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Research Article

IN VITRO MASS PROPAGATION AND SYNTHETIC SEED PRODUCTION COMBINED WITH PHYTOCHEMICAL AND ANTIOXIDANT ANALYSIS OF *BACOPA CHAMAEDRYOIDES*: AN ETHNO-MEDICINALLY IMPORTANT PLANT

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ABSTRACT

Objective: This study was carried out to optimize adventitious shoot buds induction from the surface of the leaf and internode explants along with investigation of the phytochemical constituents and *in vitro* antioxidant potential of aerial parts of *Bacopa chamaedryoides*, an ethno-medicinally important plant.

Methods: All the analysis was performed according to standard protocols.

Results: Maximum 26.3±05 shoots per leaf explant and 32.3±0.41 shoots per internode explants were induced on MS basal medium supplemented with 3.0 mg/L TDZ and 15% *Aloe vera* gel (AvG) fortified fortified medium within 30-32 days. Multiplied shoots were successfully rooted with maximum 16.8±0.35 root per shoot on half strength MS medium supplemented with 50% of AvG and reached 2.1±0.24 cm length within 15 days. Finally, the rooted plants were hardened and transferred to soil with 87.8% success rate. Micro-shoots were encapsulated in calcium alginate beads for the production of synthetic seeds (SSs). The best result was observed in 3.0% sodium alginate by means of proper globular shaping of SSs with 90.0% germination rate. The ethanolic extracts were evaluated for their potential antioxidant activities using different *in vitro* systems and exhibit better scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (IC_{50} = 1150.446 µg/mL), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (IC_{50} = 13.781 µg/mL) and ferric reducing antioxidant power (IC_{50}=5.663 µg/mL). High-performance thin layer chromatography was performed to corroborate the biochemical analysis of antioxidant activity.

Conclusion: The results of this study indicate that the aerial part of *B. chamaedryoides* is a valuable source of natural antioxidants.

Keywords: *Bacopa chamaedryoides*, phytochemical analysis, high-performance thin layer chromatography, micropropagation, synthetic seeds.

INTRODUCTION

Bacopa chamaedryoides (Kunth) Wettst., a member of the Scrophulariaceae family is non-succulent, creeping, glabrous herb whose habitat includes wet, damp and marshy areas. This ethno-medicinally important plant is widely distributed in India, Bangladesh, South Florida, Brazil, Uruguay, Mexico and other regions of a tropical world [1,2]. Since long, the tribal people and now a day the common people of India have been using this traditional medicinal herb *B. chamaedryoides* as a neuro-stimulant [3]. Due to ethno-medicinal use, the demand of these plant species is continually increasing. Mass propagation of uniform, healthy plants through tissue culture is the only viable technique for production of large numbers of clonal plants in a short time to fulfil the demand [4]. "Synthetic seed (SS) technology" is an applied execution of modern plant biotechnology, which offers tremendous potential in micropropagation and germplasm conservation of medicinal plants along with its easy handling and transportation [5,6]. The effort to produce SSs has intensified during the last two decades for various medicinal plants [7,8].

Oxidative stress has been implicated as a potential contributor to the pathogenesis of several diseases, such as cancer, diabetes and heart disease [9]. To overcome this hazard, nature has provided us a defense shield in the form of dietary antioxidants from plants [10]. Scientific information on antioxidant properties of endemic plants, limited to certain regions and known only by local populations, is still rather scarce [11]. Therefore, the assessment of such properties remains a curious and beneficial task, particularly to find new promising sources of natural antioxidants for functional foods and/or nutraceuticals [12,13]. The investigation of natural antioxidants from plant origin has remarkably increased in recent years due to the adverse health effects of synthetic antioxidants [14]. The antioxidants of fruits and vegetables can be considered as nutraceuticals due to their protection of the body from the toxic effect of free radicals and reactive oxygen species. The natural antioxidants are also being used in the treatment of several kinds of oxidative stress-related disorders and in cancer [15,16]. *Bacopa monnieri* L. is another species of the genus *Bacopa*, which is commercially very important plant and well-studied by many researchers for last five decades on diverse aspects, including micropropagation, phytochemical properties and antioxidant activities [17-22], but no literature is currently available to substantiate phytochemical constituent and antioxidant properties of *B. chamaedryoides*. The only report of micropropagation of *B. chamaedryoides* have also been established from the present laboratory [3] using node and shoot-tip culture, but present communication had described much higher frequency of micropropagation through direct shoot organogenesis. To the best of our knowledge, there was no scientific report to give credibility to the ethno-medicinal usage of this medicinal herb for the management of various ailments. Therefore, the present study has been performed to explore the quantitative and qualitative analyses of the phytochemical and antioxidant activities of the aerial parts of this plant to justify its curative usage.

METHODS

Plant material

Mature leaf (10-12 mm) and internode (8-10 mm) were cut from the sterile *in vitro* plants of *B. chamaedryoides* previously established by the present laboratory and were used as explants for adventitious

shoot induction experiment [3]. Arial parts including stem and leaves together (approximate 5 cm long from the apical tip) of tissue culture derived field grown 40-55 days old plants were collected freshly during the experiment and used for phytochemical analysis.

In vitro propagation

In vitro adventitious shoot induction and elongation

For *in vitro* propagation purpose MS basal medium was used [23]. The pH of the medium was adjusted to 5.6 before the addition of agar. The medium was autoclaved at 1.04 kg/cm pressure and 121°C for 18 min. Internodes (8-10 mm) and intact leaf lamina without petiole (10-12 mm) were used as explant. The explants were implanted in culture tubes (25 mm × 150 mm) containing MS basal medium supplemented with 0.8% agar, 3.0% sucrose and different concentrations of 6-Benzylaminopurine [BAP], 6-(γ,γ-dimethylallylamino) purine [2iP] and TDZ alone or in combination with four different concentration of AvG (Table 1). For shoot elongation, cytokines are totally withdrawn and only 0.5 mg/L IAA are supplemented along with 15% AvG. The cultures were incubated inside the growth chamber maintained at 24±2°C under a 16 hrs photoperiod with a photosynthetic photon flux density of approximately 50 µmol/m^2/s emitted from cool fluorescent tubes (Philips India Ltd.). The cultures were sub-cultured in their respective fresh media at every 4 weeks intervals.

In vitro root induction

For *in vitro* root induction, we follow the method previously optimized by the present laboratory [3]. Elongated adventitious shoots were cut into about 2.5 cm pieces and implanted on half strength MS media supplemented with 50% (v/v) AvG. AvG is the odourless, colourless mucilaginous gel, obtained from parenchymatous cells of the fresh leaves of *Aloe vera*.

Synthetic seed preparation and short-term storage

Shoot tips of about 4-5 mm length (i.e. micro-shoots) were cut from *in vitro* elongated plants and used for encapsulation. The encapsulation medium was prepared by a slight modification of the MS medium for SS preparation that is devoid of Ca^{2+} ions and agar-agar but supplemented with 3.0% sucrose and three different concentrations (1.0, 3.0 and 5.0%) of sodium alginate. The pH of the medium was adjusted to 5.6 before the addition of sodium alginate. The medium was autoclaved at 1.04 kg/cm pressure and 121°C for 18 minutes. Separately, 3.0% calcium nitrate [$Ca(NO_3)_2$] solution was autoclaved, which was required for the ion exchange reaction during encapsulation. Micro-shoots were mixed with encapsulation medium and were dropped into a conical flask containing 3.0% $Ca(NO_3)_2$ solution using a wide glass dropper and shaken well. The drops (beads) containing a single shoot tip were kept in $Ca(NO_3)_2$ solution for 20 minutes for polymerization. Thereafter, the beads were recovered by decanting the $Ca(NO_3)_2$ solution and washing three times with liquid MS basal medium. Then, few seeds were sown immediately on slant MS medium for germination and the rest of the seeds were transferred into Petri dishes containing a very small amount (1.0 ml) of liquid MS medium (without PGR) and sealed with parafilm for short time storage. Three sets were kept at three different temperatures (24, 15, and 4°C) under 16:8 hrs light/dark cycle. After storage, the germination capability of these stored SSs was assessed every 2-weeks interval (at 2, 4, 6 and 8 weeks) for up to 8 weeks by sowing them into slant MS medium for germination and complete plantlet regeneration.

Table 1: Effect of three different cytokines and *Aloe vera* leaf gel (AvG) supplemented with MS basal medium on adventitious shoot induction

Concentration of cytokines (mg/L) and AvG (%)				No. of adventitious shoot per leaf explant	No. of adventitious shoot per internode explant
2iP	TDZ	BAP	AvG		
-	-	-	-	0±0.0^{a}	0±0.0^{a}
1.0	-	-	-	9.2±0.55^{bc}	11.0±0.55^{c}
2.0	-	-	-	13.1±0.53^{de}	14.6±0.57^{de}
3.0	-	-	-	15.9±0.42^{efg}	18.1±0.59^{fghi}
4.0	-	-	-	15.2±0.43^{ef}	17.2±0.67^{fgh}
-	1.0	-	-	13.7±0.53^{de}	15.9±0.42^{efg}
-	2.0	-	-	15.1±0.37^{ef}	21.6±0.52^{ij}
-	3.0	-	-	19.3±0.67^{hi}	24.3±0.45^{jkl}
-	4.0	-	-	17.5±0.43^{fgh}	22.1±0.37^{ijk}
-	-	1.0	-	12.1±0.53^{cd}	14.5±0.43^{de}
-	-	2.0	-	16.9±0.42^{fg}	17.3±0.50^{fgh}
-	-	3.0	-	18.1±0.59^{fghi}	20.0±0.61^{hij}
-	-	4.0	-	15.4±0.42^{efg}	18.2±0.43^{fghi}
-	3.0	-	10.0	21.7±0.50^{j}	29.1±0.53^{lm}
-	3.0	-	15.0	26.3±0.50^{k}	32.3±0.41^{n}
-	3.0	-	20.0	24.6±0.50^{jk}	30.9±0.53^{m}
-	3.0	-	25.0	20.4±0.33^{hij}	28.4±0.33^{lm}

The data were recorded after 30 days of culture. Each value represents the mean±standard error, n=30. Mean followed by the same letters in each column are not significantly different at p<0.05 according to Tukey's multiple range tests

Hardening and field transfer

A total of 45 *in vitro* rooted plantlets (Fig. 1e) and 45 SS derived plantlets (Fig. 1g) were hardened by follow the method previously optimized by the present laboratory [3]. Rooted plantlets were transferred into plastic buckets containing a "mixture of soil and organic manure" (2:1 volume ratio). The soil was submerged in water like submerged aquatic vegetation. The distance between the soil-bed and the water surface was 1.0-1.5 cm. The setups were cultivated in a hardening chamber for 18-20 days. Thereafter, the hardened plants were transplanted into earthen tubs containing a mixture of soil and vermin compost (3:1 volume ratio) and maintained inside the shade-net house.

Phytochemical constituent's analysis

Qualitative screening of phytochemical constituents

The qualitative analyses of phytochemical including the presence of alkaloids, anthraquinones, cardiac glycosides, coumarins, saponins, tannins and terpenoids were performed using standard protocols [24].

Estimation of total carbohydrate and protein

Carbohydrate content was determined in the aqueous solution with anthrone sulfuric acid reagent [25]. The total protein content was determined according to a previously described procedure [26]. The carbohydrate and protein content was expressed as mg/g fw.

Determination of total flavonoid and phenolic content

The total flavonoid content was determined according to a previously described procedure [27]. Total phenolic content was measured following Folin–Ciocalteau method [28]. Total flavonoid and phenolic content were expressed as mg/g fw.

Estimation of ascorbic acid

The ascorbic acid was estimated by using Folin phenol reagent according to a previously described procedure [29]. The ascorbic acid concentration was calculated by comparison with the standard and result was expressed as mg/g fw.

Determination of antioxidant capacity

Free radical scavenging by the use of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical

The DPPH radical scavenging capacity of the ethanolic extract was determined according to a previously described procedure [30]. Briefly, 50 µL of different concentrations of ethanolic extract was mixed with 1950 mL of 6.34 × 10^{-5} M DPPH radical solution in ethanol. The mixture was allowed to stand for 30 minutes in the dark. The absorbance of the solution was measured at 517 nm. The ascorbic acid was used as a standard. The antiradical activity was expressed as IC_{50} (µg/mL), the extract dose required to cause a 50% decrease of the absorbance at 517 nm. A lower IC_{50} value corresponds to a higher antioxidant activity.

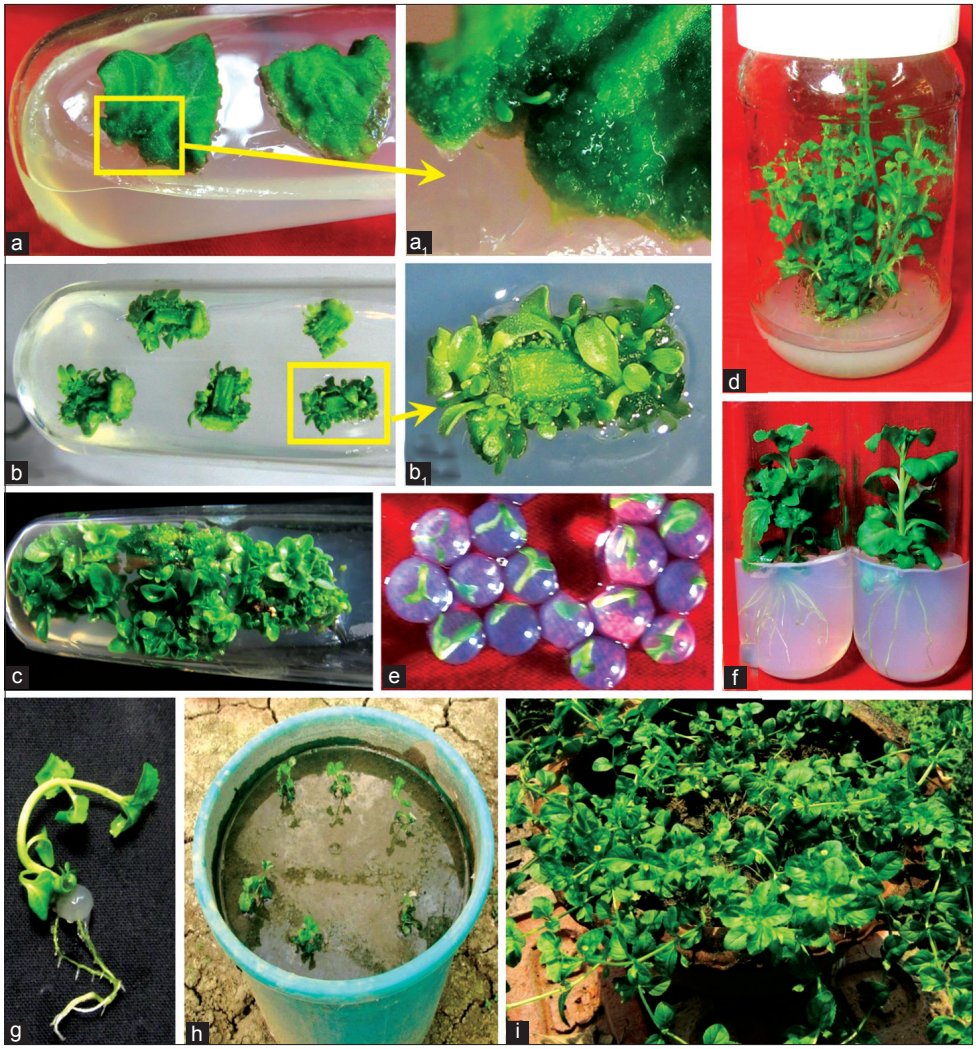

Fig. 1: Different stages of mass propagation and field performance of *Bacopa chamaedryoides*. (a) Adventitious shoots induced from adaxial surface of leaf explant on MS medium supplemented with 3.0 mg/L thidiazuron (TDZ) + 15% AvG after 15 days of culture. (a_1) enlarged portion of fig a. (b) Adventitious shoots induced from internode explant on MS medium supplemented with 3.0 mg/L TDZ + 15% AvG after 30 days of culture. (b_1) enlarged portion of fig b. (c) 45 days old culture with cluster of multiple adventitious shoot induced from internode explant. (d) Elongated shoots cultured on MS medium supplemented with 0.5 mg/L TDZ + 15% AvG after 20 days of culture. (e) Complete *in vitro* plantlets with well develop root system on half strength MS medium supplemented with 50 % AvG after 15 days of culture. (f) Synthetic seeds produced by alginate encapsulation of micro-shoots. (g) Plantlet produced by germination of synthetic seed (h) Hardening of regenerated plants on submerged condition. (i) 2 months old field grown regenerated plants with flower

Free radical scavenging by the use of the ABTS radical

The free radical scavenging activity was studied using the ABTS radical cation decolorization assay [31]. ABTS radical cation was produced by reacting 7.0 mM ABTS solution with 2.45 mM potassium persulfate, and the mixture was kept in dark at room temperature for 16 hrs. For the analysis, the solution was diluted in double distilled water to an absorbance of 0.7 (±0.02) at 734 nm. Fifty microliter of the ethanolic extract of different concentrations was added to 1950 µL of ABTS solution, and the absorbance was recorded after incubation of 30 minutes at 30°C. Ascorbic acid with different concentration was used as a standard. The antiradical activity was expressed as IC_{50} (µg/mL).

Ferric reducing antioxidant power (FRAP) assay

Antioxidant activity was also determined by ferric reducing power using a spectrophotometer at 700 nm [32]. Briefly, 1 mL of extract was mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 30 minutes. Then, 2.5 mL of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 20 minutes. The 1 mL supernatant was added to the mixture of 0.5 mL of 0.1% $FeCl_3$ and 1.0 mL of double-distilled water. The absorbance was measured at 700 nm after incubation for 10 minutes. Different concentrations of ascorbic acid were used as control. Increase in absorbance was interpreted as increased ferric reducing activity. The IC_{50} value (µg/mL) is the concentration giving an absorbance of 0.5.

High-performance thin layer chromatography (HPTLC) densitometric determination and bio autography

Two microliters of the ethanolic extract (250 mg/mL) was applied (band length - 6.0 mm; distance between bands - 10.0 mm; distance from left edge - 15.0 mm; distance from lower edge - 10.0 mm) on a pre-coated silica gel 60 F_{254} plate (5 cm × 15 cm, Merck, Germany) using Linomat 5 automated TLC applicator (Camag, Muttenz, Switzerland). Prior application, the plate was pre-washed with methanol and dried at 60°C. TLC plates were developed using the mobile phase ethyl acetate/acetic acid/formic acid/water (100:11:11:27, v/v/v/v) in a Camag HPTLC twin trough chamber (20 cm × 20 cm). The chamber was saturated with filter paper for 15 minutes, and plate equilibrium was carried out for 10 minutes. Plate was developed up to 120.0 mm and dried under a stream of cold air. The bands with antioxidant property were visualized

by dipping the plate in 2.54 mM DPPH reagent [33]. Separated bands were quantified by HPTLC densitometric scanning using Camag TLC Scanner 4 in the flourescence mode at 600 nm operated by WinCATS software (version 1.4.8).

Statistical analysis

In the case of plant tissue culture, experiment each treatment contained three replicates with 10 explants per replicate. The data pertaining to the number of shoots or roots per explant, shoot and root length, were subjected to a one-way Analysis of Variance. The differences among the means were compared by high-range statistical domain using Tukey's test with the standard software SPSS 19.0 version. In the case of phytochemical and antioxidant analysis, all experiments were repeated thrice, and data are reported as mean ± standard deviation.

RESULTS AND DISCUSSION

In vitro propagation

In vitro adventitious shoot induction and elongation

Among three cytokines tested, all three can induce direct shoot organogenesis, but optimum response observed in TDZ containing medium followed by BAP and 2iP (Table 1). Leaf explants are slightly stretched up and internode explants are swelled up with the slight lightening of color after 5-8 days of implantation.

After 12-15 days, very small outgrowths of shoot primordial are observed in naked eyes on the surface of the explants and first 'leaf initials' observed within 20 days of implantation (Fig. 1a, a_1). Internode proves to be superior to leaf explant in terms of shoot induction rate. Highest 19.3±0.67 adventitious shoot per leaf explant and 24.3±0.45 per internode explant were induced through direct shoot organogenesis within 30-32 days of implantation on MS medium containing 3.0 mg/L TDZ; whereas, this number significantly increase to maximum 26.3±0.50 and 32.3±0.41 per explant accordingly within same time duration (30-32 days) when 15% (v/v) AvG are added with optimum concentration (3.0 mg/L) of TDZ (Fig. 1b and b_1). Two to three leaves are induced from each of these adventitious shoots on the same medium within 42-45 days of culture in asynchronous manner, but elongation of these shoots are failed on this medium (Fig. 1c). Shoot elongation occurred when cytokines are totally withdrawn but 0.5 mg/L IAA and 15% (v/v) AvG are supplemented with MS basal medium and elongated up to 5.3±0.36 cm within next 20-22 days of culture in asynchronous manner (Fig. 1d). In general, our results confirmed earlier observations on *Phaseolus* where TDZ and BAP played a crucial role, but TDZ prove to be best on adventitious shoot bud induction [34]. Present findings of failure of shoot elongation on TDZ containing medium also corroborate with previous studies on *Pisum* where TDZ suppresses the shoot growth of apical meristems and instead induces profuse formation of lateral shoot meristems resulting in multiple shoots [35]. Coconut water is an undefined complex mixture of organic substances that has been successfully employed for culturing different plant species [36]. Adventitious shoot bud formation from leaf of *Leucaena* was positively influenced by use of 15 % (v/v) coconut water [37].

In the present study we have used AvG, an undefined complex mixture of organic substances containing 5.43% (w/w) total sugar [38]. In addition to the different carbohydrates, the AvG also contains over 75 active ingredients including proteins, lipids, amino acids, vitamins, enzymes, inorganic compounds and small organic compounds [39,40] which serve as a source of functional food and as an ingredient in other food products of *in vitro* grown plants.

In vitro root induction

According to the fruitful result found in our previous work [3], we use 50% (v/v) AvG along with half-strength MS medium for *in vitro* rooting. Root induction was initiated from the cut ends of excised micro-shoots within 8 days of implantation. Cent percent response was observed within 15 days of culture by way of 16.8±0.35 number of roots per micro-shoots with 2.1±0.24 cm root length (Fig. 1e). AvG is a less expensive PGR-like natural complex and which have potentiality to promote the root induction of *B. chamaedryoides* [3]. Not only on *B. chamaedryoides* but also the root inducing properties of AvG are previously reported on *Aloe vera* [4,41].

Effect of sodium alginate concentration on germinability of SS

Polymerization of sodium alginate in $Ca(NO_3)_2$ resulted in the formation of hydrogel capsules with propagules inside. Out of the three different concentrations (1.0, 3.0, and 5.0%) of sodium alginate examined, the optimum concentration was evaluated on the basis of germination percentage and days taken for germination of SSs. The highest germination of 96.7% was noted for the SSs prepared with 1.0% sodium alginate, followed by 91.1% and 63.3% germination with 3.0 and 5.0% sodium alginate accordingly. Time required for germination is directly proportionate with concentration of sodium alginate, generally 21 days required for germination of SSs prepared with 5.0% sodium alginate followed by 10 days at 3.0% and 6 days at 1.0% sodium alginate. Though the earliest germination with highest percentage was noted with 1.0% sodium alginate, but it did not polymerize accurately to give a proper shape and was too soft for handling purposes. On the other hand, the SSs with 5.0% sodium alginate caused reduction of germination rate as well as extensive delay in germination taking an excess of 15 days for germination as compared to lower concentration due to over-hardening of the beads. Therefor, 3.0% sodium alginate considered as suitable for encapsulation give a proper globular shape (Fig. 1f). Present findings are corroborate with the previous reports where medium concentration (3.0%) of sodium alginate was found suitable for all aspect including proper shaping of the beads (i.e. SSs) along with satisfactory germination rate [42-44]. "SS technology" is an important tools of modern plant biotechnology, used for germplasm conservation as well as germplasm exchange between countries [5,6].

Effect of storage temperature and storage period on viability of SSs

Among three different storage temperatures (4, 15, and 24°C) examined, 15°C proved to be the best followed by 24°C with 73.3% and 56.7% germinability respectively even after 6 weeks of storage (Fig. 2). However, only 15.5% of SSs retain their germinability after same duration of storage at 4°C. Though the highest germination (86.7%) were noted on 24°C for up to 4 weeks of storage, but interestingly the SSs are start to germinate spontaneously during 2nd weeks. As a result, all the moisture and nutrient present in encapsulation medium are used up by the germinated SSs and they start to dry during 5th weeks of storage. Due to this difficulties, the germination percentage of SSs stored at 24°C were fallen dramatically to 56.7% after 6 weeks and data was not found due to drying of all SSs after 8 weeks of storage (Fig. 2).

Therefore, we considered 15°C temperature is suitable for successful short term storage of SSs of *B. chamaedryoides*. Our results are

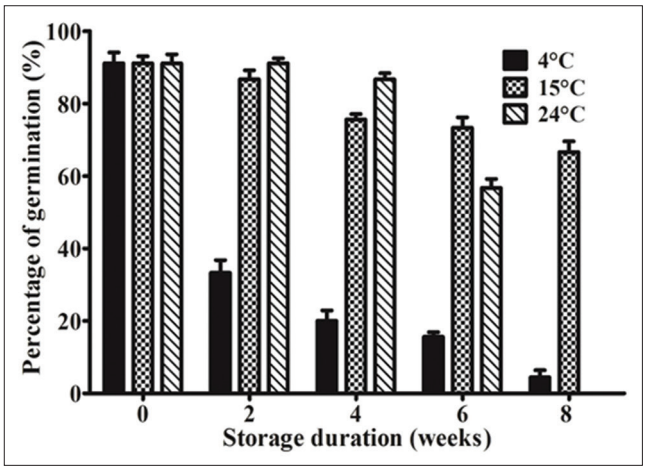

Fig. 2: Effect of storage temperature and storage duration on the germination percentage of synthetic seeds

corroborate with the findings on *Cannabis* and *Drimiopsis*, where medium temperature (15°C) proved to be optimal over low (4°C) and high (24°C) temperatures for storage of SSs [44,45]. The effective role of low storage temperature on short-term storage of encapsulated shoot tips/nodes of different plants were investigated by many researchers [6,42,43,45].

Hardening and field transfer

A total of 90 platelets was hardened (Fig. 1h) for 20 days with 87.8% (79 out of 90 plantlets survived) survival rate. Hardening was performed by following the method optimized by the present laboratory [3]. The hardened plants were then transferred to earthen tubs filled with mixture of soil and vermin compost at 3:1 ratio and maintained inside the shade-net house for next 2 months with 97.5% (77 out of 79 plantlets survived) survival rate. All the survived plants produced flower (Fig. 1i).

The phytochemical analyses

The qualitative chemical analysis of the aerial part of *B. chamaedryoides* showed the presence of bioactive components including alkaloids, terpenoids, tannins, saponins, coumarins, anthroquinones and cardiac glycosides (Table 2).

The quantitative analysis of phytochemical is represented in Table 3. The results indicate that aerial part is a rich source of phenolic compounds with about 18 mg gallic acid equivalent g^{-1} fw including flavonoids and ascorbic acid that may be the major contributor for the antioxidative efficacy. Similar amounts of total phenolics have also been reported from different important herbs and medicinal plants [46]. The ascorbic acid content of the aerial part of the plant is comparable to commonly consumed vitamin C rich vegetables and fruits [47].

Antioxidant activity of ethanolic extract

DPPH and ABTS assay

The ethanolic extract of the aerial part of *B. chamaedryoides* showed enhanced scavenging activity over DPPH free radicals. A dose–dependent increase in scavenging activity was recorded with 66.866% at 1750 µg/mL dose (Table 4).

The IC_{50} value of the extract was 1150.446 µg/mL. The ethanolic extract showed maximum ABTS radical scavenging activity (97.858%) at 50 µg/mL concentration (Table 5). The activity was concentration specific. The IC_{50} value of the extract in scavenging ABTS radical was found to be 13.781 µg/mL. It has been reported by several researchers that there is a positive correlation between the total phenolic content and antioxidant activity [48,49]. Our results clearly revealed that the higher phenolic content in the aerial part of *B. chamaedryoides* coincided with higher antioxidant activity.

Table 2: Qualitative screening of phytochemical components

Sl. no.	Biochemicals	Results
1	Alkaloids	Positive
2	Anthraquinones	Positive
3	Cardiac glycosides	Positive
4	Coumarins	Negative
5	Saponins	Positive
6	Phlobatannins	Negative
7	Tannins	Positive
8	Terpenoids	Positive

Table 3: Quantitative analysis of phytochemical components

Component	*mg/g fw
Total carbohydrate	4.336±0.330
Total protein	21.103±0.672
Total phenolic content	17.905±0.449
Total flavonoid	0.485±0.015
Ascorbic acid	1.148±0.034

*Values are mean±standard deviation of three observations

Reducing power of aerial part

The reducing power of ethanolic plant extract and ascorbic acid (standard) are shown in (Fig. 3). It was observed that the reducing power of the extract increased in a concentration dependent manner. The extract showed comparable absorbance readings (IC_{50}=5.663 µg/mL) with the positive reference standard (IC_{50}=4.817 µg/mL) and considered to have high reducing power. Previous studies have pointed out that the antioxidant capability is always related to the increase in reducing power [50].

HPTLC densitometric determination of antioxidant constituents

HPTLC was performed to corroborate the biochemical analysis of antioxidant activity. HPTLC fingerprint obtained from HPTLC image, the R_f value, peak height and peak areas of the antioxidant bands confirmed from TLC bio autography were represented in Table 6.

It is evident from the densitometric analysis that ethanolic extract contains six antioxidant bands at 600 nm after derivatization with 2.54 mM DPPH solution (Fig. 4). Similar result with six antioxidant bands was found in ethanolic extract of *Bauhinia purpurea* [51].

CONCLUSION

In the present study, high frequency of plantlets production through direct shoot organogenesis along with their *in vitro* rooting as well as encapsulation of micro-shoots to produce SSs with their *in vitro*

Table 4: DPPH radical scavenging activity

Sl. no.	Samples	Concentration (µg/mL)	Percentage of inhibition (mean±SD)	IC_{50} (µg/mL)
1.	*B. chamaedryoides*	250	18.996±0.746	
		500	30.984±0.613	
		750	37.714±0.658	
		1000	47.153±0.768	1150.446
		1250	53.883±0.478	
		1500	60.653±0.897	
		1750	66.866±0.881	
2.	Ascorbic acid (Standard)	1	12.027±0.813	
		2	42.374±0.730	
		3	27.280±0.301	
		4	57.029±0.813	3.613
		5	70.928±0.613	
		6	85.066±0.632	
		7	92.672±0.565	

SD: standard deviation, *B. chamaedryoides: Bacopa chamaedryoides*, DPPH: 2,2-diphenyl-1-picrylhydrazyl

Table 5: ABTS radical scavenging activity

Sl. no.	Samples	Concentration (µg/mL)	Percentage of inhibition (mean±SD)	IC_{50} (µg/mL)
1.	*B. chamaedryoides*	5	29.238±0.951	
		10	48.078±0.970	
		20	63.768±0.476	13.781
		30	77.694±0.567	
		40	86.894±0.476	
		50	97.858±0.289	
2.	Ascorbic acid (Standard)	1	29.679±0.682	
		2	47.574±1.075	
		3	56.081±0.764	2.624
		4	63.201±0.664	
		5	72.590±0.756	
		6	84.121±0.824	

SD: Standard deviation, *B. chamaedryoides: Bacopa chamaedryoides*

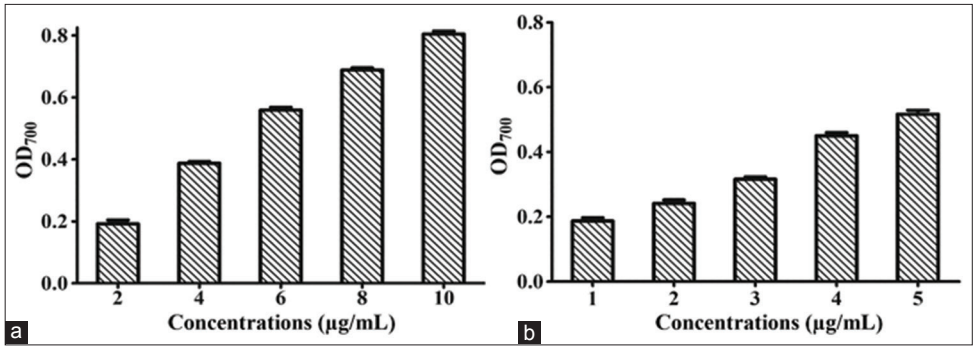

Fig. 3: Ferric reducing antioxidant power (a) The ethanolic extract of *Bacopa chamaedryoides* and (b) ascorbic acid

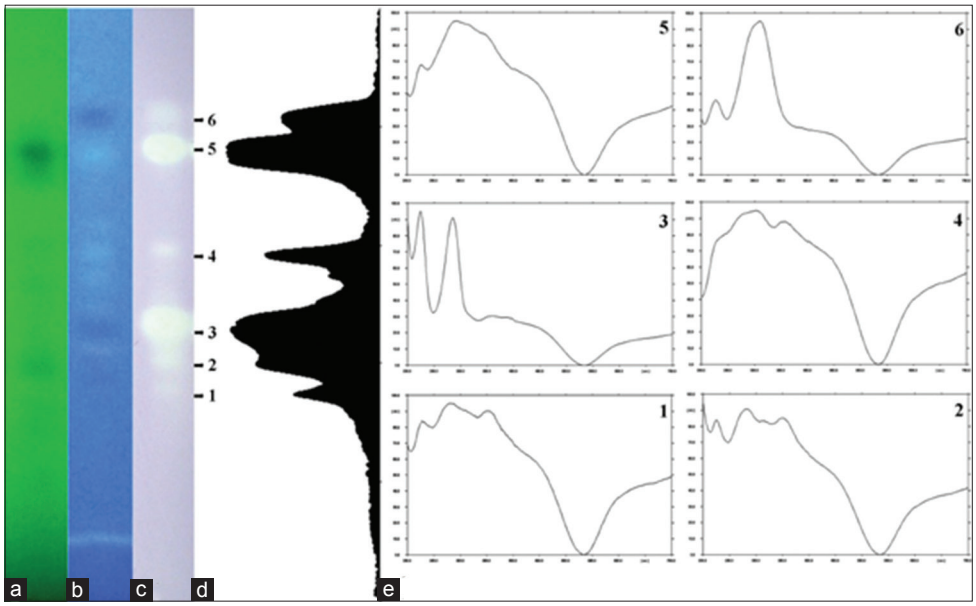

Fig. 4: High performance thin layer chromatography (HPTLC) profile of ethanolic extract of *Bacopa chamaedryoides* after derivatization with 2.54 mM 2,2-diphenyl-1-picrylhydrazyl. (a) Under UV 254 nm; (b) Under UV 366 nm; (c) Under day light and (d) HPTLC densitogram at 600 nm. (e) Absorption spectrum of the six antioxidant bands

Table 6: R_f value and peak height and area of antioxidants present in HPTLC densitogram at 600 nm after derivatization

Peak	Start R_f	Start height (AU)	Max R_f	Max height (AU)	Max %	End R_f	End height (AU)	Area (AU)	Area %
1	0.30	28.6	0.35	80.3	10.45	0.36	53.8	7323.4	10.89
2	0.36	53.9	0.40	102.0	13.26	0.41	99.2	8007.2	11.90
3	0.41	99.4	0.45	114.6	14.91	0.46	112.9	11481.0	17.06
4	0.56	53.3	0.58	100.5	13.07	0.62	20.1	8807.7	13.09
5	0.67	19.3	0.74	116.5	15.15	0.75	112.6	10916.8	16.23
6	0.80	73.3	0.81	81.2	10.55	0.86	1.6	7459.8	11.09

HPTLC: High performance thin layer chromatography

germination and hardening up to establishment on field condition was achieved. In addition, the ethanolic extract of the *B. chamaedryoides* have a potent DPPH and ABTS free-radical scavenging properties. The HPTLC analysis has provided chromatographic fingerprint profile of the antioxidants present in the extract. So the plant can be utilized as easy available source of natural antioxidants and rich source of ascorbic acid in order to alleviate oxidative stress related disorders. Additional studies are desirable to characterize the active compounds of this plant so that they can be utilized to develop new drugs for reducing oxidative stress related ailments.

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