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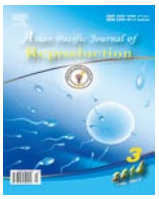

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High frequency *in vitro* plantlet regeneration and antioxidant activity of *Enicostema axillare* (Lam.) Raynal ssp. *littoralis* (Blume) Raynal: An important medicinal plant

Kousalya Loganathan[1*], V. Narmatha Bai[2]

[1]*Plant Tissue Culture Laboratory, Department of Botany, Bharathiar University, Coimbatore – 46 India*

[2]*Department of Botany, Bharathiar University, Coimbatore – 46 India*

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ABSTRACT

Objective: To develop a method for high frequency plantlets regeneration protocol for *Enicostema axillare* (Lam.) Raynal ssp. *littoralis* (Blume) Raynal (*E. axillare*) without intermediate callus phase and to determine the content and correlation of phenolic compounds and their antioxidant activity of both the plants derived from nature and nodal culture by DPPH assay. **Methods:** The nodal explants were cultured on MS basal medium fortified with different concentration of various growth regulators such as BAP, KIN, TDZ and 2iP (0.5-2 mg/L) individually and in combinations with or without GA_3 for shoot bud induction and multiplication. Total phenol and flavonoid content was determined in both plants from nature and nodal culture and antioxidant activity was determined by DPPH assay. **Results:** The highest number of multiple shoot (108.00±3.55 shoots/explants) was obtained on MS medium supplemented with BAP (2 mg/L) in combination with KIN (0.5 mg/L) and GA_3 (2 mg/L). Rooting was optimized on half-strength MS medium supplemented with IAA (0.5 mg/L). The rooted plantlets were transferred to paper cups containing vermiculite and hardening was successfully attained with 75% survival. Among the four extract of methanol and water extract from both the plantlet from nature and nodal culture, the concentration of flavonoid was found to be higher in methanol extract of the plants from nature (146.57±1.68 mg rutin/g extract) and phenol content was higher in the water extract of plant from nature (52.53 ±1.67 mg GAE /g extract). The radical scavenging activity of four extracts. Methanol extract of plant grown in nature showed the highest radical scavenging activity (IC_{50} = 87.10 μg/mL) was investigated by DPPH test. **Conculsion:** The present study not only enables reinforcement of wild plant populations using *ex situ* growth of individuals, but it also helps for high scale production of plantlets. A high correlation between antioxidant capacities and their total phenolic contents indicated that flavonoid compounds were a major contributor of antioxidant activity of both plants grown from nature and nodal culture.

1. Introduction

Enicostema axillare (*E. axillare*), a member of the Gentianaceae family is an important medicinal plant. The plant is pungent and very bitter, antihelmintic, cures fever and vata diseases. It is also used as stomachic, laxative, antidiabetic, and crushed plant material is applied to snake-bites[1,2]. The whole plant is dried powdered and given with honey as a blood purifier and in dropsy, rheumatism abdominal ulcer, hernia, swellings, itches, filariasis, tapeworm infestation and insect poisoning[3]. It is used in the treatment of leucoderma[4], veterinary

*Corresponding author: Kousalya Loganathan, Ph.D Research Scholar, Plant Tissue Culture Laboratory, Dept. of Botany, Bharathiar University, Coimbatore – 46, India.
E-mail: lkousalya25@gmail.com

diseases[5], anti-diabetic[6,7] anti-inflammatory[8] and anticancer drug[9]. Various secondary metabolites were reported from this plant such as iridoid glycoside swertiamarin[10], monoterpene alkaloid gentiocrucine and erythrocentaurin[11], triterpenesapogenin[12] and seven flavonoids such as apigenin, genkwanin, isovitexin, swertisin, 5-o- β -D-glycoside[13].

Seed setting of this plant is very high, yet germination frequency is too poor under natural conditions[14]. *In vitro* propagation represents a possible strategy not only to provide a continuous source of *E. axillare* plant material but also for the conservation of rare and endangered species. The present study was taken to develop a protocol for mass propagation using nodal explants.

This article describes the *in vitro* plant regeneration of *E. axillare*, along with quantitative study of total flavonoids and phenol content in the plants obtained from *in vitro* culture as well as from plants collected in natural environment. Furthermore, the free radical scavenging activity of methanol and water extract was evaluated using DPPH radical scavenging activity assay.

2. Materials and methods

2.1. Micropropagation

2.1.1. Chemicals and reagents

Bavistin, 6-Benzyladenine purine (BAP), Kinetin (KIN), N-phenyl-N′-1·2·3-thiadiazol-5-ylurea (TDZ), 6-(gamma, gamma Dimethylallylamino) purine (2ip), Gibberellic acid (GA_3), Indole-3-aceticacid (IAA), Indole-3-butyric acid (IBA), α -Naphthalene acetic acid (NAA), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), Butylated hydroxytoluene (BHT), Butylated hydroxyanisole (BHA), ascorbic acid (ascorbic), Gallic acid equivalence(GAE) sodium carbonate, Folin-Ciocalteu reagent, Aluminium chloride and Methanol extract(MeOH) were obtained from Himedia (Mumbai, India) and Sigma (St.Louis, USA). All other chemicals and reagents used were of analytical grade.

2.1.2. Preparation of explants

The plant material *E. axillare* was collected from Pattanam in Coimbatore, Tamil Nadu, India. An authentic sample was identified by BSI (Botanical Survey of India), Southern Circle, Coimbatore, India, and a voucher specimen (Accession no. 957) has been deposited in the herbarium of BSI. Nodal segments (3 cm long) were washed with running tap water followed by 0.5% of Teepol (detergent) treatment for 5-10 min and then treated with fungicide (1% Bavistin) for 5 min. Then treated explants were washed thoroughly with sterile distilled water and treated with 1% of Streptomycin for 10 min. The treated explants were washed thoroughly with sterile distilled water. The explants were then disinfected with 0.1% mercuric chloride for 3-5 min and finally rinsed with sterile distilled water.

2.1.3. Culture media and growth conditions

The culture medium consisted of MS medium[15] supplemented with salts, vitamins and 3% (w/v) sucrose was used in all experiments. The pH of the medium was adjusted to 5.7 before adding 0.8% (w/v) agar (Hi Media). Media (15 mL) were poured into (25×150) mm culture tubes (Borosil, Mumbai) & autoclaved at 121 ℃ and 1.06 kg/cm^2 pressure for 20 min. The cultures were incubated at (25±2) ℃ under a 16 h photoperiod of 50-60 μ mol/m-2/s-1 flux density provided by cool white fluorescent tubes (Philips, India).

2.2. Multiple shoot induction

In order to achieve multiple shoot regeneration, the following experiments were conducted as described below.

2.2.1. Different concentration of cytokinins on multiple shoot induction

Surface sterilized nodal segments were cultured on MS medium supplemented with various cytokinins such as BAP, KIN, TDZ and 2iP (0.5-2 mg/L) for multiple shoot induction. Total number of shoots and their length were calculated after 65 days of culture. For root induction, the microshoots were transferred to half strength MS medium supplemented with auxins such as IAA, IBA and NAA at different concentrations (0.5-2 mg/L). Total number of roots and their length were recorded after 35 days of culture.

2.2.2. Different combinations of cytokinins

The synergistic effect of cytokinins on in vitro derived nodal explants for multiple shoot induction was tested. In vitro derived nodal explants were cultured on MS medium with BAP (0.5-1 mg/L) in combination with various concentrations of cytokinins like KIN, TDZ, and 2iP (0.5-2 mg/L). The maximum number of shoot and root and their length was calculated after 65 days of culture.

2.2.3. GA_3 in combination with cytokinins

To enhance the rate of shoot multiplication and shoot elongation from in vitro derived nodal explants using the best combinations of cytokinins of BAP (2 mg/L) with KIN

(0.5 mg/L), BAP (1 mg/L) with TDZ (0.5 mg/L) and BAP (1 mg/L) with 2iP (0.5 mg/L) was tested along with GA3 (0.5-2 mg/L). The maximum number of shoot and roots were calculated after 7 weeks of culture.

2.2.4. Hardening

Plantlets with well developed roots were removed and gently washed under sterile distilled water to remove adhering medium. Subsequently, they were transferred to paper cups containing sterile vermiculite. Initially the plantlets were maintained at 25±2 ℃ under a 16 h photoperiod of 50-60 μmol/m-2/s-2 flux density. Then the plantlets were successfully established in garden soil.

2.3. Antioxidant studies

2.3.1. Preparation of the plant extract

The plant material was air dried in shade for ten days and the in vitro plants were dried in oven at 60 ℃ for one to two days. The material was powdered by using electric blender and stored in clean labeled airtight bottles. A hundred grams of each powder was extracted by maceration in 300 mL of methanol for 3 days with frequent agitation followed by 300 mL water. The mixture was filtered through Whatman No. 1 filter paper and the filtrate was concentrated by pouring in glass Petri dishes and brought to dryness at 60 ℃ oven. Tissue cultured shoots were also dried at 60 ℃ oven and extracted by the above said maceration methods.

2.3.2. Determination of total flavonoids

The flavonoid content of the *in vitro* and *in vivo* methanol and water extracts were determined according to Chang CC [16]. In brief, a dilute solution of the extracts in methanol (0.5 mL) was mixed with 4.5 mL of methanol and 5.0 mL (0.01 M aluminium chloride prepared in methanol). Then the mixture was allowed to stand for 10 min at room temperature. The absorbance of the reaction mixture was measured at 400 nm with an ultraviolet visible spectrophotometer. Again the blank consisted of all reagents and solvents, but without the sample. The total flavonoids content was expressed in mg of rutin per g of extract.

2.3.3. Determination of total phenolic content

Total phenolic constituents of the *in vitro* and *in vivo* methanol and water extracts were determined by Folin-Ciocalteu reagent[17] and were expressed as Gallic acid equivalents (GAE). 100 μL test sample was taken in a test tube, then 1 ml of distilled water and 500 μL (1/10 dilution) of the Folin-Ciocalteu reagent was added and test tube was shaken thoroughly. After 1 min, 1500 μL of 20% sodium carbonate (Na_2CO_3) solution was added. The final mixture was shaken and then incubated for 2 h in the dark at room temperature. The absorbance of samples was measured at 760 nm and the results were expressed in mg of Gallic acid (GAE) per g of extract.

2.3.4. DPPH free radical-scavenging activity

The DPPH-scavenging activity was determined according to the modified method of Duan X[18]. The *in vitro* and *in vivo* methanol and water extract (12.5, 25, 37.5, 50 & 62.5 μg/mL) was added to 5 mL of 0.1 mM DPPH solution in ethanol. The absorbance at 517 nm of samples was measured after 30 min of incubation at 25 ℃. The IC_{50} value was defined as the concentration (in μg/mL) of the extract required to scavenge the DPPH radical by 50 %. The antioxidant activity of *E. axillare* extracts was calculated as an inhibitory concentration of the DPPH radical at 50%.

2.4. Statistical analysis

All the experiments were conducted with 5 replicates per treatment. The significance of differences among means were calculated using Duncan's multiple range test (DMRT) at P=0.05. The data obtained from the laboratory experiments represent Mean ±SD of the three repeated experiments with 5 replications. Mean in a column with the different letter (superscript) are significantly different according to DMRT (P<0.05). The data obtained from the antioxidant assays were expressed as mean (n=5) and the IC_{50} values were calculated using SPSS software. P<0.05 was considered as statistically significant.

3. Results

3.1. Micropropagation

3.1.1. Multiple shoot induction

The nodal explants failed to respond morphogenetically on growth regulators free MS medium. BAP at most of the concentration tested was more effective in shoot induction compared to KIN, TDZ and 2iP. All the concentrations of BAP induced multiple shoots after 3 weeks of culture. However, BAP at 1 mg/L proved to be optimal concentration for producing an average of 6.00±0.70 shoots per explants with an average shoot length of (1.80±0.11) cm (Table 1).

Table 1
Effect of cytokinins on multiple shoot induction from nodal explants of *E. axillar* after 7 weeks.

Plant growth regulator (mg/L)				Number of shoots	Shoot length (cm)
BAP	TDZ	KIN	2iP		
0.5	–	–	–	4.00±0.31^{b}	0.80±0.11^{d}
1.0	–	–	–	6.00±0.70^{a}	1.80±0.11^{a}
2.0	–	–	–	3.00±0.70^{c}	1.20±0.12^{b}
–	0.5	–	–	0.00±0.00^{f}	0.00±0.00^{i}
–	1.0	–	–	2.00±0.31^{d}	0.30±0.10^{h}
–	2.0	–	–	4.00±0.54^{b}	0.50±0.09^{f}
–	–	0.5	–	0.80±0.15^{e}	0.24±0.04^{h}
–	–	1.0	–	2.00±0.31^{d}	0.60±0.07^{e}
–	–	2.0	–	4.00±0.31^{c}	0.60±0.07^{e}
–	–	–	0.5	4.80±0.60^{b}	0.80±0.12^{d}
–	–	–	1.0	3.89±0.90^{c}	0.96±0.17^{c}
–	–	–	2.0	1.80± 0.83^{d}	0.45±0.19^{g}

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

3.1.2. Root induction

The microshoots developed on all the above media were transferred onto MS medium supplemented with either or IAA, IBA or NAA for rooting. Among the three auxins tested, IAA was the most effective and the numbers of roots were produced more on the medium containing 0.5 mg/L IAA (90.20±7.60) than NAA and IAA (Table 2).

Table 2
Effect of various auxin on root induction of *E. axillare*.

Growth regulator mg/L			Number of roots	Root length (cm)
IAA	NAA	IBA		
0.5	–	–	90.20±7.61^{a}	3.56±0.35^{a}
1.0	–	–	48.40±10.02^{bc}	1.80±0.33^{b}
2.0	–	–	51.40±4.35^{b}	1.60±0.10^{bc}
–	0.5	–	31.80±3.07^{bcd}	0.76±0.12^{b}
–	1.0	–	29.40±5.78^{cd}	2.08±0.09^{b}
–	2.0	–	27.80±5.26^{cd}	1.10±0.18^{cd}
–	–	0.5	37.80±9.09^{bcd}	1.84±0.13^{b}
–	–	1.0	25.20±8.48^{d}	0.58±0.08^{d}
–	–	2.0	26.00±3.22^{d}	2.06±0.29^{b}

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

3.1.3. Effect of different combinations of cytokinins

The *in vitro* derived nodal explants (45 days) were excised and cultured on MS medium containing BAP in combination with cytokinins such as KIN, TDZ and 2iP (0.5–2 mg/L) (Table–III). Among all the combinations, BAP at 2 mg/L in combination with 0.5 mg/L of KIN was found to be the best for inducing maximum number of shoots (102.00±6.24) along with roots (3.40±0.24) after 7 weeks of culture (Table 3). BAP (1 mg/L) in combination with 2iP (1 mg/L) produced more number of roots of 123.60±10.24 (Table 3) than the shoot number (45.20±7.09). All the combinations of BAP with other growth regulators like KIN, 2iP and TDZ resulted in both shoot and root induction on the same medium.

Table 3
Synergistic effect of cytokinins on multiple shoot induction from in vitro derived nodal explants of *E. axillare*.

Plant Growth regulators(mg/L)				Number of shoots	Shoot length(cm)	Number of roots	Root length(cm)
BA	KIN	TDZ	2iP				
0.5	0.5	–	–	90.20±1.98^{ab}	3.56±0.15^{a}	5.20±0.37^{d}	3.80±0.37^{cd}
0.5	1.0	–	–	61.20±4.77^{cde}	2.10±0.15^{c}	3.40±0.50^{d}	3.00±0.31^{d}
1.0	0.5	–	–	38.60±3.05^{fg}	1.52±0.01^{de}	4.00±0.00^{d}	2.00±0.00^{d}
1.0	1.0	–	–	36.00±2.21^{fg}	1.40±0.07^{e}	3.00±0.00^{d}	3.08±0.10^{d}
2.0	0.5	–	–	102.00±6.24^{a}	3.36±0.20^{a}	3.40±0.24^{d}	1.92±0.18^{d}
2.0	1.0	–	–	76.60±8.68^{bc}	2.64±0.13^{b}	2.40±0.24^{d}	1.68±0.07^{d}
0.5	–	0.5	–	73.80±10.02^{bc}	1.92±0.08^{cd}	66.20±5.03^{c}	3.90±0.20^{bcd}
0.5	–	1.0	–	27.00±2.387^{g}	2.22±0.01^{bc}	69.20±4.46^{c}	6.88±0.83^{a}
1.0	–	0.5	–	90.00±10.05^{ab}	1.80±0.03^{cde}	57.80±4.60^{c}	2.94±0.26^{d}
1.0	–	1.0	–	68.40±4.63^{cd}	1.90±0.04^{cd}	75.60±5.88^{b}	7.16±0.43^{a}
2.0	–	0.5	–	51.22±0.76^{def}	1.40±0.07^{e}	2.40±0.24^{d}	1.92±0.18^{d}
2.0	–	1.0	–	36.12±0.31^{fg}	1.40±0.07^{e}	3.40±0.50^{d}	1.68±0.17^{d}
0.5	–	–	0.5	46.20±7.63^{efg}	2.02±0.12^{cd}	118.80±25.3^{a}	6.22±0.66^{ab}
0.5	–	–	1.0	47.40±7.23^{efg}	2.68±0.44^{b}	83.60±8.72^{ab}	6.66±0.43^{a}
1.0	–	–	0.5	51.00±6.83^{def}	2.64±0.11^{b}	32.60±2.78^{cd}	1.94±0.69^{d}
1.0	–	–	1.0	45.20±7.09^{efg}	2.60±0.13^{b}	123.60±10.4^{a}	5.50±0.85^{abc}
1.0	–		0.5	38.60±3.05^{fg}	1.90±0.04^{cd}	66.20±5.0^{c}	3.90±0.20^{bd}
1.0	–	–	1.0	27.00±2.39^{g}	1.90±0.04^{cd}	32.60±2.78^{cd}	1.68±1.17^{d}

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

3.1.4. Effect of GA_3 in with combination with cytokinins

The microshoots developed on all the above media failed to elongate when subcultured onto the same medium. In order to induce elongation, GA_3 (0.5–2 mg/L) was added to the above media. Among all the concentrations, BAP (2 mg/L) and KIN (0.5 mg/L) in combination with GA3 (2 mg/L) not only resulted in shoot elongation (4.02±0.95 cm) but also increased the number of shoots (108.0±3.55) (Table 4).

Table 4
Effect of cytokinins and GA_3 on multiple shoot induction and elongation from *in vitro* derived nodal explants of *E. axillar*.

Plant Growth regulator mg/L					Number of shoots	Shoot length (cm)	Number of roots	Root length(cm)
BA	KIN	TDZ	2iP	GA3				
2	0.5	–	–	0.5	62.00±0.86^{b}	2.44±0.58^{de}	27.80±0.36^{a}	2.08±0.90^{ab}
2	0.5	–	–	1	78.40±0.11^{b}	2.02±0.21^{ef}	16.20±0.21^{bc}	2.72±0.51^{a}
2	0.5	–	–	2	108.0±3.55^{a}	4.02±0.95^{ab}	7.60±0.46^{d}	2.66±0.54^{a}
1	–	0.5	–	0.5	38.60±1.02^{c}	4.32±0.23^{a}	11.00±0.23^{cd}	1.96±0.29^{b}
1	–	0.5	–	1	36.40±1.68^{c}	2.48±0.65^{de}	11.80±0.84^{cd}	1.12±.67^{cd}
1	–	0.5	–	2	22.40±0.85^{cd}	3.48±0.12^{bc}	9.60±0.25^{cd}	1.68±0.24^{bc}
1	–	–	0.5	0.5	18.20±.041^{d}	1.42±0.21^{f}	0.00±0.00^{e}	0.00±0.00^{e}
1	–	–	0.5	1	30.80±0.11^{cd}	1.28±0.24^{f}	19.60±0.24^{b}	0.9±0.11^{d}
1	–	–	0.5	2	76.00±0.84^{b}	1.82±0.23^{cd}	31.20±0.22^{a}	2.26±0.29^{ab}

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Table 5
Determination of total phenol and flavonoid content *in vitro* and *in vivo* methanol and water extract of *E. axillare*.

Solvent	Total flavonoida		Total phenolb	
	In vitro	*In vitro*	*In vitro*	*In vitro*
Methanol	63.40±9.63	146.57±1.68	19.55±2.97	31.69±0.91
Water	35.75±1.60	37.10±4.48	40.02±1.58	52.53±1.67

In a column, means followed by a common letter are not significantly different at $P<0.05$ by SPSS. [a]Expressed as mg Gallic acid/g of dry plant material, [b]Expressed as mg rutin/g of dry plant material.

3.1.5. Acclimatization

The microshoots obtained from all the above combinations of growth regulators were treated with fungicide Bavistin (0.4%) for 2 min and washed thoroughly in sterile double distilled water. The rooted plantlets were successfully acclimatized in paper cups with vermiculite. About 75% of the plantlets survived. The *in vitro* regenerated plants grew well in the vermiculite without any morphological changes.

3.2. Antioxidant studies

The present work was aimed to explore the potential properties of the plants derived from nature and nodal culture (tissue culture plantlets) of the methanol and water extract of *E. axillare* subsps littorale.

3.2.1. Determination of flavonoid content

Typical phenolics that possess antioxidant activity are known to be mainly phenolic acids and flavonoids. Table 5 indicates that the yield of the *E. axillare* extracts significantly varied with the culture condition and the solvent applied, in order of MeOH (10.3%) > water (8.75%). Among the four extract of MeOH and water from both the plants derived from nature and nodal culture, the flavonoid was found to be high for the MeOH extract (146.57±1.68 mg & 63.40±9.63 mg rutin/g extract for both plants derived from nature and nodal culture, respectively) than the water extract (37.10±4.48 mg & 35.75±1.60 mg rutin/g extract plants derived from nature and nodal culture, respectively).

3.2.2. Determination of phenol content

For the extract compositions, the total phenol content was significantly greater for the water extract (52.53±1.67 mg & 40.2± 1.58 mg GAE/g extract for both the plants derived from nature and nodal culture, respectively) than for the MeOH extract (31.69±0.91 mg & 19.55±2.97 mg GAE/g extract for both the plants derived from nature and nodal culture, respectively). Water extract of *E. axillare* reveals that the total phenol content was higher than the methanolic extract.

3.2.3. DPPH scavenging activity

The DPPH assay provides information about the intrinsic free radical scavenging power in solution irrespective of the physicochemical environments encountered in biological systems. The model system of scavenging DPPH free radical is a simple method to evaluate the antioxidant activity of antioxidants. DPPH was used to determine the free radical scavenging ability to bound phenolics of *E. axillare* as it possess a lone pair of electron (free radical) and shows a characteristic absorption at 517 nm. The purple color of DPPH solution would fade rapidly when it accepts an electron from an antioxidant source to be become a stable

molecule[19]. Figure 1 shows the comparative efficacy of bound phenolics of methanol and water extract of *E. axillare*. At various concentration examined (12.5, 25, 37.5, 50 & 62.5 μg/mL), a general tendency of MeOH extract > water extract was found for inhibition on DPPH scavenging activity for both the plants derived from nature and nodal culture. The antioxidant activity of the MeOH extract of *E. axillare* showed most promosing IC_{50} value of 87.10 μg/mL and 144.75 μg/mL for both the plant from nature and nodal culture, respectively. Whereas, the water extract has lower inhibition activity with IC_{50} value of 150.28 μg/mL and 167.94 μg/mL for both the plant from nature and nodal culture, respectively. The ability of *E. axillare* of two extract was also compared with BHA, BHT, Quercetin, rutin and ascorbic acid in which the DPPH scavenging activity of *E. axillare* was lower than the synthetic antioxidants.

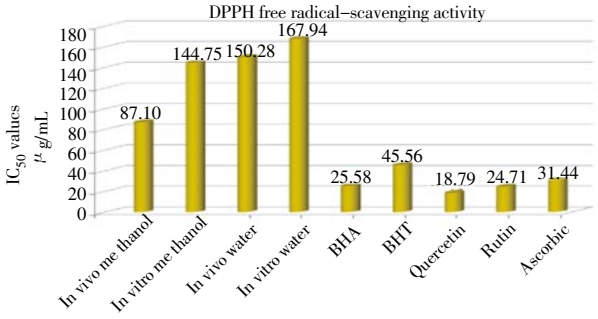

Figure 1. DPPH scavenging activity of *E. axillare* of methanol and water extract of the plant derived from nature and nodal culture.

However, the activity of both extract from *in vitro* condition was comparatively lower than the plants from nature. The yield of secondary metabolites in *in vitro* condition may be enhanced by trigger stress response like using elicitors, precursors and biotransformation, change in environment conditions, change in medium constituents etc.

4. Discussion

Shoot initiation from the nodal segments was mainly a cytokinin effect because the explants in cytokinin free medium did not respond. Cytokinins have been shown to be most critical growth regulator for shoot multiplication in many medicinal plant sp. Like *Gentiana kurroo* (*G. kurroo*) [20]. In present study, medium with BAP acted as trigger for initiating multiplication of nodal explant. This observation is in agreement with *Swertia chirayita* (*S. chirayita*)[21], *Swertia chirata* (*S. chirata*)[22], *Exacum affine* (*E. affine*)[23], and *Feronia limonia* (*F. limonia*)[24], where BAP significantly increased in the shoot number. In contrast, TDZ was found to be best growth regulator for multiple shoot induction in *Swertia angustifolia* (*S. angustifolia*)[25] and *Exacum tranavancoricum* (*E. tranavancoricum*)[26]. Any further increase in concentration more than optimum level of all cytokinins tested did not improve any parameters of shoot multiplication. Optimum rooting response using IAA has been reported for several plants including *S. chirata*[27], *Eustoma grandiflorium* (*E. grandiflorium*)[28] and *Gentiana pneumonanathe* (*G. pneumonanathe*)[29].

BAP (2 mg/L) in combination with KIN (0.5 mg/L) increased multiple shoots induction among the combination of cytokinin used. Our results are in agreement with earlier findings in *S. chirata*[27, 30] where BAP and KIN resulted in marked increase in multiple shoot proliferation. When increasing BAP concentration with decreasing KIN concentration led to a increase in the number of shoot multiplication is well-documented for medicinal plants, such as *Kaempferia galangal* (*K. galangal*)[31], and *Piper longum* (*P. longum*)[32] and *S. chirayita*[21]. Both shoot and root were formed on the medium with combination of cytokinin or with GA_3. These results are in agreement with *Canscora decurrens* (*C. decurrens*) where both shoot and roots were obtained in the same medium[33].

BAP (2 mg/L) and KIN (0.5 mg/L) in combination with GA3 (2 mg/L) produced highest number of multiple shoot along with elongation of shoots. Also, GA_3 promoted shoot development in *Gentiana triflora* (*G. triflora*)[34] and shoot elongation in *S. chirata* along with BAP[30]. The culture of gentians and related species, GA_3 affects the shoots elongation and their multiplication[35].

Antioxidant plays an important role in protecting the human body against the damage caused by reactive oxygen species. Increased oxidative stress has been postulated in the diabetic state. Oxygen free radicals can initiate peroxidation of lipids, which in turn stimulate glycation of proteins, inactivation of enzymes and alteration in the structure and function of collagen, basement and other membranes, and play a role in the long-term complications of diabetes mellitus[36].

Flavonoids as one of the most diverse and widespread group of natural compounds, are likely to be the most important natural phenolics[37]. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties. The total flavonoid was found to be high for the MeOH extract for both plants derived from nature (*in vivo*) and nodal culture (*in vitro*) than the water extract. Our results are in agreement with[38] who reported that total phenol and flavonol content in methanol extract was higher than in other extract (petroleum ether, chloroform and ethyl acetate) of same plant, *E. axillare*. In case of *Halenia elliptica* (*H. elliptica*) belongs to the same family, the total phenol content was found to be high in the MeOH extract[39].

The total phenol was found to be high for the water extract for both plants derived from nature (*in vivo*) and nodal culture (*in vitro*) than the methanol extract. The aqueous extract of *E. littorale* showed the blood glucose lowering effect and glycosylated haemoglobin in alloxan induced diabeteic rats[40] and blood glucose lowering effect of the extract in streptozotocin-treated rats[41]. The insulin secretory effect of an aqueous extract of *E. littorale* has also been reported by[42]. The above said activities of aqueous extract of the genus Enicostema may be due to the presence of alkaloids, sterols, catechins, phenolic acids & coumaric acid and also contains minerals and aminoacids[41] and compounds that may exert a synergistic effect. By this reports, we can conclude that the activity of water extract may be due to the presence of those phytochemicals present in the water extract of *E. axillare*.

In present study, DPPH radical scavenging activity was found to be high in methanol of plant collected in nature condition (*in vivo*). Similar results were obtained in *H. elliptica* in which the highest DPPH free radical scavenging activity was reported in methanol extract[39]. Flavonoid antioxidants function as scavengers of free radicals by rapid donation of a hydrogen atom to free radicals[43]. The superior activity of methanol extract to that of water extract could be due to the high flavonoid content. Our results are in agreement with[38] in which due to the presence of alkaloids, phenols, saponins, iridoid glycosides and tannins in methanolic extract may also contribute to the total antioxidant activities in *E. axillare*. Also, DPPH radical scavenging activity was found to be higher in ethanol extract of *S. chirayita*[44] which belongs to the same family.

The micropropagation method described provides reproducible and effective method for mass multiplication. Shoot multiplication from leaf and nodal explants of *E. littorale* reported earlier[14] yielded 86.8±3.9 shoots per explants from segmented leaf callus. However, a microprogation system that includes a callus phase is undesirable due to the possible introduction of somatic genetic variability in the resulting propagules[45]. In the present study, there was a significant increase in the shoot multiplication with the added benefit that both shoot and root were induced in the same medium. This *in vitro* protocol can serve as a tool for mass multiplication of elite genotype and also for extraction of bioactive compounds of interest from this plant species. Also, there is a strong correlation between phenolic compounds implied that antioxidants were capable of scavenging free radicals in both the plants developed from nature and nodal culture of *E. axillare*(Lam.) Raynal ssp. *littoralis* (Blume) Raynal.

Conflict of interest statement

We dclare that we have no conflict of interest.

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