar, Singapore and Thailand (Tushar *et al.*, 2008). In India, the plant is widely spread across the Western Ghats regions of Tamil Nadu (Kanniyakumari, Tirunelveli and Nilgiri districts), Kerala (Thiruvananthapuram, Wynaad, Thrissur, Idukki and Palakkad districts) and Karnataka (Kodagu, Udupi, Dakshina and Uttara Kannada districts) (Sumy *et al.*, 2000; Mohanan and Sivadasan, 2002). This tree is also known as False Calumba or Tree Turmeric due to presence of yellow colored alkaloid berberine (an isoquinoline), a medicinally active compound with numerous bioactivities (Jayaweera, 2006; Warakagoda and Subasinghe, 2014). Other constituents like protoberberine, jatrorrhizine, magnoflorine, berberrubine, thalifendine, palmitine, sitosterol, palmitic acid, oleic acid and oxyberberine are present in stem and roots of this plant (Siwon *et al.*, 1980; Pinho *et al.*, 1992; Augusta, 2003; Anonymous, 2005). Due to presence of various constituents, the plants have various therapeutic activities like treating digestive disorders, chronic fevers, wounds, ulcers,

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jaundice, burns, skin diseases, abdominal disorders, diabetes, fever and general debility (Warrier *et al.*, 1994; Agusta, 2003). Apart from that, it is also used in cosmetic industry and other ayurvedic products (soap, bath gels, face wash and bath oil), and have antidiabetic (Shirwaikar *et al.*, 2005), anti-inflammatory (Caius, 1992), antioxidant and anthelmintic activities (Das *et al.*, 2018). These activities are possible when the plants are grown abundantly and sufficient raw materials are procured and dried woody stem has high demand in the crude drug market. Generally, propagation occurs through sexual method, *i.e.*, done naturally by seeds but the plant takes around 13-15 years to mature. Seed germination takes longer time (6-8 months) due to hard seed coat (Harinarayanan *et al.*, 1994; Tushar *et al.*, 2008) and, hence the seed germination was found around 30%. Research article revealed that around 12% of the fresh fruits contain non-viable seeds (Senerath, 1991) and also vegetative propagation through stem cuttings are unsuccessful (Gunatillake *et al.*, 2002). These all together resulted relatively slow growth rate, degradation of natural habitats, habitat specificity, no proper domestication through cultivation, illegal over exploitation and destructive collection natural populations. These difficulties are only overcome through *in vitro* cultivation. There are few reports on cell and suspension culture of this plant by which shoots and roots are developed (Parthasarathy, 2007; Staden *et al.*, 2008) and also reported increased amount of berberine content, estimated by HPLC method (Talat *et al.*, 2009; Senarath, 2010). In another study, it is revealed that petiole and leaf explants of CF formed callus on vermi-compost extract media along with coelomic fluid (Kashyap *et al.*, 2016), but no such literature is available on development of stem callus on MS medium, containing coconut water in combination with plant growth hormones, followed by impact of zinc sulphate for early rooting of the stem as well as estimation of the berberine content through HPLC and HPTLC methods. Further, very scanty reports or no such reports on SEM study (Scanning Electron Microscopy) on proliferation of parenchymatous cells for callus growth of explants. In view of that, the present study has undertaken to establish the stem callus in MS medium, supplemented with various concentrations of plant hormones, followed by SEM study for the mechanism of callus growth and estimation of berberine content through various chromatographic methods such as TLC, HPLC and HPTLC.

2. Materials and Methods

2.1 Plant material, surface sterilization and explants selection

Seeds of *C. fenestratum* are collected from Dr. P.E. Rajasekharan, Principal Scientist, Plant Biotechnology Department, Indian Institute of Horticultural Research, Hessaraghatta, Bangalore. Prior to use, seeds are *ex vitro* germinated in plastic cup container filled with sand: coir dust (1:1) medium, placed inside laboratory and drained using 0.5 g/l topsin fungicide solution to avoid fungal infection of seeds (Figure 1). After 24 h, surface sterilized and pretreated seeds by various solvents like 3% potassium nitrate, 2000 mg/l gibberelic acid (GA₃), 2250 mg/l GA₃ and kept for germination (Figure 2). Germinated plants are used as explants for the present study.

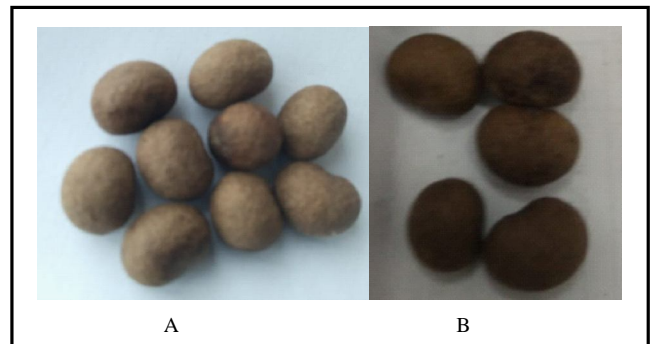


Figure 1: A: Hard *Coscinium* seed, B. Fruits containing seeds.

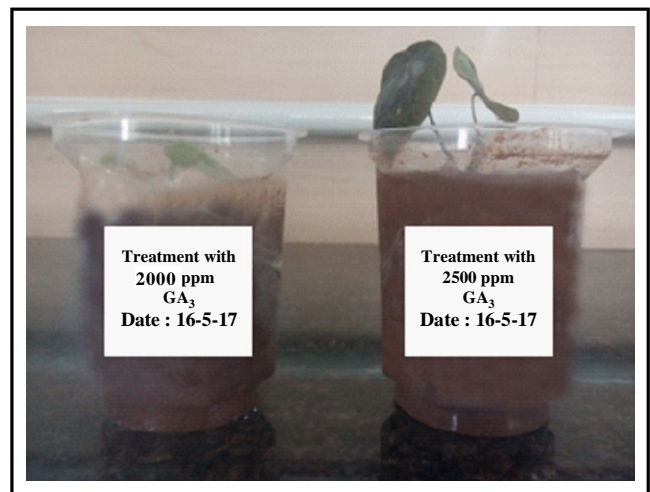


Figure 2: Seed germination with GA₃ at 2000 ppm and 2500 ppm.

2.2 Culture media

Murashigae and Skoog medium (MS) is procured from Hi-media, India. Full strength and half strength medium prepared for initiation of callus growth and organogenesis of the plants. Growth regulators are procured from Himedia, India and used in different concentrations for this present study, *viz.*, 2,4-Di chlorophenoxy acetic acid (2,4-D), kinetin, indole acetic acid (IAA), Indole butyric acid (IBA), gibberellic acid (GA₃), 6-benzylaminopurine (BAP) and naphthalene acetic acid (NAA), zinc sulphate (ZnSO₄) and coconut water.

2.3 Preparation of culture medium

Various combinations of MS media are prepared with double distilled water, using sugar concentration of 3g/l along with different concentrations of required growth hormones. 0.7% agar is used as gelling agent. The media is then dispensed into culture tubes, 100 ml EM flask, 200 ml bottles with 15, 40 and 60 ml media and coconut water of 2.5, 5 and 7.5 ml, respectively on plugs or Laxbro plastic caps and made air tight. They are then sterilized in an autoclave at 121°C temperature and 103.4 kPa pressure for 20 min.

2.4 Preparation of explants for inoculation and incubation

The stems of germinated explants is washed thoroughly with running tap water for 15 min then treated with bavistin (0.1%) for

15 min. The treated plant materials are subjected to sterilization using 0.1% mercuric chloride for 5 min. and repeated washing with sterile double distilled water under aseptic environment. A known weight of stem part is dissected and inoculated to the medium. The inoculated tubes, flasks and bottles are then incubated in the culture room at $25 \pm 2^\circ\text{C}$ under fluorescent light with an intensity of 60 UE/m²/sec. A photoperiod of 16 h of light and 8 h of darkness is maintained through an automatic timer and a constant relative humidity of 65-75% using an air-cooling system is also maintained.

2.5 Growth measurement

In each replication of different treatments for an individual experiment, uniform explant tissues are inoculated and incubated at $25 \pm 2^\circ\text{C}$. A fixed number of replications are taken for the record in each time. Weight of the callus and its maintenance are also observed 60 days after inoculation and after every 16 days of sub-culturing, respectively.

(a) Effect of different combinations of auxin and kinetin on callus induction

Different strengths of MS basal medium along with auxins (IAA, IBA, NAA, 2, 4-D) and cytokinin (kinetin) at various concentrations are used in combinations. Stem at 1.5 to 2 cm length are sterilized and inoculated in culture tubes containing 15 ml media. Observations are recorded after 45 days of inoculation.

(b) Effect of different combinations of auxin, cytokinins and ZnSO₄ for the rooting of callus

MS half strength medium prepared and supplemented with IBA at 0.1 mg/l and cytokinins at 1 and 2 mg/l are used in combinations. Further, IBA at various concentrations and ZnSO₄ at 1, 2 and 3 mg/l are used for organogenesis of callus, using coconut water in the media. Observation upto 60 days, the rooting of callus under different treatments as well as direct rooting of the stem parts is recorded.

2.6 Mechanism of proliferation of callus

Growth of callus is occurred due to the multiplication of parenchymatous cells. Hence, callus is known as an abnormal, uncontrolled growth of parenchyma tissues. This proliferation was clearly visible under SEM study.

2.7 Preparation of extract for chemical analysis

Obtained calli (5 g dried) is refluxed 3-4 h with methanol and solvent water and analyzed for various phytochemicals present in the calli as per the standard method (Harborne, 1973; Evans, 2002).

2.8 TLC identification

Presence of berberine was identified in methanol and aqueous callus extracts by using mobile phase n-butanol, acetic acid and water (8:1:1) and silica gel G as stationary phase. Standard berberine hydrochloride is used for comparison. The plates are then derivatised in iodine chamber (Figure 5).

2.9 Estimation of berberine from extracts

HPLC and HPTLC were carried out for estimation of berberine content in both the extracted callus samples. HPTLC (CAMAG) is used for separation and estimation of berberine content in both the extracts. n-butanol, acetic acid and water (8:1:1) are used as mobile

phase and silica gel GF₂₅₄ as stationary phase. The results are compared against standard berberine hydrochloride at wavelength 450 nm. The linearity of the concentration observed from 12-80 nanogram ($r^2 = 0.9988$). Sample and standard prepared 1 mg/ml concentration.

Further, efficient HPLC (Shimadzu, India) system is applied for estimation of berberine content. The condition for HPLC as column: SS Wakosil II C-18 (250 X 4.6 mm), mobile phase: methanol and water (90: 10), flow rate 1.0 ml per min and compound detected at 220 nm. Sample and standard prepared 1 mg/ml concentration using methanol as solvent. Sample further diluted within the limit of standard area for the calculation.

2.10 Statistical analysis

Callus growth was analyzed by one-way ANOVA study, followed by Tukey's multiple comparison post test and $p < 0.05$ is considered as significant.

3. Results and Discussion

3.1 Seed germination

Seed of CF is very hard and germination rate is also very slow due to high content of alkaloids in the seed. Hence, various techniques are adopted earlier for breaking the dormancy (Anil Kumar *et al.*, 2010; Ramasubbu *et al.*, 2012; Warakagoda and Subasinghe, 2015). The same method is tried in this experiment and is resulted GA₃ at dose of 2500 ppm showed better result than others. Germination of seed occurred faster within 4 months but same in concentration 3000 ppm showed lower germination which is similar to the earlier reports.

3.2 Growth measurement

i. Effect of different combinations of auxin and kinetin on callus induction

The effect of different combinations of auxins and kinetin on callus induction of *C. fenestratum* in different strengths of MS medium is given in Table 1. The results suggest that the growth of callus initiated in full strength of MS media, supplemented with 2, 4-D at 0.1 mg/l and kinetin at 2 mg/l. The highest growth of callus is observed in full strength MS media (5.48 cm) than half strength MS media (2.10 cm) (Negative results are not reflected in Table 1). It was revealed that a combination of 2,4-D, BAP and kinetin (2.0, 2.0, 1.0 μM , respectively) in MS medium enhances the callus production in *C. fenestratum* (Khan *et al.*, 2008) in 46 days. Our present study is also followed the same trend for the callus development (Figure 3).

Table 1: Effect of plant growth regulators for callus initiation

Treatments (mg/l)	Callus growth		Diameter of callus (cm)	
	½ strength	Full strength	½ strength	Full strength
2,4 D 1+ Kinetin 0.1	No	No	---	---
2,4 D 1+ Kinetin 0.2	Yes	Yes	0.80 ± 0.11	1.12 ± 0.22
2,4 D 1+ Kinetin 0.3	Yes	Yes	0.90 ± 0.12	1.00 ± 0.31
2,4 D 0.1+ Kinetin 1	Yes	Yes	1.00 ± 0.20	2.20 ± 0.11*
2,4 D 0.1+ Kinetin 2	Yes	Yes	2.10 ± 0.10*	5.48 ± 0.20**
2,4 D 0.1+ Kinetin 3	Yes	Yes	1.43 ± 0.10	3.42 ± 0.31**

n = 6; readings ± SEM; significant at $p < 0.05^{**}$



Figure 3: Initiation of callus and organogenesis (root formation).

ii. Effect of different combinations of auxin, cytokinins and ZnSO₄ for the rooting of callus

MS half strength medium prepared and supplemented with IBA at 0.1, 0.2, 0.3, 1, 2 and 3 mg/l and kinetin at 1 mg/l are used in combinations. Further, IBA at concentration 2 mg/ml and ZnSO₄ at various concentrations 1, 2 and 3 mg/l are used for organogenesis of callus, using coconut water in the media. It is seen that IBA is effective against rooting of callus whereas added application of ZnSO₄ at concentration 3 mg/ml along with IBA 2 mg/ml in combinations showed direct rooting in stem within 18 days in half strength MS medium mixed with coconut water (Table 2, Figure 4). Literature survey revealed that root initiation occurred after the 3rd week of transferring the shoots to the rooting medium and the rooting percentage greatly depends on strength of MS medium (Senarath, 2010). Our results showed rooting of stem occurred within 18 days which is lesser time than that of reported earlier. This may be due to added zinc sulphate which acts synergistic activity along with IBA. It was reported that 50 µm zinc sulphate concentration significantly caused boosting of leaf area and both fresh and dry weight of shoot and root development (Nejad *et al.*, 2014) which is also positive in our present study.



Figure 4: Rooting of direct stem of *C. fenestratum*.

Table 2: Effect of plant growth regulators on rooting of *Coscinium* stem

Treatments (mg/l)	Root growth		Length of Root (cm)	
	½ strength	Full strength	½ strength	Full strength
IBA 1+ Kinetin 1	No	No	---	---
IBA 2+ Kinetin 1	Yes	Yes	2.01	0.67
IBA 3+ Kinetin 1	No	No	No	No
IBA 0.1+ Kinetin 1	No	No	No	No
IBA 0.2+ Kinetin 1	No	No	No	No
IBA 0.3+ Kinetin 1	No	No	No	No
IBA 1+ ZnSO ₄ 1	No	No	No	No
IBA 2+ ZnSO ₄ 1	No	No	No	No
IBA 3+ ZnSO ₄ 1	No	No	No	No
IBA 1+ ZnSO ₄ 2	No	No	No	No
IBA 2+ ZnSO ₄ 2	Yes	No	1.02 ± 0.10	No
IBA 3+ ZnSO ₄ 2	Yes	No	0.92 ± 0.20	No
IBA 1+ ZnSO ₄ 3	No	No	No	No
IBA 2+ ZnSO ₄ 3	Yes	Yes	2.6 ± 0.11**	1.10 ± 0.10*
IBA 3+ ZnSO ₄ 3	Yes	Yes	No	No

n =6; readings ± SEM; significant at $p < 0.05^{**}$

3.3 Mechanism of proliferation of callus

Scanning Electron Microscopy is carried out first time to observe the proper proliferation of parenchyma cells for growth of callus. It is seen that uncontrolled abnormal growth (Figure 5). There are various scale used for observation of growth mechanism of callus.

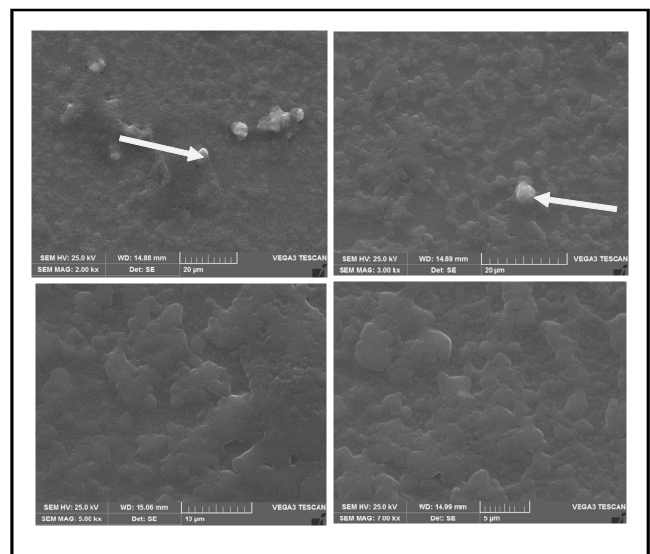
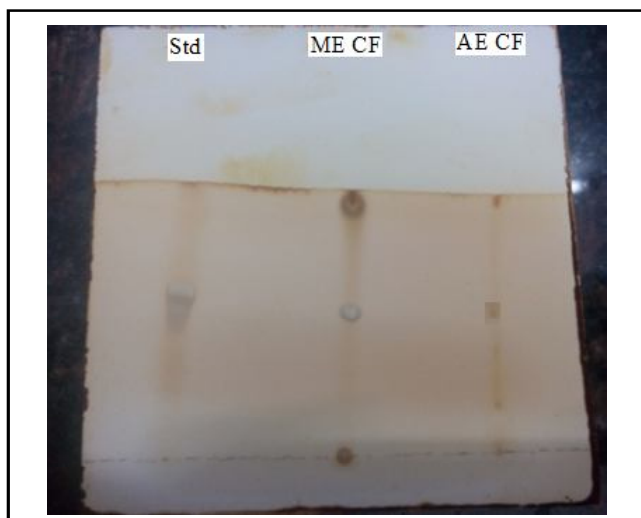


Figure 5: SEM study of *C. fenestratum* stem callus.

3.4 TLC identification of CF extracts

Two different extracts, *viz.*, methanolic and aqueous extracts were prepared and resulted higher percentage yield in methanol extract (24.8% w/w) than aqueous one (16.3% w/w), but both contain saponins, alkaloids, flavonoids, resins, *etc.* Thereafter, TLC study has carried out with the various solvent systems and revealed the n-butanol, acetic acid and water is the mobile phase that identified and separated chemical constituents when compared with the

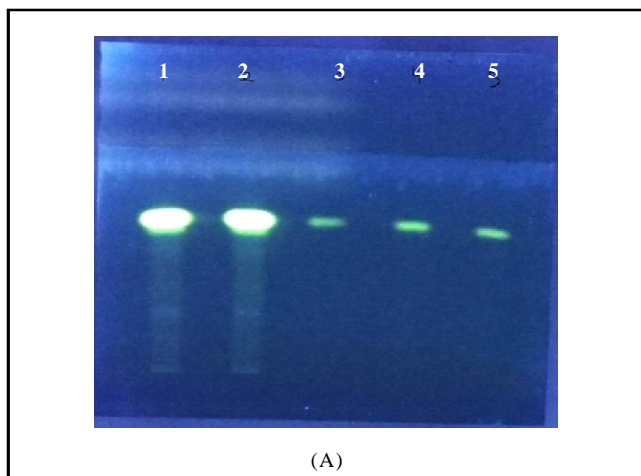
standard berberin hydrochloride. The R_f is calculated and resulted 0.63 (Figure 6) after derivatised in iodine chamber.



Std= standard berberine; MECF= methanol extract of CF; AECF = aqueous extract of CF

Figure 6: TLC plate of extracted CF stem callus.

Earlier scientific literatures evident that berberine identification was carried out using various TLC solvent systems (Rojangsa *et al.*, 2006; Krishna *et al.*, 2011), looking at that we are also used different solvent system with standardized ratio for the separation and identification of berberine in the CF extracts.

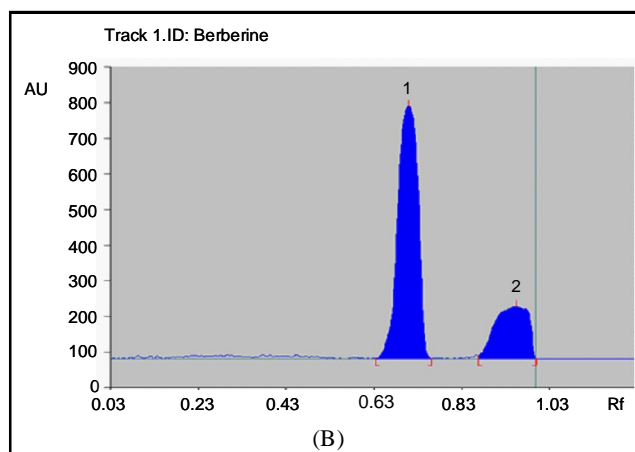


A = HPTLC plate at UV light (1, 2 are CF extracts and 3, 4 and 5 are standard berberine).

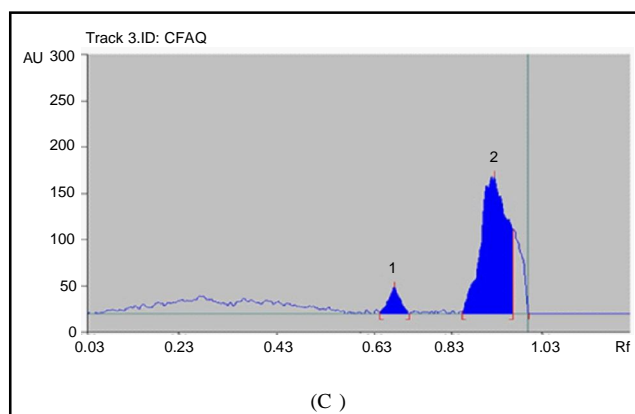
Figure 7: HPTLC of callus extract of CF.

3.5 Estimation of berberine in the extract

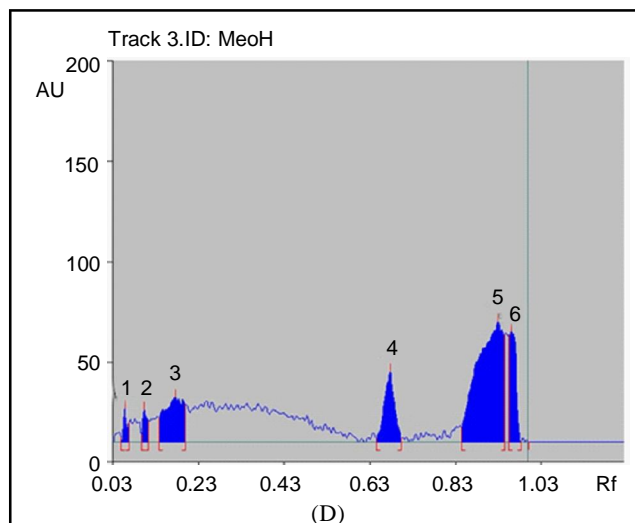
HPTLC method is applied for the separation and estimation of the berberine content in both the MECF and AECF using same solvent system as TLC used. Results showed that R_f values are coincided at 0.63 when scanned at 450 nm. The finger printing as well as the tracks are showed in Figures 7 A, B, C and D. Many literatures also revealed the estimation of berberin through HPTLC method using various solvent systems (Rojangsa *et al.*, 2006; Krishna *et al.*, 2011; Jayaprakasam and Ravi, 2014).



·B = HPTLC peak of standard berberine at R_f 0.63.



·C = HPTLC peak of AECF at R_f 0.63.



·D = HPTLC peak of MECF at R_f 0.63

3.6 HPLC method

Further, effective separation and estimation of CF extracts are carried out using HPLC method using methanol and water solvent system at 220 nm. Finally the berberine content is estimated by calculated R_t of standard and sample ($R_t = 3.074$ min.) (Figures 8 a, b and c).

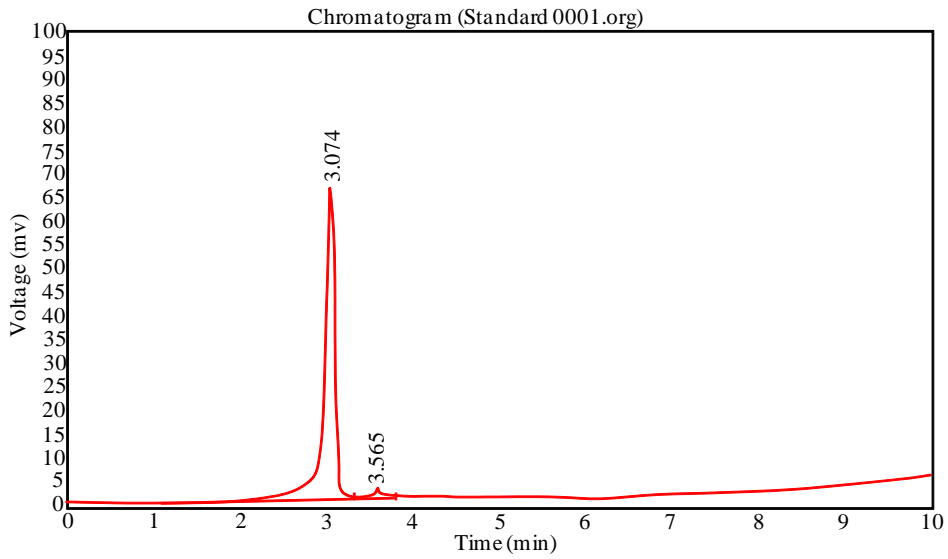


Figure 8(a): HPLC of standard berberine hydrochloride.

Results of standard graph

Peak No.	Peak ID	Ret. time	Height	Area	Conc.
1		3.074	65154.414	565060.625	95.7911
2		3.565	1352.586	24827.801	4.2089
Total			66507.000	589888.426	100.0000

System evaluation

Peak No.	Peak ID	Ret. time	Half peak width	Theoretical levels	Resolution	Tail factor	Asymmetry
1		3.074	0.100	5147.950	0.000	0.647	0.500
2		3.565	0.297	800.002	1.303	0.964	0.928

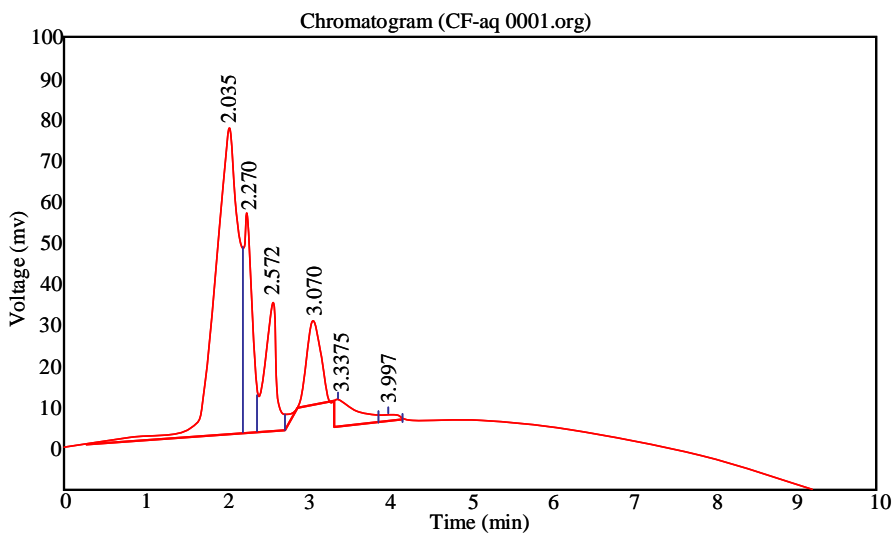


Figure 8(b): HPLC graph of AECE..

Results of AECF graph

Peak No.	Peak ID	Ret. time	Height	Area	Conc.
1		2.035	73666.961	1444833.125	57.4882
2		2.270	52032.164	364193.375	14.4908
3		2.572	30384.494	306517.625	12.1960
4		3.070	19667.855	224781.406	8.9438
5		3.375	6232.493	124087.844	4.9373
6		3.997	1832.170	28167.869	1.9439
Total			183816.137	2492581.244	100.0000

System evaluation

Peak No.	Peak ID	Ret. time	Half peak width	Theoretical levels	Resolution	Tail factor	Asymmetry
1		2.035	0.337	203.408	0.000	0.726	0.498
2		2.270	0.115	2164.899	0.517	1.382	1.763
3		2.572	0.158	1463.371	1.098	0.877	0.754
4		3.070	0.300	700.459	0.575	4.853	8.706
5		3.375	0.300	700.459	0.575	4.853	8.706
6		3.997	0.257	1344.392	1.123	2.434	3.868

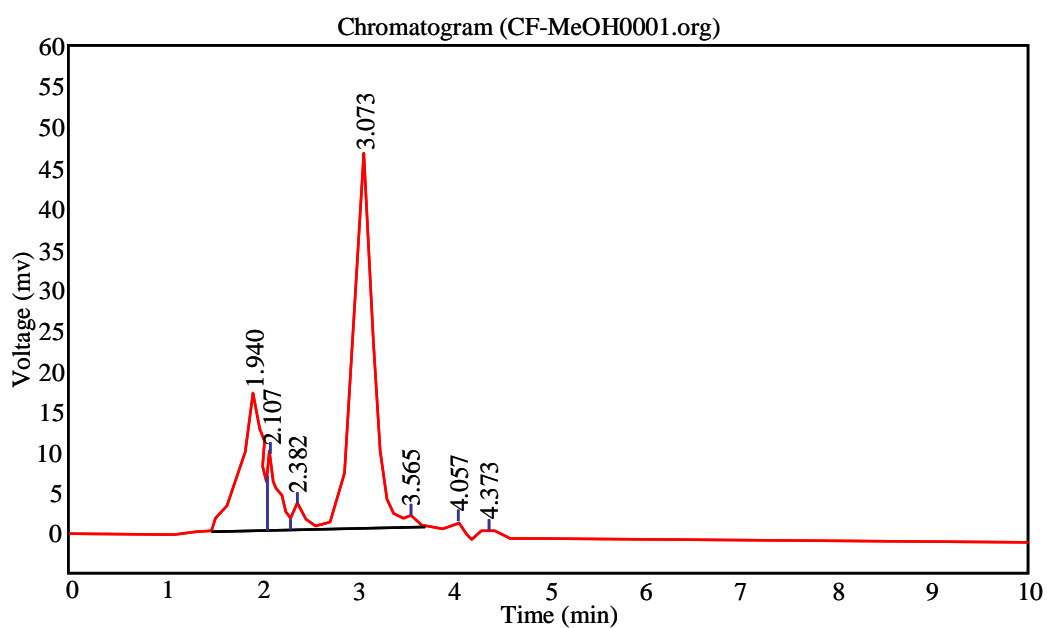


Figure 8(c): HPLC graph of MECF.

Results of MECF graph

Peak No.	Peak ID	Ret. time	Height	Area	Conc.
1		1.940	16974.535	266202.688	21.0077
2		2.107	10625.940	84754.539	6.6885
3		2.382	4371.208	39732.684	3.1356
4		3.073	46628.488	437294.688	66.0761
5		3.565	667.818	3631.600	0.2866
6		4.057	1595.774	12497.084	1.7487
7		4.373	960.333	13391.933	1.0568
Total			81824.096	857505.216	100.0000

System evaluation

Peak No.	Peak ID	Ret. time	Half peak width	Theoretical levels	Resolution	Tail factor	Asymmetry
1		1.940	0.232	388.496	0.000	0.649	0.321
2		2.107	0.133	1383.005	0.457	3.409	5.818
3		2.382	0.137	1682.466	1.019	2.153	3.306
4		3.073	0.210	1186.562	1.995	0.987	0.993
5		3.565	0.093	8082.366	1.621	1.067	1.093
6		4.057	0.125	5834.657	1.318	0.823	0.644
7		4.373	0.272	1435.689	0.798	0.949	0.926

We observed that both the HPTLC and HPLC graphs, the amount of berberine content is estimated for both the extracts and the results are tabulated in Table 3.

Table 3: Estimation of berberine content in various extracts

Methods	Extract	Berberine present (mg)	%RSD
HPTLC	MECF	1.23	1.12
	AECF	0.97	
HPLC	MECF	1.34	1.54
	AECF	0.99	

From these two methods, it is observed that methanol extract of CF showed higher amount of berberine content than aqueous extract. HPLC showed better amount of berberine (1.34 mg) content than HPTLC (1.23 mg) for MECF. Earlier literature also revealed the same results where methanolic extract showed higher percentage of berberine content than other extract (Jayaprakasam and Ravi, 2014). Further, it is also revealed that percentage yield of extract also higher in methanol extract than others (Arawwawala and Wickramaarachchi, 2012; Akowuah *et al.*, 2014) and the same trend followed in the present study where methanol extract of callus showed higher percentage of yield than aqueous extract. It is also proved that content of active constituents are also depends on the percentage of yield (Dent *et al.*, 2013; Das *et al.*, 2016; Das *et al.*, 2017). Our present study is also reported the similar results that influence direct correlation with the amount content of the active constituents.

4. Conclusion

The present study established the *in vitro* culture of *C. fenestratum* stem in full strength MS medium, supplemented with various plant growth regulators (combinations of 2, 4-D at 0.1 mg/l and kinetin

at 2 mg/l) in various concentrations within 46 days. First time, SEM study is reported the mechanism for callus growth *via* abnormal proliferation of parenchyma cells. Thereafter, organogenesis of stem callus developed in combinations of IBA and kinetin combinations in half strength MS medium. Further, direct rooting of stem in half strength MS medium is observed within 18 days when IBA combined with zinc sulphate is used along with coconut water in the medium (IBA 2 mg/l and ZnSO₄ 3 mg/l combination). Grown callus further extracted with methanol and aqueous solvents and estimated for berberine content by HPLC and HPTLC where methanol extract showed higher content of berberine than aqueous extract. Further, study required hardening of plant in huge quantities for conversion of endangered species to cultivated plant and to discover new biomolecules for various therapeutic activities.

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Conflict of interest

We declare that we have no conflict of interest.

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