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Phytochemical and molecular profiling of tissue culture derived *Bacopa monnieri* (L.) Wettst clones against field grown plants

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Abstract
There has been overexploitation of *Bacopa monnieri* from natural habitats due to growing commercialization and urbanization. Inspite of being found in the wetlands, the plant has a very low drug content i.e., 0.2% and hence for extraction of these drugs, large amount of plant material is needed which can be accomplished via tissue culture. Phenylalanine when used as a precursor in optimum concentration (0.25 – 0.5 mg/ml) showed rapid enhancement of the phytoconstituents in Brahmi. Upon protein profiling, maximum protein content was found in the tissue culture produced samples in comparison to the field grown ones.

Keywords: *Bacopa monnieri,* elicitor, *In vitro*, phenylalanine, protein, saponin

Introduction
In India, all sections of people use medicinal plants either directly as folk medicines or indirectly as Pharmaceutical preparations of modern medicines [1]. India has great diversity of medicinal plants among which *Bacopa monnieri* (L.) Wettst holds immense importance. *Bacopa monnieri* (L.) Wettst (Family Plantaginaceae) commonly called Brahmi, is a neuroprotective herb that is mainly dominant in warm wetlands of the Asian countries and is native to Australia and India [2].

It is widely recommended as a nerve tonic and is recognized in Ayurvedic materia medica for its memory enhancing properties [3]. Brahmi has also been used as a remedy for curing and various cardiovascular ailments like insomnia, depression, psychosis and amnesia along with memory impairment [4]. In silico studies and pre-clinical application of Brahmi extract showed its therapeutic activity against treatment of cancer, glioblastoma tumor cell, cognitive impairment in Parkinson's and Alzheimer's disease along with hypothyroidism [5]. These neurotropic effects of *Bacopa monnieri* (L.) Wettst are mainly due to the presence of Bacoside A, Bacoside B and two dammarane tetracyclic triterpenoid saponins on the basis of detailed analysis using 1D and 2D NMR spectroscopic methods, and chemical evidences [6, 7]. Medicinal importance of *Bacopa monnieri* (L.) Wettst and its continuous depletion from natural habitat [8] are enough reasons for applying tissue culture approaches especially micropropagation which can be used as alternative techniques for rapid growth and germplasm conservation of this nootropic herb for ensuring a continuous supply of bioactive metabolite producing plant sample to the pharmaceutical industry. Variations in growth regulators, alteration of nutrients and introduction of certain metabolic intermediates can enhance the secondary metabolite production of *in vitro* plants.

Earlier, many reviews have discussed pharmacological properties of *Bacopa monnieri* in a broad perspective; however, very few comprehensive articles have shown its effect on molecular level. Hence, in this study, we have made an attempt to conserve the medicinally important herb, *Bacopa monnieri* (L.) Wettst through *in vitro* methodology and evaluated the phytoconstituents (responsible for its therapeutic nature) of the tissue cultured samples and compared them with field grown plants (Fig 1.) along with protein profiling of this therapeutic herb.

Materials and Method
Collection of plant material
Two weeks old young and healthy plants of *Bacopa monnieri* (L.) Wettst were collected from the departmental garden of Lady Brabourne College, Kolkata, West Bengal 700017. The plants were identified by the experts of Calcutta University Herbarium (CUH), and the herbarium specimens were submitted at CUH, Kolkata (Acc No. 20041) [25].

Multiplication of shoot buds
For explants, apical buds (1-2 cm) of field grown plants were taken. The explants were then surface sterilized. The nutrient media that was used for tissue culture of *Bacopa* was modified Murashige and Skoog's (MS) basal medium supplemented with sucrose and Gelrite® [2] along with varying concentrations of growth regulators such as IAA (Indole-3-acetic acid) for root formation and BAP (6-benzylaminopurine) for shoot formation.

Callusing and Organogenesis
For callusing, leaves were used. MS basal media along with 5 mg/ml BAP 5 + 0.5 mg/l 2,4-D were used [9]. The culture tubes containing media and the instruments required for culture were packed and autoclaved for sterilization. In a laminar air flow chamber under aseptic conditions, the explants were inoculated in sterilized modified basal media and maintained in a culture room at 24°C. After every four weeks, sub-culturing was performed under aseptic conditions in order to keep maintaining the growth of the plant sample.

Fig 1: *In vivo* plant of *v* (L.) Wettst. (Samanta *et al.*)

Biochemical Assays
Selecting plants for Control and Elicitation
For elongation, the *in vitro* shoots were sub-cultured in MS basal medium for 4 weeks. These culture derived *Bacopa* shoots were taken as control while the rest were subjected to elicitation. Experiments was carried out in triplicates. L-Phenylalanine is a precursor of anthocyanin biosynthesis via phenylpropanoid and flavonoid metabolic pathways [10]. It is added as an elicitor during culture. Methanol was used for preparation of stock solutions. In the control set, instead of elicitor, water was added. Varied concentrations of phenylalanine (0.25, 0.5, 1 and 2 mg/ml for clones E1-E4, respectively) was added to the media with the control having no phenylalanine in it. After maintaining them for 2 months, several parameters were observed from the *in vitro* regenerated shoots.

Preparation of the plant extract
Fresh leaves of *Bacopa* (2 g) were crushed using 1 ml of 80% methanol in a mortar and pestle. The macerated tissue was then taken in an Eppendorf tube and cold centrifuged at 15000rpm. The supernatant was carefully collected and rotary evaporator was used to vaporize the methanol overnight, in order to get the crude plant extract.

Total saponin content
The total saponin content in the plant sample was estimated using the vanillin-sulphuric acid method of [11] as modified by [12]. Methanolic extract of the plant sample was added to 8% vanillin solution followed by 72% H_2SO_4. The reaction mixture was incubated in a water bath for 10 min followed by cooling. The absorbance was recorded at 544 nm in Systronics UV-Vis spectrophotometer (Shimadzu) [3]. Bacoside A was used as standard and the results were expressed in terms of mg bacoside A equivalent/g plant material [12].

Phenolics Estimation

The total phenolic content in the plant sample was determined using Folin-Ciocalteau reagent [13]. 1 mg/ml methanolic stock solution was taken to which Folin-Ciocalteau reagent and 7.5% Na_2CO_3 was added [14]. This reaction mixture was incubated in dark for 30 minutes and the absorbance was recorded at 760 nm in Systronics UV-Vis spectrophotometer (Shimadzu). Gallic acid was used as the standard and the results were expressed as mg gallic acid equivalents/g of fresh weight (mg GAE/g FW).

Flavonoid Estimation

Flavonoid content was estimated using Aluminum chloride colorimetric assay [8]. In this case, quercetin was used as standard and the concentration of flavonoid was expressed as quercetin equivalence from the standard curve in mg/ml QE/g FW. 1 mg/ml of plant extract was taken to which distilled water and 5% sodium nitrite was added followed by addition of 10% $AlCl_3$ and sodium hydroxide [1]. The absorbance was recorded at 510 nm by Systronics UV-Vis spectrophotometer (Shimadzu).

Total antioxidant activity

A method derived by Prieto *et al* was used for measuring total antioxidative activity [15]. Methanolic extract was taken in a test tube in which reagent solution was added. This reaction mixture was incubated at 95ºC for 90 minutes and then cooled down [2]. The absorbance was measured at 695 nm using Systronics UV-Vis spectrophotometer (Shimadzu). Ascorbic acid was used as a standard and the total antioxidative capacity was expressed as ascorbic acid equivalent (mg/mL Asc AE/g FW).

Free radical scavenging activity

The antioxidative capacity was assessed by their ability to scavenge DPPH [16]. To evaluate the antioxidative capacity of these extracts, they were allowed to react with DPPH in a methanolic solution and kept in the dark for an hour. The absorbance was measured at 517 nm using Systronics UV-Vis spectrophotometer (Shimadzu). Methanol was used instead of plant extract in the blank set. DPPH radical scavenging activity can be calculated by: Free radical scavenging activity (%) = $100 \times (1-A_{sample}/A_{blank})$ Where, A_{blank} is the absorbance of the control and A_{sample} is the absorbance of the extract or ascorbic acid [17].

Total Protein content

The total protein content in the plant sample was estimated using Bradford reagent where bovine serum albumin (BSA) was used as a standard [18]. The reaction mixture was incubated at 37°C for 10-12 minutes. Absorbance was recorded at 595 nm using Systronics UV-Vis spectrophotometer (Shimadzu). The total protein content was expressed as mg protein/g of fresh weight (mg/g FW).

Total protein profile

2 g of tissue was taken and mixed with protein extraction buffer (PEB) and pulverized in a pre-chilled mortar and pestle. The crushed material was taken in Eppendorf tubes and centrifuged at 12000 rpm in a cold centrifuge. The supernatant was collected and re-centrifuged. Following this, the supernatant having protein submerged in buffer solution was carefully decanted off and stored at –20°C for further analysis [16].

SDS-PAGE electrophoresis

Sodium dodecyl sulfate (SDS) is a detergent which is commonly used in electrophoresis for estimating the purity of the sample and its molecular weight. The gels consist of acrylamide, bisacrylamide, SDS and a Tris-HCl buffer. TEMED and Ammonium persulfate (APS) were freshly added. Gels were polymerized in a gel caster. Initially the resolving gel (15%) was poured and allowed to polymerize. Then the stacking gel (5%) was poured and a comb was placed to create the wells. After polymerization, the comb was removed and the gel was ready for electrophoresis. Samples (50 µg protein) were mixed with equal amount of 1x SDS-gel loading buffer (SLB) and added to the wells in the gel caster using a pipette. The setup was then placed on a vertical electrophoresis system with 1x Tris-Glycine electrophoresis buffer. Finally, the apparatus was hooked up to a power source under appropriate running conditions to separate the protein bands. After 2-3 hours of electrophoresis, the gel was immersed in staining solution overnight and destained with 5:4:1 mixture of methanol, water and acetic acid. After visualizing bands and zones clearly, the gel was withdrawn from the destaining solution and washed with distilled water and fixed in 20% glycerol. The image was taken under illuminator and analyzed using Gel Analyzer software.

Results

Shoot bud multiplication

Shoot bud multiplication is dependent upon meristem initiation and its activity which in turn is hormonally controlled mainly by cytokinin (Banerjee *et al* 2008) [25]. Maximum shoot induction was augmented in the medium with 2 mg/l BAP (Fig 2.). Root formation in *in vitro* generated shoots is performed after adding auxin exogenously. Full-strength rooting was observed in MS medium containing 1mg /l of IAA.

Fig 2: *In vitro* plant (control) of Bacopa monnieri (L.) Wettst. After 2 weeks

Callusing and Organogenesis

The soft callus was green to yellowish in color. After 10 days of culture, the initial swelling for callus formation was seen (Fig 3.) followed by plantlet formation after 15 days when the leaf callus was transferred into MS basal media augmented with 5 mg/l BAP. After a period of 7-12 weeks, each callus showed a regeneration of 60-70 plantlets from the same media (Fig 4.). Thus, this protocol proved to be more cost effective and less time consuming as it showed plantlet regeneration from callus in a media containing single hormone and within a considerable period of time.

Fig 3: Organogenic callus of *Bacopa monnieri* (L.) Wettst. After 2 weeks (Samanta *et al*.)

Fig 4: Regenerated plantlets of *Bacopa monnieri* (L.) Wettst. after 7 weeks

Total saponin content

The tissue culture derived samples showed 18% increase in the saponins content (146.464 ± 0.0007 mg bacoside A equivalent/g FW) as compared to its field growing counterparts (123.489 ± 0.0089 mg bacoside A equivalent/g FW) (Table 1). Upon elicitation by phenylalanine, the saponin content increased by 1.05 folds whereas in the callus and regenerated plantlets, the content slightly declined.

Table 1: Determination of Total Saponin, Total Phenol and Total Flavonoid in methanolic extract of *Bacopa monnieri*

Plant sample	Total Saponin (mg bacoside A equivalent/g FW)	Total Phenol (mg GAE/g FW)	Total Flavonoid (mg/ml QE/g FW)
In vivo plants (Field grown)	123.489 ± 0.0089	19.0449 ± 0.019	20.582 ± 0.416
In vitro plants (Control)	146.464 ± 0.0007	20.0048 ± 0.033	23.012 ± 0.543
Callus tissue	132.461 ± 0.0009	20.0485 ± 0.065	21.065 ± 0.412
Regenerated tissue	131.591 ± 0.0007	19.0085 ± 0.019	21.874 ± 0.456
E1 (0.25 mg/ml PA)	154.445 ± 0.005	21.0056 ± 0.033	24.152 ± 0.94
E2 (0.5 mg/ml PA)	151.745 ± 0.001	21.0038 ± 0.033	24.096 ± 0.94
E3 (1 mg/ml PA)	137.385 ± 0.0003	19.0089 ± 0.033	22.0102 ± 0.543
E4 (2 mg/ml PA)	135.778 ± 0.0006	18.0057 ± 0.019	18.0118 ± 0.543

Data represented as Mean ± S.E. from 3 replicates

Total phenol content

The phenol content differs in each set. There is a 5% more increase in the production of phenolics in tissue culture derived plants (20.0048 ± 0.033 mg GAE/g FW) in comparison to *in vivo* plants (19.0449 ± 0.019 mg GAE/g FW). After the addition of low concentration of elicitor (0.25 mg/ml and 0.5 mg/ml phenylalanine) the production of total phenol increased by 1-fold whereas upon further increasing the concentration of phenyalanine (1 mg/ml and 2 mg/ml), it is seen to suppress the production of phenolics in the plant sample. (Table 1).

Total flavonoid content

The total flavonoid content also varies among different samples with *in vitro* plants having a production of 23.012 ± 0.543 mg/mL QE/g FW. After elicitation, the flavonoid content was augmented by 5% (Table 1). In laboratory conditions, experimental factors can be regulated. Hence, tissue culture derived plants had enhanced concentration of secondary metabolites as compared to the field grown plants.

Total antioxidant capacity

In the present study, the antioxidant capacity was recorded for both the field grown and tissue culture derived plants and the data obtained showed that the methanolic extract of the *in vitro* elicitated plants showed highest antioxidant activity which was obtained from regression equation (y = 0.0071x - 0.0295) of calibration curve of Ascorbic acid (Table 2). The total antioxidant capacity of *in vitro Bacopa monnieri* plants was 230.333 ± 0.001 mg/mL Asc AE/g FW. 7.6% increase in TAC was observed post elicitation whereas further elicitation (1 mg/ml and 2 mg/ml) caused suppression in antioxidative capacity.

Table 2: Determination of Total Antioxidant and Free radical scavenging activity in methanolic extract of *Bacopa monnieri*

Plant sample	Total antioxidant capacity (mg/mL Asc AE/g FW)	Free radical scavenging activity (%) (1mg/mL)
In vivo plants (Field grown)	217.516 ± 0.0002	87.342 ± 0.0035
In vitro plants (Control)	230.333 ± 0.0001	89.0428 ± 0.0062
Callus tissue	228.463 ± 0.0004	87.4665 ± 0.0074
Regenerated tissue	224.634 ± 0.0002	85.615 ± 0.0095
E1 (0.25 mg/ml PA)	245.009 ± 0.0001	94.0061 ± 0.0008
E2 (0.5 mg/ml PA)	248.0115 ± 0.0008	95.297 ± 0.0715
E3 (1 mg/ml PA)	238.767 ± 0.0008	76.198 ± 0.0062
E4 (2 mg/ml PA)	236.733 ± 0.0001	69.4741 ± 0.0348
Ascorbic acid	255.4836 ± 0.0004	53.639 ± 0.0001

Data represented as Mean ± S.E. from 3 replicates

DPPH radical scavenging activity

Upon increasing the concentration of plant extract, there was an increase observed in the radical scavenging activities of DPPH. This assay is a reliable parameter for determining the *in vitro* antioxidant activity of plant extracts. This occurs due to discoloration of the purple-colored DPPH to light yellow color indicating that the free radical scavenging activity of *Bacopa monnieri* was due to its ability to donate protons. The percentage inhibition of the *in vivo* plant extract is 87.342 ± 0.0035 %, while that of the elicitated clone is 95.297 ± 0.0715 %, nearly 1.09 times higher than the field grown plants.

Quantitative analysis of Total protein content

In this assay, the absorbance maxima for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when it binds with protein. Both the ionic and hydrophobic interactions stabilize the anionic form of the dye resulting in a visible change of color. The *in vivo* leaf tissues showed a total protein content of 21.975 ± 0.0035 mg/gm Fresh Weight, whereas the *in vitro* ones had 24.583 ± 0.0009 mg/gm Fresh weight. Thus, the *in vitro* leaves of *Bacopa monnieri* (L.) Wettst showed 1.11 times more protein content than *in vivo* leaves.

Qualitative analysis of Total protein content

The protein profile gives an idea about the differential gene expression in same species, with respect to changes in the environment of a plant, as different new molecular weight proteins (represented by unique bands) are seen to be expressed in different tissues, growing in different conditions [6, 14]. *In vitro* and *in vivo* leaf tissues shows the presence of 15 bands in total. *In vitro* tissues have bands in the range of 12.3 kDa to 399.7 kDa, while *in vivo* leaf tissues have a range of 12.9 kDa to 399.7 kDa (Table 3.). Callus tissue has 16 bands in total, having a molecular mass ranging from 12.3 kDa to 377.1 kDa. Callus tissue have 2 bands in common with *in vitro* leaves (53.2 kDa and 12.3 kDa), 1 band in common with *in vivo* leaves (53.2 KDa) and 1 band in common (15 kDa) with regenerated tissue. Regenerated tissue has a total of 15 bands with molecular mass ranging from 12.6 kDa to 387.4 kDa, having differences in banding patterns with *in vivo* leaf, *in vitro* leaf and callus tissue. [19]. *In vivo* tissue, *in vitro* tissue, callus & regenerated tissues have 1 common band of molecular mass 53.2 kDa (Fig 5.). This may be due to the presence of rubisco protein [26].

Table 3: Total protein profile by polyacrylamide gel electrophoresis in *in vivo* & *in vitro* tissues of *Bacopa monnieri* (L.) Wettst.

Plant material	Total number of protein bands	Molecular weight range (kDa)
Callus tissue	16	12.3-377.1
Regenerated tissues	15	12.6-387.4
In vitro leaf tissues	15	12.3-399.7
In vivo leaf tissues	15	12.9-399.7

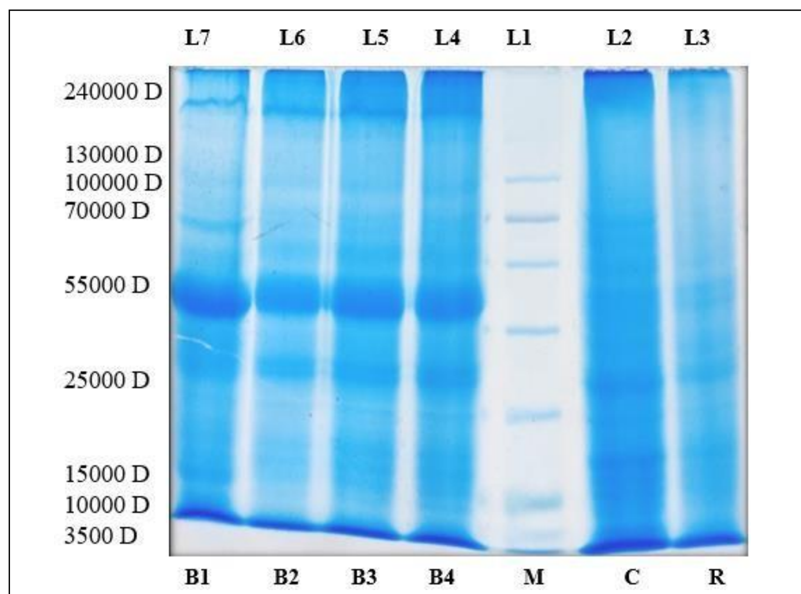

Fig 5: Total Protein profile of *Bacopa monnieri* (L.) Wettst by SDS-PAGE electrophoresis, Lane 1: M= Protein molecular weight marker, Lane 2: C= Callus protein, Lane 3: R= Regenerated tissue protein, Lane 6, 5, 4: B2, B3, B4= *In vitro* leaf protein from plant grown in BAP media, Lane 7: B1= *In vivo* leaf protein

Discussion

The aromatic amino acid phenylalanine derived from shikimic acid pathway is not only an essential component for synthesis of proteins, but also serves as precursors for a variety of secondary metabolites that are necessary for growth of plant. As evident from our results, phenylalanine when used at a concentration ranging between 0.25 – 0.5 mg/ml serves to be the optimum by enhancing the secondary metabolite production whereas further elevated concentrations of phenylalanine (1 – 2 mg/ml) interferes with the relevant enzymatic pathways causing reduced phytoconstituents in the samples. Parale *et al*. also observed the same where along with increasing concentration of phenylalanine, there was a gradual decline in the shoot and callus biomass of *Bacopa monnieri*. Bauer *et al*. (2004), also reported that there was a suppression in the growth of *Coleus blumei* transformed callus in a medium containing 1mg/l phenylalanine [20]. Saponins of *Bacopa monnieri* are significant in their role in modulating neurological disorders. Our results showed increased saponin content in *in vitro* elicitated samples than the *in vivo* counterparts. Bhardwaj *et al*. also observed variations in the saponin content of Brahmi from different geographical locations. These variations could be due to changes in their herbal formulations at different regions around the world [4]. Other group of researchers reported that the total saponin glycoside production increased by 2.6-fold in *Bacopa* after adding phenylalanine for consecutive 6 days and the saponin concentration reached its peak after adding both l-alanine and l-phenylalanine for consecutive 6 days [21]. In our results, phenol content increased by 1-fold in the presence of elicitor phenylalanine. This was in accordance with the findings of Roy *et al*. where they observed increased phenol content after addition of phenylalanine at low concentrations in *Mentha piperita*. The phenolic compounds have redox properties which are responsible for their antioxidative potency hence can play a significant role in neutralizing and absorbing free radicals either by decomposing peroxides or by quenching the singlet and triplet oxygen [22]. Previous study by Alam *et al*. showed similar radical scavenging property of *Bacopa monnieri* by using its methanolic extract. The antioxidant activity is directly proportional to the total phenolic content present in the herb i.e., the higher the total phenol content in the sample, the higher will be the total antioxidative capacity and percentage inhibition of free radicals [23]. Our results indicate that tissue culture generated plants contain comparatively greater concentration of antioxidative compounds (Table 2.). Hence, they are good free radical scavengers thus can inhibit lipid autoxidation, which is in turn is beneficial for treating several disorders where lipid peroxidation acts as an important phenomenon resulting in pathogenesis [14]. The callus and regenerated plantlets showed decreased content of secondary metabolites as compared to the *in vitro* and elicitated samples (Table 1. and 2.). This might be due to reduced production of certain enzymes in the dedifferentiated samples. The protein content in the *in vitro* plant sample was much higher as compared to the field grown plants (Table 1.). These findings matched with the SDS-PAGE electrophoresis where differential banding patterns were observed for differentially treated samples thus indicating a source of thought for future research in this arena (Fig 5.).

Conclusion

Tissue culture derived samples showed better content of phytoconstituents as they are grown under stress-free controlled laboratory conditions hence, they show higher yield within a short span of time. In environment, it is not easy to identify which particular parameter is leading to these variations. So, with the help of tissue culture methodologies, it was observed that there was a stable production of phenol, flavonoid and saponin in the shoot based clonal lines [22]. In plant tissue culture, an elicitor can be defined as a compound which when incorporated

into a biological system in minimal concentrations can enhance the synthesis of certain target metabolites [24]. L-Phenylalanine when introduced as an elicitor at low concentrations enhanced the production of secondary metabolites hence it can be used by biotechnologists to improve the yield of phytoconstituents in a short period of time. The presence of these phytocompounds is directly responsible for the neuroprotective and pharmacological activities thus establishing the applications of *B. monnieri* as an herbal medicine for preventing inflammation and ROS treatment by their antioxidative capacity by scavenging free radicals. The plant can further be introduced to chromatographic separation and purification processes for isolating these bioactive lead compounds that can result in the discovery of new therapeutic agents.

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Conflict of Interest
The authors declare no conflict of interest.

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