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±0.5 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 (IC₅₀ = 1150.446 µg/mL), 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (IC₅₀ = 13.781 µg/mL) and ferric reducing antioxidant power (IC₅₀=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 ethnomedicinally 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-medical 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 fulfill 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-medical 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]. Aerial 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/cm² 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/m²/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 medium 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 Ca²⁺ 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/cm² pressure and 121°C for 18 min. Calcium nitrate [Ca(NO₃)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(NO₃)₂ solution using a wide glass dropper and shaken well. The drops (beads) containing a single shoot tip were kept in Ca(NO₃)₂ solution for 20 minutes for polymerization. Thereafter, the beads were recovered by decanting the Ca(NO₃)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.

**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 IC₅₀ (µg/mL), the extract dose required to cause a 50% decrease of the absorbance at 517 nm. A lower IC₅₀ value corresponds to a higher antioxidant activity.
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 bioautography

Two microliters of the ethanolic extract (250 mg/mL) was applied (band length - 6.0 mm; distance between bands - 1.0 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.

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. (a1) 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. (b1) 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 developed 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.
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 fluorescence 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, but cytokine concentration (1.0, 3.0, and 5.0%) of cytokine 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% cytokine, followed by 91.1% and 63.3% germination with 3.0 and 5.0% cytokine respectively. Time required for germination is directly proportionate with concentration of cytokine, generally 21 days required for germination of SSs prepared with 5.0% cytokine following by 10 days at 3.0% and 6 days at 1.0% cytokine. Though the earliest germination with highest percentage was noted with 1.0% cytokine, 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% cytokine 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% cytokine 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 cytokine was found suitable for all aspect including proper shaping of the beads. In vitro adventitious shoot induction in Aloe vera [4,41].

Effect of sodium alginate concentration on germinability of SS
Polymerization of sodium alginate in Ca(NO₃)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, 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 following 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. In vitro adventitious shoot induction in Aloe vera [4,41].

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 week. 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

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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. 1).

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 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 IC50 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 IC50 value of the extract in scavenging ABTS radical was found to be 1.781 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.

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 (IC50 = 5.663 µg/mL) with the positive reference standard (IC50 = 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 Rf 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>
<thead>
<tr>
<th>Sl. no.</th>
<th>Samples</th>
<th>Concentration (µg/mL)</th>
<th>Percentage of inhibition (mean±SD)</th>
<th>IC50 (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. B. chamaedryoides</td>
<td>250</td>
<td>18.996±0.746</td>
<td>30.984±0.613</td>
<td>750</td>
</tr>
<tr>
<td>2. Ascorbic acid</td>
<td>Standard</td>
<td>12.027±0.813</td>
<td>66.866±0.881</td>
<td>27.280±0.301</td>
</tr>
<tr>
<td>3.</td>
<td>2</td>
<td>42.374±0.730</td>
<td>1.148±0.034</td>
<td>250</td>
</tr>
<tr>
<td>4.</td>
<td>6</td>
<td>85.066±0.632</td>
<td>72.590±0.756</td>
<td>7</td>
</tr>
</tbody>
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Table 4: DPPH radical scavenging activity

<table>
<thead>
<tr>
<th>Sl. no.</th>
<th>Samples</th>
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<th>Percentage of inhibition (mean±SD)</th>
<th>IC50 (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. B. chamaedryoides</td>
<td>5</td>
<td>29.238±0.951</td>
<td>50</td>
<td>48.078±0.970</td>
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<tr>
<td>2. Ascorbic acid</td>
<td>Standard</td>
<td>29.238±0.951</td>
<td>2.624</td>
<td>66.866±0.881</td>
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Table 5: ABTS radical scavenging activity

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

3. Haque SM, Ghosh B. Micropropagation, *in vitro* flowering and


