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ORIGINAL ARTICLE

High frequency direct shoot organogenesis of leaf explants and a comparative evaluation of phytochemicals, antioxidant potential of wild vs. in vitro plant extracts of *Lysimachia laxa*

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Abstract The present studies were attempted to develop direct shoot organogenesis from in vitro grown leaf explants of *Lysimachia laxa* and comparative evaluation of phytochemical and antioxidant potential of in vitro raised and wild plants extracts. The fresh leaves of this species are used for deworming gastrointestinal worm infection in traditional medicine. Overexploitation of this species and poor regeneration has led to rapid decline in wild population, therefore, present investigation was attempted to develop an efficient rapid mass propagation protocol for this species. Our result showed significantly ($P < 0.05$) high adventitious shoot proliferation of 17.21 ± 0.24 number per leaf explants cultured in Murashige and Skoog medium fortified with 1.25 mg L^{-1} thiadizuron and 1.0 mg L^{-1} α-naphthalene acetic acid. Further enhancement was achieved through elongation medium fortified with 1.0 mg L^{-1} 6-benzylaminopurine by average shoot number of 31.1 ± 0.80 and length of 5.96 ± 0.13 cm. Murashige and Skoog medium fortified with 0.50 mg L^{-1} Indole-3-acetic acid showed high rooting induction (100%) with average root number of 11.70 and length 7.35 cm. All rooted plants were successfully acclimatized in greenhouse and transferred to field condition with a survival rate of 97%. The contents of phenolic and flavonoid were higher in in vitro raised plant in compared to wild plant extracts. Antioxidants assay showed high radical scavenging activity of IC_{50} 1.61 ± 0.07 mg dry material and reducing power of 49.79 ± 0.11 mg/g ascorbic acid equivalent by aqueous methanol extracts of in vitro raised 3-months-old plants in compare to the wild plants. The present protocol is a viable option for pharmaceutical or nutraceutical industries for sustainable utilization of *L. laxa* with enhanced of phytochemical and antioxidant potency which is not reported elsewhere.

Keywords *Lysimachia laxa* · Direct organogenesis · Phytochemicals · Antioxidant · In vitro and wild plant extract

Introduction

Lysimachia laxa Baudo (Syn; *Lysimachia ramosa* Wall ex Duby) is a perennial herb belonging to the family of Primulaceae. About 180 species are reported from temperate and subtropical forest of Northern Hemisphere, but with a few species reported in Africa, Australia, and South America (Hu and Kelso 1996). In India, *L. laxa* is reported from Meghalaya, Arunachal Pradesh, Manipur and Nagaland at the altitude between 1800 and 2000 m (Gupta et al. 2012).

The fresh leaf of *L. laxa* is widely used for deworming gastrointestinal worm infection (Challam et al. 2010; Mao et al. 2009) and antioxidant activity was reported in different parts of *L. laxa* by Gupta et al. (2013b). Some other species of the genus viz. *L. clethroide* (Jiang et al. 2007)

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and *L. davurica* (Liang et al. 2006) are also reported to have pharmacologically bioactive compounds viz. triterpenoid, saponins, organic acid, flavones and flavonoids. For its medicinal value, *L. laxa* has been over harvested in nature, other anthropogenic activity and also poor seed germination resulted in rapid decline of population in the wild. Consequently, it is crucial demand to develop micropropagation protocols for its mass production of *L. laxa*. However, previous study on the species showed multiple shoot induction through nodal and shoots tip with moderate succeeded in term of number of shoots production (Gupta et al. 2012) while present studies demonstrated high frequency direct shoot organogenesis through leaf explants without intervening callus which is considered as having minimum risk of somaclonal variation and moreover this technique provide a vital option for transgenic implementation.

Plants are sources of diverse bioactive components like phenolic, polyphenolic, flavonoid, flavonol, saponin, terpene, etc., having significant antioxidant potential to protect from highly unstable free radical bombardment in cellular microenvironment (Gupta et al. 2013b). Thus, antioxidants are complex molecules able to prevent and cure several oxidative damages which could be chronic health problem viz. cancer, aging, atherosclerosis, ischemic injury, and neurodegeneration (Shinde et al. 2010; Surabhi and Leelavathi 2010). Antioxidant potential has been assessed in several medicinal plants species for herbal drugs, nutraceutical and cosmetic formulations (Miliauskas et al. 2004).

Therefore, it is important to search for sources of bioactive antioxidant compounds derived from natural resources, especially high altitude plants, which are adapted to various stress conditions. Plant derived polyphenolic group is also well recognized as potent dietary antioxidants agent (Aprotosoaie et al. 2016). The present investigation on antioxidant potential of *L. laxa* was undertaken with special reference to high altitude plants species. Moreover, this bioactive constituent is having diverse chemical characteristics which are responsible for selection of different polarities of solvent extraction system and antioxidant activities. Polar solvents are more frequently used for the retrieval of polyphenols from a plant matrix. Methanol, ethanol, acetone, chloroform, and ethyl acetate have been extensively used as extraction solvent of antioxidant compounds from various plants. Present investigation employed three different polarities solvent system.

The objective of present investigation on *L. laxa* was to develop high frequency direct shoot organogenesis from leaf explants and comparative evaluation of phytochemical and antioxidant potential in tissue culture raised and wild plants which is not reported elsewhere.

Materials and methods

Fresh leaves and seeds of *L. laxa* were collected from Talle valley at an altitude of 1800–2000 m, in Lower Subansiri district, Arunachal Pradesh, India. The specimen (field number Mao and Gupta/No. 19205) was identified and deposited in 'ARUN' Harbarium of Botanical Survey of India, Arunachal Pradesh Regional Center, Itanagar, India. The collected plant material was dried at room temperature and made into powder for biochemical and antioxidant evaluation. To induce direct shoot organogenesis from axenic leaves, in vitro germinated 2-months-old seedlings with expanded leaves were used as a source material. All the chemicals and solvents were used analytical grade viz. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyl toluene (BHT), ascorbic acid, and quercetin (Sigma Chemical Co., St. Louis, MO, USA) whereas Folin–Ciocalteus's phenol reagent, gallic acid, potassium ferricyanide, aluminum chloride ($FeCl_3$), and sodium carbonate were obtained from Merck chemical (Darmstadt, Germany).

Adventitious shoot bud regeneration

The leaf with petiole or without petiole explants were cultured on semisolid MS (Murashige and Skoog 1962) medium for shoot buds regeneration. The leaves with an average size 1.0–1.5 cm^{-2} were implanted in the medium with the abaxial side facing down. The MS medium with 0.8% (w/v) Bacto agar and 3% (w/v) sucrose was used as a control against to treatments supplemented with different concentrations of thiadizuron (TDZ) (0.5, 1.25 and 2.0 mg L^{-1}) either alone or in combinations with 6-benzylaminopurine (BA) (1, 2 and 4 mg L^{-1}), α-naphthaleneacetic acid (NAA), Indole-3-butyric acid (IBA) (0.5, 1 and 2 mg L^{-1}) (Table 1) were tested for initiation of direct shoot organogenesis.

Shoot proliferation and elongation

The adventitious shoot buds regenerated from leaf explants were used for further investigation of rapid shoot proliferation and elongation. The adventitious shoot clusters were fragmented and transferred to MS media supplemented with low concentrations of either alone TDZ (0.25, 0.50, 1.0 mg L^{-1}) or BA (0.50, 1.50, 2.50 mg L^{-1}) (Table 2) for shoot proliferation and elongation. Plant growth regulators (PGRs) free, basal medium was used as the control in the experiments.

Table 1 Morphogenic response leaf explants of *L. laxa* on MS medium with TDZ singly or in combination with either BA, NAA or IBA

Treatments (mg L^{-1})				*Lysimachia laxa* leaf explant	
TDZ	BA	NAA	IBA	% of response	No of shoot buds
0.5	0	0	0	38.88	1.42 ± 0.20l
1.25	0	0	0	87.77	9.12 ± 0.51fghi
2.0	0	0	0	81.11	8.18 ± 0.56hij
1.25	1	0	0	73.33	9.43 ± 0.69fgh
1.25	2	0	0	74.44	11.14 ± 0.73de
1.25	4	0	0	70	9.67 ± 0.73fg
1.25	0	0.50	0	100	15.94 ± 0.23ab
1.25	0	1.0	0	100	17.21 ± 0.24a
1.25	0	2.0	0	93.33	14.70 ± 0.51bc
1.25	0	0	0.50	100	12.26 ± 0.38d
1.25	0	0	1.0	91	10.18 ± 0.50ef
1.25	0	0	2.0	84.44	7.90 ± 0.48ijk
LSD				–	1.43

Means followed by the same letters within a column are not significantly different (Fisher's LSD; $P < 0.05$)

Table 2 Effect of lower concentrations of TDZ and BA on adventitious shoot bud proliferation and elongation

Treatments (mg L^{-1})		*Lysimachia laxa*	
TDZ	BA	Mean shoot no	Mean shoot length
0	0	7.1 ± 0.29g	3.80 ± 0.12g
0.25	0	16.4 ± 0.49f	5.19 ± 0.11bc
0.50	0	20.7 ± 0.88c	5.12 ± 0.12bcd
1.0	0	18.6 ± 0.84cde	4.95 ± 0.14cde
0	0.50	24.7 ± 0.86b	5.45 ± 0.19b
0	1.5	31.1 ± 0.80a	5.96 ± 0.13a
0	2.5	19.5 ± 0.91cd	4.75 ± 0.13def
LSD		2.11	0.39

Means followed by the same letters within a column are not significantly different (Fisher's LSD; $P < 0.05$)

Rooting induction and acclimatization

The microshoots of 1–1.5 cm length with 3–4 leaves, excised from the cluster of shoots were used for rooting experiment. The excised shoots were inoculated on MS basal medium without hormone as control and MS supplemented with Indole-3-acetic (IAA) or IBA at different concentration (0.25, 0.50 and 1.0 mg L^{-1}) (Table 3) for rooting induction. All data were recorded after 6 weeks of inoculation as percentage of rooting, roots number and roots length. Eight-weeks-old in vitro rooted plants were carefully removed from cultures media and washed thoroughly in running tap water to remove all the adhered culture medium and treated with 1% Bavistin (M/s BASF India Pvt. Ltd. India), a broad spectrum fungicide, for 10 min before being transferred to a root trainer (Agro Vision Biotech, 164 Jaipur, India) containing a mixture of 1:1 ratio leaf mold and soil. The plants were kept under greenhouse condition at 24 °C with a relative humidity of 90%. All plantlets were treated with half strength MS solution at 7 days intervals for 1 month and subsequently all acclimatized plants were transferred to field condition.

Phytochemical and antioxidant potential

One gram powdered of each plant material of wild and in vitro raised 3-month-old plants were extracted with 20 ml each of methanol, aqueous methanol (aq.methanol) (50% v/v) and acetone, with staring of 18–24 h at room temperature. All extracts filtered through Whatman No. 1 filter paper and diluted to 50 ml with respective solvent. The extracts were analyzed for total phenolic, flavonoid,

Table 3 Effect of various auxins on root induction from in vitro raised microshoots of *L. laxa* after 6 weeks of continuous culture

Treatments (mg L^{-1})		% Responses	No of roots	Root length (cm)
IAA	IBA			
0	0	48	2.86 ± 0.43def	3.23 ± 0.46cdef
0.25	0	100	8.43 ± 0.23b	6.10 ± 0.18ab
0.50	0	100	11.70 ± 0.40a	7.35 ± 0.29a
1.00	0	100	6.25 ± 0.41bc	5.06 ± 0.20bc
0	0.25	58	3.88 ± 0.47d	4.05 ± 0.47cde
0	0.50	76	3.25 ± 0.26de	4.10 ± 0.34cd
0	1.00	72	2.55 ± 0.26defg	3.40 ± 0.30cdef
LSD			2.42	1.91

Means followed by the same letters within a column are not significantly different (Fisher's LSD, $P < 0.05$)

flavonol content, reducing power, and free radical scavenging activities.

Estimation of total phenolic content

Folin–Ciocalteu method was followed for total phenolic contents of crude extracts (Singleton and Rossi 1965). 20–100 µl of each samples were taken into test tubes followed by 1.0 ml of Folin–Ciocalteu reagent and 0.8 ml of sodium carbonate (7.5%) was added and finally mix in test tube, solutions was allowed to stand for 30 min. Absorption at 765 nm was measured (UV-Visible spectrophotometer Hitachi U 2000 Japan). The total phenolic content was expressed as gallic acid equivalents (GAE) in milligram per gram (mg/g) of extract. The total phenolic content estimated by the equation of calibration curve; $y = 0.0013x + 0.0498$, $R^2 = 0.999$ where y was the absorbance and x was the Gallic acid equivalent (mg/g).

Estimation of total flavonoids content

The total flavonoid was estimated (Ordonez et al. 2006) by adding 0.5 ml of 2% $AlCl_3$ ethanol solution to the sample of 0.5 ml. After 1-h incubation at room temperature, the absorbance was measured at 420 nm (UV-visible spectrophotometer Hitachi U 2000 Japan). The presence of flavonoids were confirm with a yellow color of sample. Total flavonoid content was estimated as rutin (mg/g) using the equation based on the calibration curve; $y = 0.0182x - 0.0222$, $R^2 = 0.9962$, where y was the absorbance and x was the Rutin equivalent (mg/g).

Estimation of total flavonol content

The total flavonols in the plant extracts were evaluated (Kumaran and Karunakaran 2006) by adding 2.0 ml of 2% $AlCl_3$ ethanol and 3.0 ml (50 g L^{-1}) sodium acetate solutions to 2.0 ml of extract. The absorption at 440 nm (UV-visible spectrophotometer Hitachi U 2000 Japan) was measured after 2.5 h. Total flavonol content was calculated as quercetin (mg/g) based on the equation calibration curve; $y = 0.0049x + 0.0047$, $R^2 = 0.9935$, where y was the absorbance and x was the quercetin equivalent (mg/g).

Ferric reducing antioxidant power (FRAP)

The reducing power of plant extracts was estimated (Oyaizu 1986) by mixing 100 µl of plant extracts with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 1% potassium ferricyanide (2.5 ml). The mixture was incubated at 50 °C for 20 min. Aliquots of 10% trichloroacetic acid (2.5 ml) were added to the mixture followed by centrifugation at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.1%) of 0.5 ml. The absorbance was measured at 700 nm. Reducing power is given in ascorbic acid equivalent (AAE) in milligram per gram (mg/g) of dry material based on equation derived from the calibration curve; $y = 0.0023x - 0.0063$, $R^2 = 0.9955$ where y was the absorbance and x was the ascorbic acid equivalent (mg/g).

Determination of free radical scavenging activity

The free radical scavenging activity of the plant samples and butylated hydroxyl toluene (BHT) as positive control was determined using the stable radical DPPH (1, 1-diphenyl-2-picrylhydrazyl) (Blois 1958). Aliquots (20–100 µl) of the tested samples were decanted in test tubes and 3.9 ml of freshly prepared DPPH solution (25 mg L^{-1}) in methanol was added in each test tube and well mixed. The absorbance was measured at 517 nm (UV-visible spectrophotometer Hitachi U 2000 Japan) after 30 min.

The capability to scavenge the DPPH radical was calculated, using the following equation:

$$\text{DPPH scavenged } (\%) = \{(\text{Ac} - \text{At}) \div \text{Ac}\} \times 100$$

where 'Ac' is the absorbance of the control reaction and 'At' is the absorbance of the extracts sample. The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration in mg of dry material per ml (mg/ml) that inhibits the formation of DPPH radicals by 50%. Each value was determined from regression equation.

Statistical analysis

All the data were collected after 8 weeks for shoot regeneration, 7 weeks for shoot proliferation and elongation, and 5 weeks for rooting experiments. The experiment consist of 30 replicates for each treatment and thrice repeated for direct shoot organogenesis. While each phytochemical assay and antioxidant activities were performed three replicates. All the collected data were subjected to analysis of variance (ANOVA), and the means were compared using Fisher's least significant difference (LSD) by SPSS version 22.

Result

Direct shoot organogenesis

Both explants (leaf without petiole and leaf with petiole) responded well without any variation in TDZ supplemented medium while control or BA alone supplemented medium did not produced any responses in shoot bud induction. Morphological variations on the inoculated leaf explants were observed initially on petiole from its tip to leaf apex along the midrib region in the 2nd week of culture and finally running through entire leaf surface. Eventually, number of shoots initiated from the petiole region to midrib and further running throughout the leaf on the 4th week (Fig. 1a).

After 7 weeks of culture, among three combinations tested viz., TDZ + BA, TDZ + IBA and TDZ + NAA (Table 1); the optimal combination was observed on TDZ (1.25 mg L^{-1}) + NAA (1 mg L^{-1}) which showed significantly (LSD 1.43; $P < 0.05$) high adventitious shoot bud organogenesis in leaf explants. At this optimal medium recorded with 100% regeneration frequency and mean shoot bud number of 17.21 ± 0.24 (Fig. 1b). The present studies revealed that TDZ in combination with NAA significantly enhanced shoot buds formation than any other combinations with BA or IBA. However, cultures more than 7th week old at high concentration of TDZ (2 mg L^{-1}) produced fasciated shoot in some tested explants (Fig. 1d).

Proliferation and elongation of adventitious shoots

After 7 weeks of continuous culture, the shoot clusters were transferred to MS medium containing low concentration of either TDZ or BA. The highest mean shoots number and length per explant was recorded on MS medium with BA (1.50 mg L^{-1}) (Table 2). Of these two hormones, BA alone gave highest mean shoot number and length to 31.1 ± 0.80 and 5.96 ± 0.13 cm, respectively (Fig. 1c). Thus, BA at the optimal concentration (1.50 mg L^{-1}) shows significant (LSD 2.11; $P < 0.05$) difference in compare to TDZ treatment in proliferation and elongation medium. In addition, control treatment also show significant registered mean shoot number of 7.1 ± 0.29 and shoot length of 3.80 ± 0.12 cm.

Rooting and acclimatization

All the shoots developed rhizogenesis with various responses depend on auxins tested. Of the two auxins tested, 100% rooting was observed on IAA treatments in comparison to IBA (Table 3). The optimal concentration of IAA (0.50 mg L^{-1}) showed significantly (LSD 2.11; $P < 0.05$) high mean root number and length of 11.70 and 7.35 cm (Fig. 1e), respectively, within 6 weeks of cultured in comparison to control (Fig. 1f) or IBA treatment. In vitro grown rooted plantlets were successfully acclimatized with 100% survival rate in field condition without any morphological variation (Figs. 1g, h).

Phytochemical potential

The present investigation showed yield potential of three solvent extractive value, the extractive yield of wild plant significantly ($P < 0.05$) differs with in vitro raised 3-months-old plant extracts excluding methanol and aqueous methanol (1:1) solvent extracts yield (Table 4). The highest amount of extract yield obtained significantly ($P < 0.05$) in aq.methanol solvent extraction in both wild and in vitro plant extracts as 29.56 ± 0.29 g/100 g dry material and 22.83 ± 0.17 g/100 g dry material respectively. While lowest extracts yield obtained in acetone extracts in both wild and in vitro plant extracts.

The results of phenolic content evaluated in different solvent extracts (methanol, aq.methanol and acetone) of wild vs. in vitro plant extracts are given in Table 4. The highest amount of total phenolic was obtained significantly ($P < 0.05$) in aq.methanol extracts of in vitro grown plant (22.32 ± 0.05 mg GAE/g dry material) in compare to wild

Fig. 1 Direct organogenesis from leaf explants of *L. laxa*. **a** Initiation start on the surface of leaf lamina after 2nd week cultured. **b** Shoots proliferation in MS medium fortified with TDZ + NAA (1.25 + 1 mg L^{-1}) after 4th week cultured. **c** Shoot buds elongation and proliferation in MS medium fortified with 1.50 mg L^{-1} BA after 7th week cultured. **d** *Arrow mark* fasciation shoot at the concentration of 2 mg L^{-1} TDZ after 8th week cultured. **e** Rooting in MS medium fortified with 0.50 mg L^{-1} IAA after 6 weeks. **f** Rooting in control after 6 weeks cultured. **g** 3-months-old under greenhouse condition. **h** Acclimatized plants after 4 months in natural condition. In all figures showing *scale bar* in 1 cm length excepting figs. **g** and **h** *scale bar* 5 cm in length

plant extracts (14.69 ± 0.08 mg GAE/g dry material). The lowest amount of phenolic content was obtained in acetone extracts of both wild (1.80 ± 0.08 mg GAE/g dry material) and in vitro plant extracts (2.07 ± 0.47 mg GAE/g dry material).

The flavonoid contents of different solvent extracts varies significantly ($P < 0.05$) in all solvent extractions both in wild and in vitro grown plants (Table 4). The amount of flavonoid obtained from aq.methanol extracts of in vitro grown plant (7.93 ± 0.05 mg Rutin equivalent mg/g dry material) was significantly higher than wild plant extracts (5.63 ± 0.01 mg Rutin equivalent mg/g dry material). Aq.methanol solvent extracts as the best method for flavonoid extraction in *L. laxa*.

The flavonols contents of different solvent extracts varies significantly ($P < 0.05$) in all the solvent extractions in both wild and in vitro grown plant extracts (Table 4), the highest amount of flavonols was observed in the methanol extract of in vitro grown plant extracts (17.17 ± 0.14 mg quercetin equivalent/g dry material) and the lowest amount of flavonols content (5.20 ± 0.05 mg quercetin equivalent/g dry material) was observed in wild plant aq.methanol extracts. In addition in vitro grown plants in acetone extracts displayed higher than wild plant extract.

Table 4 Phytochemical estimation and antioxidant potential of wild vs. micropropagated (3-month-old culture) plant extracts on methanol, Aq-methanol (1:1) and acetone solvent

	Wild plant extracts			In vitro plant extracts (3-month-old culture)		
	Methanol	Aq-methanol (1:1)	Acetone	Methanol	Aq-methanol (1:1)	Acetone
Extractive value (g/100 g Dry material) (LSD 1.2; $P < 0.05$)	23.39 ± 0.17b	29.56 ± 0.29a	13.72 ± 0.06d	12.50 ± 0.29e	22.83 ± 0.17bc	7.17 ± 0.02f
Phenolic content (GAE equivalent mg/g) (LSD 2; $P < 0.05$)	11.54 ± 0.43d	14.69 ± 0.08c	1.80 ± 0.08ef	20.47 ± 0.44ab	22.32 ± 0.05a	2.07 ± 0.47e
Flavonoids content (Rutin equivalent mg/g) (LSD 0.42; $P < 0.05$)	4.53 ± 0.02d	5.63 ± 0.01c	3.84 ± 0.02e	4.92 ± 008c	7.93 ± 0.05a	6.29 ± 0.12b
Flavonols content (Quercetin equivalent mg/g) (LSD 0.48; $P < 0.05$)	6.48 ± 0.02e	5.20 ± 0.05 f	7.24 ± 0.04d	17.17 ± 0.14a	8.61 ± 0.09c	11.81 ± 0.04b
Reducing power (ascorbic acid equivalent mg/g) (LSD 1.37; $P < 0.05$)	36.62 ± 0.08d	42.76 ± 0.12c	19.69 ± 0.42 f	43.87 ± 0.26b	49.79 ± 0.11a	32.65 ± 0.10e
DPPH radical scavenging (IC_{50} mg/g dry material) (LSD 0.58; $P < 0.05$)	3.02 ± 0.001de	2.57 ± 0.11bcd	3.92 ± 0.10f	2.04 ± 0.06ab	1.61 ± 0.09a	2.22 ± 0.14bc

Means followed by the same letters within a row are not significantly different (Fisher's $P < 0.05$)

Antioxidant potential

Ferric reducing antioxidant power (FRAP), the reducing powers of these two explants varies significantly ($P < 0.05$) in the all tested solvent system and evaluated as ascorbic acid equivalent (mg AAE/g) dry material (Table 4). The highest reducing power was exhibited significantly in aq.methanol extract of in vitro grown plant extracts (49.79 ± 0.11 mg/g AAE) in compare to aq.methanol extract of wild plants (42.76 ± 0.12 mg AAE/g dry material), while acetone extract of wild plant showed lowest reducing power activity (19.69 ± 0.42 mg/g AAE) in terms of ascorbic acid equivalent. In addition, the acetone extracts of in vitro grown plant displayed higher than wild plant extracts. In the present investigation the highest radical scavenging activity was exhibited significantly ($P < 0.05$) in the aq. methanol extract of in vitro grown plant extracts ($IC_{50} = 1.61 \pm 0.07$ mg dry material) in compare to wild plant extracts ($IC_{50} = 2.57 \pm 0.11$ mg dry material).

Discussion

Direct shoot organogenesis

The effect of TDZ on direct shoot organogenesis in leaf explants of *L. laxa* has been demonstrated. The morphogenic potential of TDZ in stimulating adventitious shoot organogenesis has been proved using different parts as explants (Karakas and Turker 2013). The present result indicated that all the concentration of TDZ was able to induce adventitious shoot buds in leaf explants, α-naphthaleneacetic acid (NAA) with optimal TDZ showed cent percent response of adventitious shoot buds induction while BA alone failed to give any responses in *L. laxa*. Present results differs in responses from the earlier report of two species of *Lysimachia* (*L. christinae* and *L. nummularia*) where BA was reported as the most effective for direct shoot organogenesis from leaf explants (Zheng et al. 2009). Thus the morphogenic potential varies species to species within the same genus or need to assign a diverse range of plant growth regulators to optimized protocols for genus level. High concentration of TDZ promoted callus formation in *L. laxa*. The callus was observed embryogenic, which eventually developed into shoots after they were transferred to elongation medium but the present studies was aimed for only developing a mass production system through direct organogenesis without callugenesis. The present investigation showed that TDZ played an epicentric role in adventitious shoots induction in leaf explants of *L. laxa*. The potency of TDZ has been well known for inducing various morphogenic responses under in vitro condition. TDZ stimulates *de novo* synthesis of auxins by increasing the levels of IAA and its precursor, tryptophan (Murthy et al. 1995). TDZ has proved to be a valuable plant growth regulator for rapid plant regeneration in several recalcitrant species through organogenesis (Malik and Saxena 1992) or shoots proliferation through indirect organogenesis in *Bellis perennis* (Karakas and Turker 2013). At high concentration of TDZ and prolonged culture induced callugenesis as well as fasciation shoot

(Fig. 1d), which was also observed in *G. assamicus* (Gupta et al. 2013a). Similar observation was reported by Graham et al. (1997). This problem can be solved through frequent subculture at low concentration of cytokinins after initiation phase.

After 7 weeks of continuous culture, induced microshoots clusters were transferred to MS medium containing either TDZ or BA. Of these two hormones, BA alone at a concentration of 1.5 mg L^{-1} enhanced maximum shoot number and length in *L. laxa* (Fig. 1c). It might be interpreted as synergetic effect, which could induced endogenous hormone during initiation of culture in TDZ + NAA medium and eventually endogenous hormone interact with plant growth regulator BA on elongation medium to enhance the average shoot number and length. Further need investigation on endogenous hormone profile to understand the exact mechanism behind proliferation and elongation of shoots during transfer in low concentration in secondary medium. Thus our findings suggested that after triggering the initiation of adventitious shoot buds it is essential to transfer in low concentration in secondary medium for proliferation and elongation of shoot buds. Similar observation was made in secondary medium where *Gaultheria fragrantissima* responded better in proliferation and elongation medium (Cao et al. 2002; Debnath 2009; Ranyaphi et al. 2011). Thus our reproducible protocol, using the leaf explants without callus phase will be suitable for *Agrobacterium tumefaciens* mediated genetic transformation and for further mass production of true to type plants for industrial harvest or domestic market consumption of this important ethnomedicinal species from Northeast India.

Rooting and acclimatization

Rooting is crucial and important phase for in vitro grown micro shoots, where in some plant species rooting phase is found to be bottle neck for micropropagation. However, the uniformly grown microshoots of *L. laxa* formed roots using both IAA and IBA. The rooting response in basal MS medium could be attributed to the carry over effect or endogenous auxin level of in vitro grown microshoots. Similar findings reported in *Clerodendrum colebrookianum* (Mao et al. 1995). In terms of getting more number of healthy and lengthy roots, IAA was found to be better than IBA for *L. laxa*. Similar finding was reported in *Glossogyne tenuifolia* (Chen et al. 2014).

Phytochemical potential

Today's health care system is rapidly relocating towards natural therapy and medicine, for it has lesser side effects, cost effective and eco-friendly. Our present investigation made comparative antioxidant and phytochemical potential of wild plant extracts and in vitro 3-months-old raised plant extracts of *L. laxa* which is being used in ethnomedicine by local tribal of Northeast India. The investigation shows differences in extracted value of the plant extracts which could be access as varying nature of the components present and depends on the polarities of the solvent used for extraction (Sarwar et al. 2012). Phenolic compounds are one of the major diversified antioxidant agents having their redox properties to adsorb or quenching the free radicals (Florence et al. 2011). The present investigation showed in aq.methanol, higher phenolic content in in vitro raised plant extracts in compare to absolute methanol or acetone from wild plant extracts. It might be interpreted that in vitro raised plant produced more secondary metabolite in circumstances of various nutrient and control environment system and also phenolics are more prone to capture in higher amount in more polar solvents system as compare to aqueous methanol/ethanol with absolute methanol/ethanol or acetone (Sultana et al. 2009). Several authors had reported enhancement of secondary metabolite in micropropagated plant, tissue or cell type materials over that of the field grown plants (Dias et al. 2000; Romero et al. 2009; Shinde et al. 2010).

The investigation in total flavonoid content of different plant materials, using three different solvent systems have shown highest amount of flavonoid content in the aq.methanol extract of in vitro raised plant in compared to wild plant extracts. Moreover, in vitro raised plant extracts in acetone solvent also displayed flavonoid content enhancing in compare to wild plant extracts. It may be said that in vitro culture system could synthesis those flavonoid components which are able to dissolve in acetone solvent and consequently more extracts as compare to wild plant extract. Our present study showed that the aq.methanolic extracts were better option for flavonoid extraction. The highest amount of flavonol was obtained in the methanol extract of in vitro raised plant in compare to wild plant methanolic extracts. Similar finding reported in *Cucumis anguria* (Thiruvengadam and Chung 2015). Overall our finding shows that in vitro raised plant extracts better performed in aq.methanol solvent for the extraction of phenolic and flavonoids components from the plant whereas worthy amount of flavonol can be obtained using methanol as extracting solvent. Similar finding had been reported by Gupta et al. (2013b). Several authors had also reported that plantlet regenerated through in vitro culture system have a potential for the synthesis of bioactive compound in medicinal plant (Abbasi et al. 2010; Baskaran et al. 2014; Thiruvengadam and Chung 2015). Our findings are similar results reported that micropropagated *Moringa oleifera* is superior in nutrients content in compared to conventional grown plants (Saini et al. 2012).

Antioxidant potential

The reducing power is based on electron transfer assay involving the reduction of potassium ferricyanide followed by the generation of Prussian blue color in tested samples (Berker et al. 2007; Aprotosoaie et al. 2016). The highest reducing power was exhibited by the aq.methanol extract of in vitro raised plant in compare to wild plant extracts of *L. laxa* which is also significantly high in phenolic and flavonoid contents. In vitro culture plants on MS medium or PGRs might be enhancing or accumulating the phenolic, flavonoids and flavonol contents of *L. laxa*. Other authors also reported that in vitro culture of medicinal plants resulted in an increase in secondary metabolites like phenolic compounds and hypericin in in vitro culture of *Hypericum perforatum* L. (Savio et al. 2012).

The present evaluations of anti-radical properties of the three different solvent extracts were performed by DPPH radical scavenging assay. The antioxidant molecules can quench DPPH free radicals by providing hydrogen atom or by electron donation at the cost of forming colorless stable molecule 2, 2-diphenyl-1-hydrazine with the rate of decreasing in absorbance (at 517 nm) of the solution. Thus the more potent antioxidant reflects more decrease in absorbance and consequently IC_{50} value will be lowest (Gupta et al. 2013b). In the present study the highest radical scavenging activity was shown by the aq.methanol extract of in vitro raised 3-months-old plants in compare to wild plants extracts. The high DPPH radical scavenging activities of the aq.methanol extract of *L. laxa* may be due to the presence of high amount of phenolic components and flavonoid contents that can be provide the necessary component as a free radical scavenger. Several authors reported PGRs and media could be influence in the enhancement of secondary metabolite in cell, tissue, organ culture or entire plant (Thiruvengadam et al. 2014; Baskaran et al. 2014). Thus, enhancement in phenolic, flavonoid, and flavonols components in tissue culture regenerated plantlets as a part of accumulation in secondary metabolites which is a viable option for medicinal or nutraceutical industries without disturbance natural resources. In addition, our results show that methanolic, aq.methanolic, and acetone extracts exhibited different degree of antioxidant potential. The extracts obtained through high polar solvents (aq.methanol) were found to be more effective radical scavengers in compare to those are using less polar solvents (methanol and acetone).

Conclusion

The present study was the first attempt to evaluate high frequency direct shoots organogenesis from leaf explant of an important ethnomedicine species, *L. laxa* and its phytochemical potential beside antioxidant efficacy of in vitro raised plantlets and wild plant extracts on three solvent systems. The investigations have shown that optimal TDZ in combination with NAA proliferated maximum initiation of adventitious shoot organogenesis from leaf explants. Our finding suggested that TDZ and NAA have crucial synergistic effects in proliferation of direct shoots organogenesis from leaf explants of *L. laxa*. In addition to, it is concluded that the dual phase cultural system was best option to give high shoots multiplication in elongation and proliferation medium. The plantlets rooted well in all the tested auxine and even in control. All the rooted plantlets were acclimatized with 97% survival in field condition. The present protocol is highly efficient and suited for industrial crops production without loss of phytochemical profile in *L. laxa* which is not reported elsewhere.

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Author contributions SG implemented the all experiments, analyzed the data and wrote the manuscript. AAM, TS and SS planned the work, edited the manuscript and monitored the entire research work.

Compliance with ethical standards

Conflict of interest All authors declare that they have no any conflict of interest in this publication. This publication is approved by the author and all coauthors. The work was carried out at Plant Tissue culture Lab, Botanical Survey of India, ERC, Shillong.

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