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EFFICIENT REGENERATION AND ANTIOXIDANT ACTIVITY OF THE ENDANGERED SPECIES *SWERTIA CHIRAYITA*

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ABSTRACT

An efficient in vitro regeneration protocol was established for *Swertia chirayita*. The present study describes the regeneration of *S. chirayita* from shoot tip explants collected from wild grown plantlets. The highest number of shoots (26.10 ± 0.73) were obtained on Murashige and Skoog basal medium supplemented with 6-benzyladenine (BA) (0.5mg/l) in combination with gibberellic acid (GA_3) (1.0 mg/l). The mean number of roots (11.46 ± 1.56) was obtained on MS medium supplemented with IAA (1.0 mg/l). Well rooted plantlets were acclimatized and transferred to soil successfully. An assay of the antioxidant potential of the in vitro grown tissues revealed that the antioxidant activity of the regenerated shoots was significantly higher than wild-grown plantlets.

KEY WORDS: ***Swertia chirayita*; Shoot tip; MS medium; Regeneration**

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INTRODUCTION

The *Gentianaceae* family, one of the most diverse plant families, is also one of the most vulnerable due to its habitat destruction and high harvest rates. *Swertia chirayita* (Roxb. ex Fleming. H. Karst.) is an indigenous, medicinal, endangered, *Gentian* species native to temperate Himalayas and found at an altitude of (4000-5000 ft). *S. chirayita* is used as herbal medicine for various health ailments including liver disorders, malaria, gastrointestinal infections and diabetes and it has been used in Unani medicine [1]. The natural propagation of *S. chirayita* by seed distribution is restricted mainly because of severe seed dormancy, non-viability and poor seed germination [2]. *S. chirayita* has also been prioritized by National Medicinal Plant Board (Government of India) for conservation and cultivation [3]. Conventional approaches of propagation alone cannot guarantee the re-establishment and recovery of this important plant species. Consequently, the application of alternative reproducible regeneration strategies has become inevitable for mass propagation and sustainable utilization of this age-old medicinal plant. Direct organogenesis without intervening callus phase proves to be advantageous over indirect organogenesis where chances of somaclonal variation are closely encountered. In vitro regeneration in *S. chirayita* has been reported by few authors from different explants including root derived callus culture [4], in vivo nodal explants [1], in vitro leaf [5] and immature seed culture [6], from in vitro seedling [7], and from in vivo leaf explants [8]. So far, there are very few reports available on in vitro regeneration of *S. chirayita* from in vivo explants and no reports are available for the regeneration from in vivo shoot tip explants. *S. chirayita* plants contain several active constituents such as xanthones, flavonoids, iridoids and secoiridoid glycosides that are responsible for its therapeutics properties. Xanthones are also described as strong scavengers of free radicals. Few reports have been conducted on the antioxidant activity of *S. chirayita* [9]. Therefore, the main objective of this study was to establish an efficient and rapid protocol for the regeneration from in vivo shoot tip explants and to evaluate the antioxidant activity from in vitro and in vivo plantlets of *S. chirayita.*

MATERIALS AND METHODS

Plant material

Authenticated *S. chirayita* plant samples were purchased from Ms. Pramila Choudhary, Director, Organoindia Organisation, Tung, Darjeeling, West Bengal, India during the month of November. In vivo grown shoot tips harvested from 6 month old plant were used as explant to study regeneration potentiality.

Culture establishment

Shoot tips were obtained from 6 month old wild grown plantlets. Excised axillary shoots tips were washed thoroughly in tap water and soaked in 0.2% Bavistin (fungicide) solution followed with Tween 20, (5-6drops/100ml) solution for 20 min. Finally, the shoot tips were surface sterilized with 0.1% $HgCl_2$ w/v for 5 min and thoroughly rinsed 4-5 times with sterilized distilled water. Sterilized shoot tip explants about 2 cm in length, were transferred into MS medium [10] supplemented with various PGRs such as BA (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) alone and in combination with Kn (0.1 mg/l), 2,4-D (1.0mg/l) and GA_3 (1.0 mg/l) with 3% sucrose (w/v), and 0.7% agar. Before autoclaving the medium for 20 min at 121ºC, the pH was adjusted to 5.8. Cultures were maintained in a growth chamber with a 16 h/8 h light/dark photoperiod at 24 ± 1ºC (day) and 20 ± 1ºC (night) Light was supplied at intensity of 25 µmol $m^{-2}s^{-1}$ by cool-white fluorescent lamps. Subcultures were performed at an interval of 3-4 weeks.

Multiple shoot induction and shoot elongation

Shoot tips excised from the culture were inoculated in the MS medium supplemented with BA (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) individually and in combination with Kn (0.1 mg/l), 2,4-D (1.0 mg/l) and GA_3 (1.0 mg/l) for multiple shoot induction. Finally shoots were subcultured in MS medium with 3% (w/v) sucrose containing BA (0.5mg/l) and GA_3 (1.0

mg/l) for multiple shoot induction. Subsequent subculturing was done after every 4 weeks. After two subculture in the same medium, explants were transferred to the PGRs free MS basal medium for further elongation.

Rooting and acclimatization

Regenerated shoots with 3-4 compound leaves were excised from each culture and transferred to full strength MS medium supplemented with IAA (1.0mg/l, 2.0mg/l and 3.0mg/l) and IBA (1.0mg/l, 2.0mg/l and 3.0mg/l) separately. The numbers of roots developed per shoot were recorded after 4 weeks. Rooted plantlets were removed from in vitro culture, washed properly with distilled water and transferred to plastic pots containing soilrite : sand (1:1; 1:2; 1:3 v/v), and covered with transparent plastic bag to maintain a humidity of approximately 80-90%. Plantlets were grown under a natural light environment at 24 ± 1°C (day) and 20 ± 1°C (night). After 2 week, the plastic covers were removed progressively to allow for the acclimatization of the plants.

Determination of antioxidant activity of in vitro and in vivo plantlets

In the present work, in vitro and in vivo antioxidant properties of the methanolic extract of *Swertia chirayita* was investigated using the method [11] with minor modifications. Plant materials from both the samples were shade dried and ground to fine powder. Dried samples were extracted at a concentration of 1 mg/ml for the antioxidant assays. Total antioxidant activity in in vitro and in vivo plants of *S. chirayita* was assessed using 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) for determination of free radical-scavenging activity. Different concentrations of extract were added at an equal volume to methanolic solution of DPPH. In brief, an aliquot (10, 25, 50, 100 and 200 μl) of each sample extract was diluted with methanol (990, 975, 950, 900 and 800 μl respectively) and added to a methanolic DPPH solution (4ml) in each sample. The reaction mixture was incubated for 30 min in the dark after which the absorbance reading was taken at 517 nm using a spectrophotometer. The radical scavenging activity was calculated as the percentage of DPPH discolouration using the equation % scavenging DPPH free radical= $100 \times (1- A_E / Ac)$ Where A_E is the absorbance of the extract at a particular level and Ac is the absorbance of the DPPH solution with nothing added.

Statistical analysis

Each treatment consisted of five Erlenmeyer flasks, each containing five explants (n = 25). All experiments were repeated three times. Data obtained from all experiments were presented as the means ± SE of three replications.

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RESULTS AND DISCUSSIONS

Figure 1
Regeneration of Swertia chirayita from shoot tip explants

a.Shoot bud induction in MS medium with 1.0 mg/l BA and 0.1 mg/l Kn; b-c. Multiple shoot induction after 4 weeks of culture in the MS medium supplemented with 0.5mg/l BA and 1.0mg/l GA_3; d. Showing shoot length of complete plantlet; e. In vitro rooting from elongated shoots; f. Regenerated plantlets in plastic cups containing soilrite and sand in 1:2 ratio in growth room; g. Complete establishment of plantlet after transferring to earthen pots containing same hardening material.

In the present study, complete regeneration was successfully achieved from in vivo shoot tip explants of *S. chirayita* (Fig. 1). Shoot tips collected from wild grown plantlets produced varied number of multiple shoots in MS medium supplemented with PGRs such as BA (0.5.1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) alone and with combination of Kn (0.1 mg/l), GA_3 (1.0mg/l) and 2,4-D (1.0mg/l). The type and concentration of PGRs influenced the average number of shoots per explants as well as mean length of shoots (Fig. 3 and 4). The highest number of shoot bud initiations was obtained within 4 weeks of culture when the explants were placed on MS medium supplemented with BA (0.5 mg/l) and combination of GA_3 (1.0mg/l).

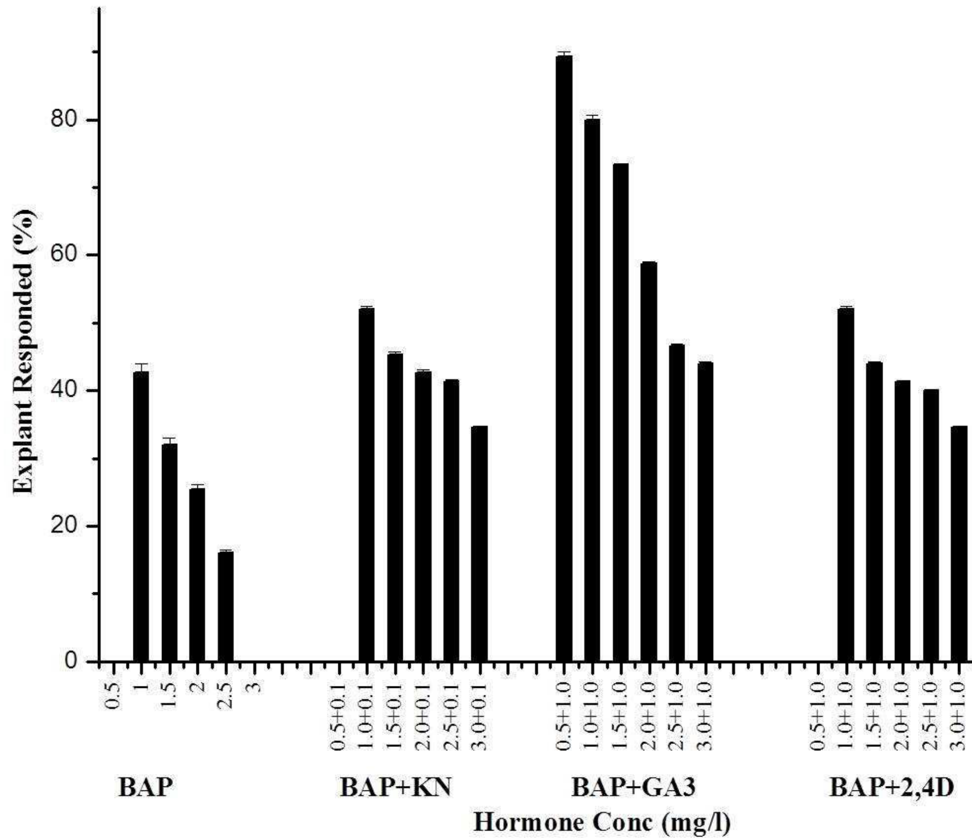

Figure 2

Effects of various concentrations of, 6-benzyladenine (BA) alone and in combination with Kinetin (Kn), Gibberellic acid (GA_3) and 2,4-Dichlorophenoxyacetic acid (2,4-D) on percentage shoot induction of S. chirayita.

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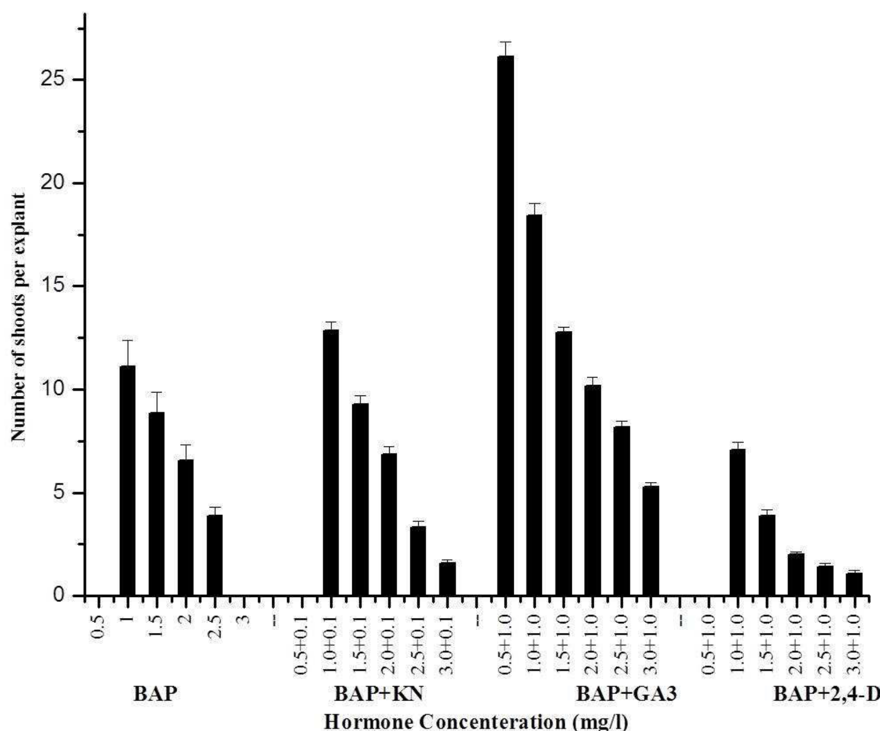

Figure 3

Effects of various concentrations of 6-benzyladenine (BA) alone and in combination with Kinetin (Kn), Gibberellic acid (GA_3) and 2,4-Dichlorophenoxyacetic acid (2,4-D) on number of shoots per explants of S. chirayita. Values represent means ± SE of three replications.

The contamination of *S. chirayita* explants was high (approx. 80%) as shoot tip explants were obtained from wild grown plantlets. Sterilization with 0.2% Bavistin (fungicide) solution followed with Tween 20, (5-6drops/100ml) solution for 20 min and 0.1% $HgCl_2$ significantly reduced the level of contamination. The explants did not respond well when BA alone was used. GA_3 plays a very significant role in the mass multiplication as a PGR. In the present experiment without GA_3 number of shoots was decreased, which explains the vital role of GA_3 in shoot multiplication as reported for other plants [12]. GA_3 at 0.3-10.0mg/l has been shown to promote mass propagation of shoot buds in *Saussurea lappa* [13] and *Ocimum* species [14]. GA_3 had also been found conducive for in vitro shoot regeneration in floret explants of chrysanthemum [15] or for promotion of growth, biomass production and xylem fiber length in transgenic trees [16]. In this context Sekioka and Tanaka [17] are of the opinion that GA_3 can act as a replacement for auxin in shoot induction, and thus a ratio of cytokinin-GA_3 may be decisive for differentiation in certain plant tissues. Rajender et al. [18] observed 76% shooting response in MS medium with GA_3 (1.0mg/l) with combination of BA (0.5mg/l) in *Stachytarpheta jamaicensis*. Similar results were found in our study also among all combinations of PGRs tested, GA_3 (1.0 mg/l) with combination of BA (0.5 mg/l) showed 89% shoot induction (Fig. 2) response with yield a cluster of highest numbers of shoots (26.10 ± 0.73) per explants (Fig. 3). Highest shoot buds was found when explants were inoculated in MS medium with 0.14 µM gibberellic acid (GA_3), with combination of 0.89 µM (BA) [19]. These results suggest that GA_3 concentration is one of the important factors regulating in vitro regeneration from shoot tip of *S. chirayita*. Direct shoot regeneration in *S. chirayita* was observed in

MS media supplemented with BA (2.0 mg/l) from nodal explants for the development of axillary shoot buds after 30 days [20]. In their study, the reduced BA (0.5 mg/l) concentration was found to be effective for multiple shoot induction. Similarly, in our study also, least concentration of BA (0.5mg/l) was found to be the best for multiple shoot induction and proliferation from in vivo shoot tip explants after 4 weeks. Studies of Chaudhuri et al. [1, 5] reported only 18 shoots per explant in *S. chirayita* using nodal explants with MS + 4.65μM Kn + 0.44μM BA +75mg/l CH +10mM KNO_3 after 10 weeks of time period and more than seven shoot buds per explants for the first time when the explants were placed on MS medium supplemented with 2.22 μM N-6-benzyladenine, 11.6 μM kinetin, and 0.5 μM a-Naphthalene acetic acid respectively. Maximum number of 5.9, 5.8 and 4.9 shoot buds per nodal explant of *S. chirayita* was found using 3μM each of BA, 2iP and Kn respectively [21]. According to Balaraju et al. [9] maximum multiple shoot induction was observed from shoot tip explants obtained from in vitro grown seedlings in MS medium supplemented with BA (1.0 mg/l) and Kn (0.1 mg/l) in *S. chirayita.* An increase in BA concentration with constant Kn led to a decrease in the number of shoots per explant. In our study, an increase in BA concentration with constant GA_3 led to a decrease in the number of shoots per explant. Microshoots of maximum length (40.66 ± 2.70 mm) were produced (Fig. 4) when shoots were transferred from culture flask containing BA (0.5 mg/l) and GA_3 (1.0 mg/l) to MS basal medium without PGRs after 6 weeks of culture. After subculturing, the shoots attained a height of 4 cm and possessed 3–4 nodes and about 10-12 leaves in 4 weeks of time.

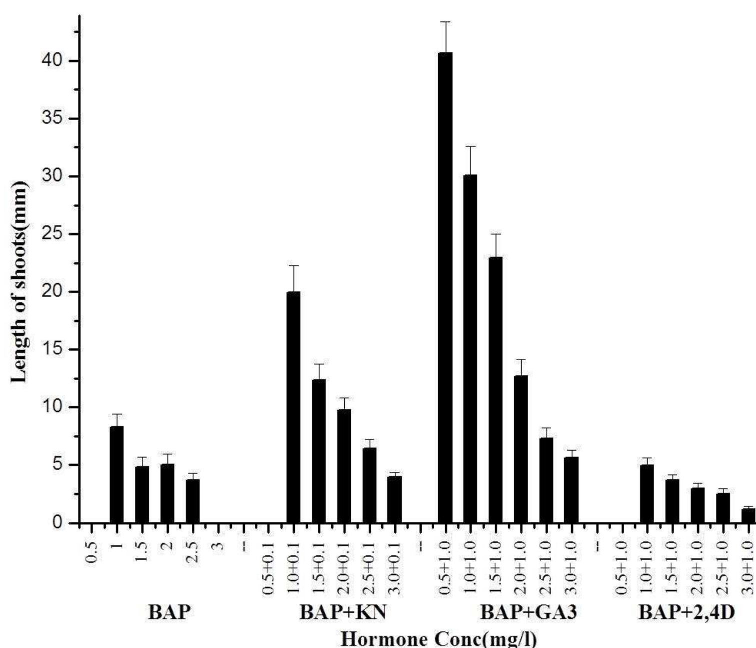

Figure 4

Effects of various concentrations of 6-benzyladenine (BA) alone and in combination with Kinetin (Kn), Gibberellic acid (GA_3) and 2,4-Dichlorophenoxyacetic acid (2,4-D) on mean shoot length of S. chirayita. Values represent means ± SE of three replications

Proliferated shoots when excised and cultured on full-strength MS medium containing different concentrations of IAA and IBA showed varied results for rooting. IAA was found to be more effective for root induction than IBA. The percent response was 80%

(Fig. 5) and the highest number of roots (11.46 ± 1.56) per shoot (Fig. 6) with a maximum length (16 ± 1.90 mm) was produced in MS medium containing IAA (1.0 mg/l) in 4 weeks of time. Maximum rooting (60–65%) was observed in the time duration of 8 weeks on MS media supplemented with IAA ranging between 1-5 mg/l [22]. After 4 weeks of root induction, the plantlets with fully expanded leaflets with a height of 4-5 cm were washed to remove all adhering culture medium and then successfully hardened in the culture room (22 ± 2ºC), with sterilized planting substrates soilrite : sand (1:1; 1:2; 1:3 v/v) for 3-4 weeks. Of the different types of planting substrates examined, percentage survival of the plantlets was highest (80%) in soilrite and sand in 1:2 ratio. Roots developed by excised shoots of *S. chirayita* were viable, and plantlets were successfully acclimatized to ex vitro conditions.

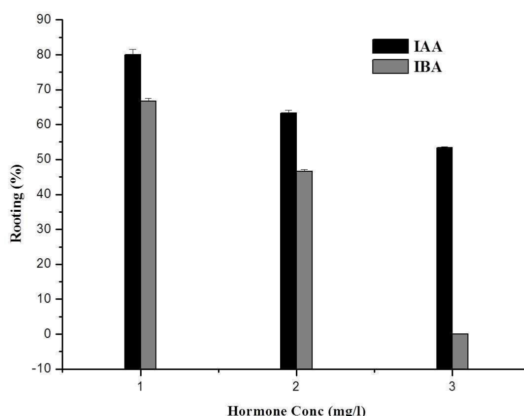

Figure 5
Effects of different auxins (IAA and IBA) on percentage rooting of S. chirayita. Values represent mean ± SE of three replications.

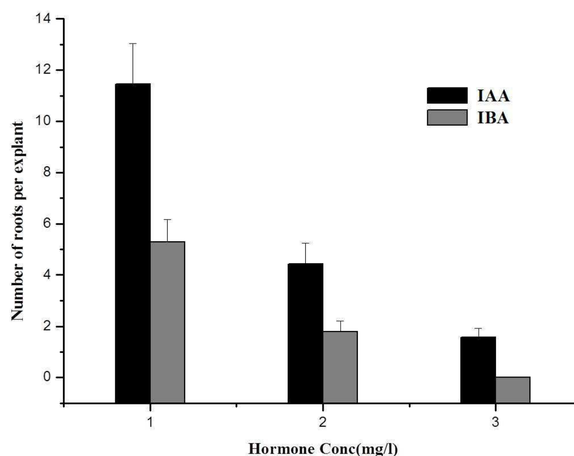

Figure 6
Effects of different auxins (IAA and IBA) on rooting of regenerated S. chirayita per shoots. Values represent mean ± SE of three replications.

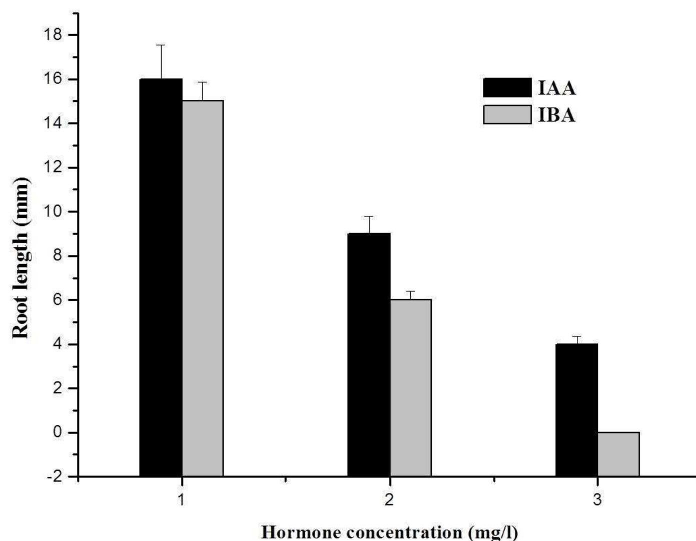

Figure 7
Effects of different auxins (IAA and IBA) on root length of S. chirayita. Values represent mean ± SE of three replications.

Free-radical scavenging activity (FRSA) was determined to evaluate the antioxidant potential of regenerated plantlets, and these were compared with levels in in vivo plantlets. In vitro plantlets had significantly higher capacity to detoxify DPPH free radicals than in vivo plant samples collected from wild conditions (Fig. 8). Our results are consistent with numerous previous findings in which antioxidant activity for *S. chirayita* plant species was reported [9, 23]. They found that in vitro plantlets had significantly higher capacity to detoxify DPPH free radicals than in vivo collected from wild condition.

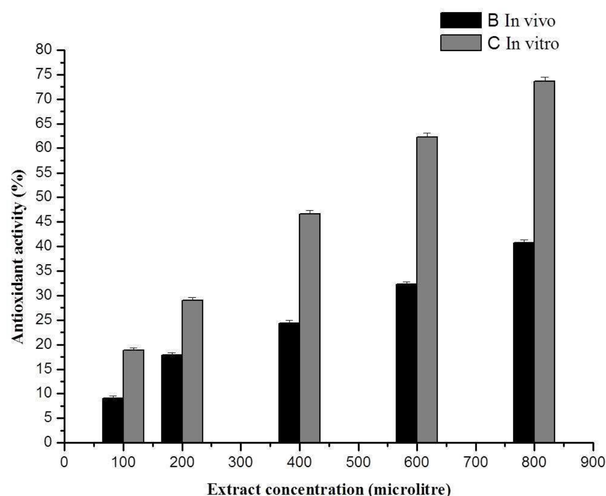

Figure 8
Antioxidant activity of in vitro and in vivo plantlets of S. chirayita. The activity was determined using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) as free radical. Values represents means ± SE of three replications.

CONCLUSION

The above results demonstrated that wild-grown shoot tip explants of *S. chirayita* are amenable to in vitro culture. This the first report showing the procedure for direct organogenesis and establishment of complete plantlets from shoot tip explants of wild grown *S. chirayita* plantlets. Further, the results highlight the efficiency of lower concentrations of BA and GA_3 used for efficient regeneration in a short period of time compared to the conventional methods of propagation of *S. chirayita* plantlets taking more than a year time to mature completely in nature. Regenerated plantlets could be useful sources of tissues for biochemical characterization of medicinally active constituents and for selection and cloning of superior individual genotypes. The protocol could be useful for large scale production of uniform healthy plantlets and provides a possible system towards genetic improvement of plant. As antioxidant activity levels were high in in vitro plantlets, this suggested that commercial production of in vitro derived cultures of *S. chirayita* for biologically active compounds were possible.

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