ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Micropropagation and Comparative Phytochemical, Antioxidant Study of *BacopaMonnieri* (L.) Pennell.

SK Mishra[1]*, KN Tiwari[2], PL Shivna[1], and AK Mishra[2].

[1]Department of Pharmaceutics, IIT (BHU), Varanasi, India.
[2]Department of Botany, MMV, BHU, Varanasi, India.

ABSTRACT

Bacopa monnieri an important medicinal plant of Scorphulariaceae family and is distributed in the wet and marshy lands throughout India, Nepal, Sri Lanka, China, Taiwan, and Hawaii. It shows the presence of various phytoconstituents namely, alkaloids, saponins, phenolics and flavonoids. These phytoconstituents are responsible for anti oxidant and various other activity of the plant. The protocols for tissue culture has been developed for regeneration of the BM plant from small part (nodal segment). The regenerated plant having the similar potential in terms of phytoconstituents and antioxidant activity. The *In vitro* (leaf and stem) regenerated plants have slightly less antioxidant activity as compared to the *In vivo* but comparable. The protocols (MS+BAP5µg/ml) may used for the clonal regeneration of plant on large scale through which it can be protected and preserved for long duration in small space. A good response was observed in a soil ratio 3:2:1 (Soil rite: red sand: garden soil) for acclamatization.

Keywords: Bacopa, phenolics, antioxidants, flavonoids, regeneration etc.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Bacopa monnieri is a highly valuable medicinal plant from the Scorphulariaceae family has been proven its importance majorly in relation to botanical, commercial, ethnophamacological, phytochemical, pharmacological and toxicological studies. It is distributed in the wet and marshy lands throughout India, Nepal, Sri Lanka, China, Taiwan, and Hawaii. It shows the presence of alkaloids (nicotine, brahmine, herpestine), saponins (hersaponin, betulic acid and bacosides) and other chemicals like stigmastanol, beta-sitosterol and stigmasterol. It is found to be effective in several neurological disorders like anxiety and neurosis. It is also used in anti-inflammatory, analgesic and antipyretic activity, treat asthma, insanity, epilepsy, hoarseness, enlargement of spleen, snake bite, rheumatism, leprosy, eczema and ring worm, it is also used as a diuretic, appetitive and cardio tonic. The plant has gained vast therapeutic and commercial value, so it is necessary to prevent it from being endangered. (Sharuti et al., 2013) Now a days it is used for medicinal purpose in fresh form and main constituents in different pharmaceutical products, but it lost from their natural habitats due to overexploitation. (Richa Jain et al. 2013). On the basis of medicinal uses, commercial value and potential for further research and development, *B. monnieri* is designated as the second most important medicinal plant of India (Shrivastava *et. al.*, 1999). The annual demand of *B. monnieri* during the year 2004-2005 was 6621.8 tonnes with an annual growth rate of 7%.

The requirement of raw materials of *B. monnieri* is expected to increase further, which may be due to the popularity of using *Bacopa* based “memory enhancing” drugs. Most importantly, *B. monnieri* has been enlisted among 178 species of medicinal plants of India with high trade requirement (≥100 million tonnes/year). Unfortunately, the requirement of this plant material is fulfilled mainly by collection from wastelands (Ved DK and Goraya GS, 2007). Thus, development of methods for ensuring the availability of raw material of a consistent quality from regular and viable sources is imperative (Shrivastava *et. al*., 1999). The seeds of *B. monnieri* have short viability of two months and thus, are poor propagules. Besides, two-leaved stage is the crucial period for this plant as frequent seedling death has been observed during this time. Thus, raising plants from seeds is a difficult task (Tiwari *et. al.* 2001, Rathore *et. al.* 2013). Vegetative propagation by stem is a slow process (Tiwari *et. al.* 2001, Shah JD, 1965). Besides, it was observed that the growth of *B. monnieri* is dependent on seasonal changes (Sharma N, 2005). Thus, conventional method of propagation is inadequate to meet the demand of raw material of *B. monnieri*. The unsustainable collection of raw material from natural population has already placed the plant under threatened category (Tiwari *et. al.* 1998, Ceasar *et. al.*, 2010). Thus, for constant supply of raw materials and reduction of pressure on natural/wild population, development of an efficient and reliable *in vitro* plant regeneration protocol for this wonder medicinal herb is essential. Plant tissue culture is a powerful weapon for propagation and conservation of medicinal herbs as well as other economical herbs. The method is based on using a plant part or single cell or group of cells in artificial aseptic conditions is called "Tissue Culture" and these help without affecting the main feature of plants or herbs. Tissue culture technology has been known as an effective tool to propagate several valuable medicinal plants. The technique may be suitably applied in case of brahmi where natural regeneration through seeds or cuttings is slow and require good irrigation facilities. The present study was taken up with the aim to develop a clonal propagation protocol for bacopa with elite plant as the starting material, resulted in large scale production of superior quality clones of the ex plant. The present study is also focused compare the antioxidant and photochemical content difference in the *in-vivo* and *in-vitro* cultivated plant of *Bacopa monnieri.*

MATERIALS AND METHODS

Selection of plants

Bacoapa monnieri is an herb of medicinal importance which has many proven beneficial pharmacological activity. Due to its pharmacological importance and commercial value of the plant over exploited from natural resources has taking place and make this herbs as an edge of endangered species.

Collection and authentication of plant

Plant of *Bacoapa monnieri* was collected from field of Botanical garden of Department of Dravyaguna, Banaras Hindu University, Varanasi, U.P in the month of August and September 2014. The plant was

authenticated by Dr. V. K. Joshi, Head, Department of Dravyaguna, BHU, Varanasi and was kept in shade for about ten days. The dried plant part was then powdered for further phytochemical and antioxidant study.

Preparation of Media

Murashige and Skoog's (MS) medium was used for the cultivation of *B. monnieri*. at *in vitro* condition. The MS medium was prepared by adding required amounts of stock solutions and final volume was made up with distilled water. The pH of the medium was adjusted to 5.8 using 1 N NaOH/KCl. About 50 ml of the medium was poured into sterile culture bottles. The culture bottles with MS medium was autoclaved at 121°C for 20 min. at 15 lbs pressure and transferred to the media storage room where they were kept under aseptic condition for further experimental study.

Development of aseptic culture

Bacopa Moniarae twigs with 3-4 nodes were collected, were washed thoroughly in running tap water to remove the superficial dust and mud, then explants were washed with dilute detergent (1 - 2% Cetrimide) solution for 10 minute and then washed well in running tap water. Explants were surface sterilized with 5 min treatment with 0.1% (w/v) $HgCl_2$. BM was extremely sensitive to surface sterilizing agent, therefore, the surface sterilization procedure was optimized and this helped in preventing blackening of tissues and establishment of clean cultures. During surface sterilization treatment, it was found that treatment with 0.1% mercuric chloride leads to blackening of the explants. Hence limited treatment of 0.1% mercuric chloride was given to plants 4 - 5 min, and it was proved to be the best sterilent. Finally, the explants were washed thoroughly (4 - 5 times) with sterilized distilled water. Throughout the experiments, MS medium with 3% (w/v) sucrose and gelled with 0.7% (w/v) agar is used. The pH of all media was adjusted to 5.8 before autoclaving at 121°C (15 min). The cultures were incubated in a culture room at 25 ± 2°C under 16/8 hrs (light/dark) photoperiod maintained by light supplied by cool-white fluorescent tubes at an intensity of 2500 lux.

***In- vitro* establishment of cultures**

Surface sterlised nodal explants were inoculated onto full strength MS medium (Murashige and Skoog, 1962) having 3% sucrose and supplemented with different concentrations and combinations of plant growth regulators viz. simple MS medium, MS + BAP (1µg/ml), MS + BAP (3µg/ml) and MS + BAP (5µg/ml) with other regulators.

***In- vitro* shoot multiplication**

Shoots induced from nodal were excised and placed on fresh medium of same composition for establishment of initial stock of shoots. Single shoots were separated and further used to perform experiments to standardize best medium composition for optimal shoot multiplication. For the purpose, MS medium supplemented with cytokinin BAP (5µg/ml) alone was tried. *In vitro* generated healthy shoots were maintained by regular sub culturing of propagules of 3 shoots each after every 3 weeks of culture.

***In-vitro* Rooting**

In-vitro developed healthy shoots were transferred to root induction medium supplimented with 2% sucrose and different concentrations of auxins hormones NAA/IAA (0.35-0.95 mg/l).

Acclimatization and Hardening

Acclimatization is the process in which an individual plants adjusts to a gradual change in its environment (such as a change in temperature, humidity, photoperiod, or pH), allowing it to maintain performance across a range of environmental conditions. The acclimatization condition in this experiment are as follows: temperature 24 ± 2^0 C; photoperiod 12hrs light and 12hrs dark, irrigation salt solution (MS medium without organic supplement) supply up to two weeks. *In-vitro* plantlets were washed under running tap water to remove all traces of agar. There after the plantlets were transferred directly to pots containing a mixture of soil: sand: manure and kept in Polyhouse for about 4 weeks where they were periodically watered. Well

developed plants with healthy shoots were hence successfully transferred to field conditions. Experiments were repeated thrice and each experiment consisted of a minimum of ten replicates. Observations on mean number of shoots/ roots and mean shoot length/root length were recorded after 4 weeks of culture. Data collected is represented as mean ±standard error (SE).

Extraction of *In-vitro and In-vivo plant parts*

Soxhlet extraction has been done with alcoholic (ethanol) solvent at 45^0C for 48 hrs. The dried *In-vitro and In-vivo* plant material (250 gm) was taken separately (Leaf and Stem) in powdered (Coarse size) form. The extract obtained was dried in rota vapor and stored in a desiccator for further phytochemical and antioxidant study.

Phytochemical study

The ethanolic extract of different plant part was screened for following phytoconstituents such as alkaloids, glycosides, flavonoids and saponins, sterols, carbohydrates, Saponins, proteins and amino acids and Phenol by the use of several reagents. (Khandelwal, 2007 & Kokate, 1986). The extract was further evaluated for phenols (Hagerman et al., 2000b) and flavonoids (Kumaran & Karunakaran, 2006) content.

Estimation of Phenolics (Price et. Al., 1980)

5 gm sample was homogenized in acetone and kept overnight in a flask. Supernatant was collected and residues were extracted with acetone, filter and centrifuge. Supernatant was used for estimation of phenolics. DW was added to extract and add ferric ammonium sulphate kept at room temperature. Potassium ferricyanide was added and absorbance was measured at 720 nm (concentration in µg/ml of extract.)

Estimation of Flavonoids (Harborne JB., 1975, Lamaison JL and. Carnt A, 1990)

5 gm sample was acid hydrolyzed with sulphuric acid and neutralized with sodium hydroxide. Ethyl acetate was added and shake well, ethyl acetate portion is collected.(repeat thrice) Ethyl acetate was palled and evaporated to dryness residues are reconstituted with methanol and assayed for flavonoids content. Extract was mixed with methanolic AlCl3 and
absorbance was measured at 430 nm.

In-vitro Anti-Oxidant study

DPPH radical scavenging activity

The free radicle scavenging activity of the extract, based on the scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radicle, was determined by the method described by Braca et al. (2001). Concentration 1mg/ml of Ascorbic acid is taken as stock solution. Prepare different dilution of Ascorbic acid (10, 20, 60, 80, 100, 200, 400 and 500µg/ml). And then take 0.5ml from above dilution and add 3ml of DPPH methanolic solution in each test tube. And take absorbance against blank (Methanol). Plant extract (0.1ml) was added to 3ml of a 0.004%MeOH solution of DPPH. Absorbance at 517nm was determined after 30minute, and the percentage inhibition activity was calculated from $[(Ao-A_1)/ Ao]*100$, where Ao is the absorbance of control, and A_1 is the absorbance of the extract/standard.

Reducing power

The reducing power of hydro-alcoholic extract was determined according to the method of Oyaizu (1986). Phosphate buffer was prepared as Buffer A and B. Different concentration of ascorbic acid were mixed with phosphate buffer (2.5ml)0.2M, pH 6.6 and potassium fericynide ($K_3Fe(CN)_6$) 2.5ml of 1%. The mixture was incubated at 37ºC for 20minute. After that 2.5ml TCA (10%) was added to the mixture, which was then centrifuged at 1000 rpm for 10 minute. The upper organic layer of solution 2.5ml was taken and mixed with TDW 2.5 ml and add $FeCl_3$ (0.5 ml), 0.1% and absorbance of reaction mixture was measured at 700nm against blank. Different amounts of alcoholic extract (50-250 mg) in 1ml of methanol were mixed with phosphate buffer (2.5 ml, 0.2 mol/l, and pH 6.6) and potassium ferricyanide $[K_3Fe (CN)_6]$ (2.5ml) of tri-chloroacetic acid

(TCA) (10%) was added to the mixture which was then centrifuged at 1000 rpm at room temperature for 10minute. The upper layer of solution was mixed with distilled water (2.5ml) and $FeCl_3$ (0.5ml, 0.1%), and the absorbance was measured at 700nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Superoxide dismutase (SOD) (Nishikimi, 1972)

Although superoxide anion is a weak oxidant, it ultimately produces powerful and dangerous hydroxyl radicals as wellas singlet oxygen, both of which contribute to oxidative stress (Meyer and Isaksen, 1995). The superoxide anion scavengingactivity can be measured as described by Robak and Gryglewski (1988). Different concentration of alcoholic extract (Leaf/Stem) of *Bacopa monnieri*were mixed with phosphate buffer (1.25ml), pH 7.8 and then add 600µl methionine after that 150µl EDTA, 300µl NBT, and 200µl riboflavin were added respectively. The mixture was kept aside for 6min and then absorbance was taken at 560nm against blank.

Lipid peroxidation assay (S. Sinha, R. Saxena et al., 2006)

Egg homogenate 10% in TDW (v/v) and 50µl alcoholic extract were mixed in test tube and then volume make up to 500µl by adding TDW. Finally 25µl $FeSO_4$ (0.07M) was added to the above mixture. And incubate for 30min to induce lipid peroxidation. There after 750µl 20% acetic acid (AA) pH 3.5 and 750µl 0.8% TBA prepared in 1.1% Sodium dodecyle sulphate (SDS) and 25µl 20% TCA were added, vortex and then heated in boiling water bath for 60 minute. After cooling 3.0µl Butanol was added to each test tube and centrifuge at 3000rpm for 10minute. The absorbance of the organic upper layer was measured against 3ml butanol at 532nm. For blank 50µl butanol was used in place of extract.

RESULTS

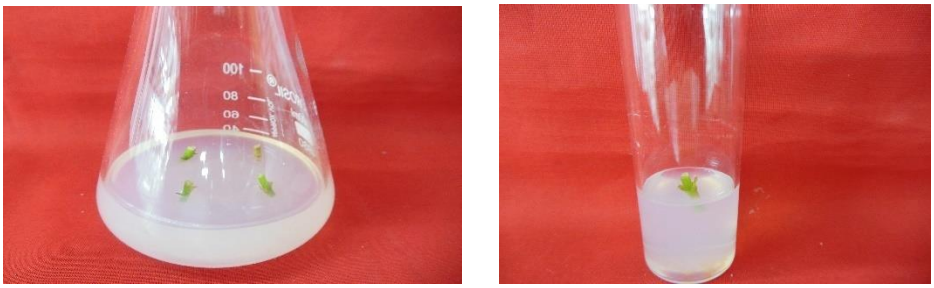
Micro propagation

It was observed that best medium for initiation and development in terms of bud breakage (%age) and maximum shoot length were MS+ BAP (5µg/ml) with shoot length of 5.46 cm; 80% bud breakage. In the other three mediums: simple MS medium, MS +BAP(1µg/ml), MS+BAP(3µg/ml), shoot length was less as compared to above defined mediums. (Table 1), (Fig. 1).

Table 1: Effect of Growth hormones on ex plant of BM.

Medium	No. of Shoots ± 3 cm	Avg. shoot Length (cm)	Inter nodal Distance(cm)	Average live culture tube
MS medium	3.2± 0.28	3.13	0.72± 0.01	20
MS+BAP(1µg/ml)	3.3 ± 0.07	4.15	0.75 ± 0.04	27
MS+BAP(3µg/ml)	5.5± 0.50	4.75	0.76 ± 0.02	33
MS+BAP(5µg/ml)	6.5 ± 0.10	5.46	0.80 ± 0.06	34

Fig 1: Initiation of culturing process with ex plant of BM.



ISSN: 0975-8585

In vitro shoot multiplication

Observations were taken for evaluating the growth of explants by taking parameters like internodal distance (inter-nodal distance was measured form the third node and measured up to sixth node from the shoot tip), average shoot length and number of nodes (15 shoots randomly selected per medium). The experiment was carried out in four mediums having different concentrations of growth regulators each with five replications; only results of best medium are given. The medium showing best results was MS+BAP(5µg/ml)average shoot length of 5.46 cm. Sub-culturing was carried out after 25-30 days using the same medium combinations as for initiation and establishment stages. (Fig. 2, 3).

Fig 2: Shoot proliferation after 15 days of nodal BM ex plant.

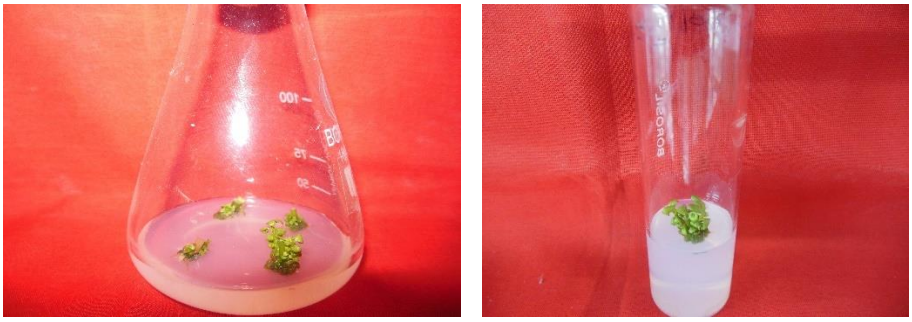


Fig 3: Shoot proliferation after 30 days of nodal BM ex plant.

Acclimatization

The result of acclimatized plants after 1 month at different concentration and ratio of soil was observed. A good response was observed in a soil ratio 3:2:1 (Soil rite: red sand: garden soil). (Table 2), (Fig. 4, 5)

Table 2: Effect of soil ratio on shoot height and branches of ex plant of BM.

No. of plant	Average shoot height			No of branches		
Soil ratio	3:2:1	3:1:2	3:0:1	3:2:1	3:1:2	3:0:1
1	4.15	2.45	4.14	8	4	7
2	3	4.94	3.5	7	5	5
3	4.75	2.96	4	8	6	9
4	5.46	Dry	5.1	3	Dry	5
5	3	5.3	4.7	8	6	8
6	33	3.43	4.8	10	8	9
7	3.28	1.66	6.4	14	3	9
8	Dry	5	4.1	Dry	3	7
9	3.83	3.02	4.3	3	4	9
10	3.38	5.2	2.6	9	2	10
11	5.5	2	2.7	7	3	6

ISSN: 0975-8585

Fig. 4: Acclimetization of *in-vitro* cultured BM plants of BM.

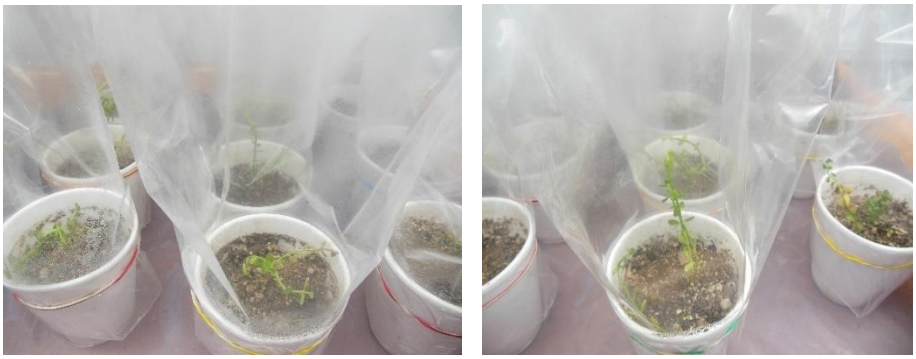


Fig. 5: Acclemetized *in-vitro* BM plant after 1 month.

Phytochemical study

The extracts of different parts of *In vitro* and *In vivo plant* (Leaf & stem) was phytochemically screened with suitable reagent and presence of Alkaloids, Glycosides, Phenolics, Flavonoids, Tannins, Saponin and Terpenoids was found. (Table 3) Further both (*In vitro* and *In vivo)* the extracts were estimated for phenolics and flavonoids. *In vivo* and *In vitro* parts (Leaf and stem) of the plant contain 0.019±0.001 mg/gm (Leaf) 0.025±0.0005 mg/gm (Stem), 0.022±0.002 mg/gm (Leaf), 0.021±0.0008 mg/gm (Stem) and 0.012±0.005 mg/gm (Leaf), 0.075±0.011 mg/gm (Stem), 0.013±0.003 mg/gm (Leaf), 0.070±0.008 mg/gm (Stem) of Phenolics and flavonoid. (Table 4)

Table 3: Phytochemical screening of BM

S. No	Test	Alcoholic extract(Leaf)	Alcoholic extract(Stem)	Alcoholic extract of in-vitro grown(Leaf)	Alcoholic extract of in-vitro grown (Stem)
1	Alkaloids	+ ve	+ ve	+ ve	+ ve
2	Amino acids	- ve	- ve	- ve	- ve
3	Glycosides	+ ve	- ve	+ ve	- ve
4	Cardiac glycosides	- ve	- ve	- ve	- ve
5	Carbohydrate	- ve	- ve	- ve	- ve
6	Phenolic	+ ve	+ ve	+ ve	+ ve
7	Flavonoids	+ ve	+ ve	+ ve	+ ve
8	Proteins	- ve	- ve	- ve	- ve
9	Organic acids	- ve	- ve	- ve	- ve
10	Steroids	+ ve	+ ve	+ ve	+ ve
11	Tannins	+ ve	+ ve	+ ve	+ ve
12	Saponin	+ ve	+ ve	+ ve	+ ve
13	Terpenoids	+ ve	+ ve	+ ve	+ ve

ISSN: 0975-8585

Table 4: Phenolics and flavonoid content of BM

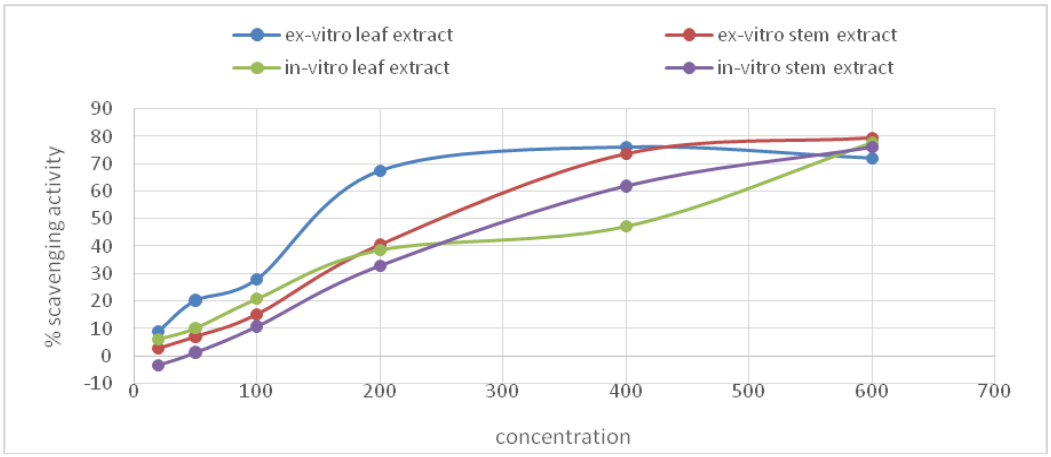
S. No.	In vivo		In vitro	
	Leaf	Stem	Leaf	Stem
Phenolics (mg/gm)	0.025±0.0005	0.022±0.002	0.021±0.0008	0.019±0.001
Flavonoids (mg/gm)	0.075±0.011	0.013±0.003	0.070±0.008	0.012±0.005

Antioxidant Assays

DPPH free radical assay

The reduction capability of DPPH radical is determined by the decrease in absorbance at 517nm induced by antioxidants. The scavenging effects of extract increased with their concentrations to similar extents in both *In vivo* and *In vitro* condition. The percentage inhibitions of Ascorbic acid (taken as standard) is 85.110 at 400 µg/ml. The comparison of *In vivo* and *In vitro* extract is shown in figure 1; it is showing that the extract is able to inhibit the DPPH 72.04611 (In vivo leaf), 79.5389 (In vivo stem) 77.8098 (In vitro leaf), 76.08069 (In vitro stem) at 600 µg/ml. (Fig. 6)

Fig. 6: % inhibition of *in-vivo* and *in-vitro* plant extract of BM.



Reducing power

Fig. 7: % reduction of *in-vivo* and *in-vitro* plant extract of BM.

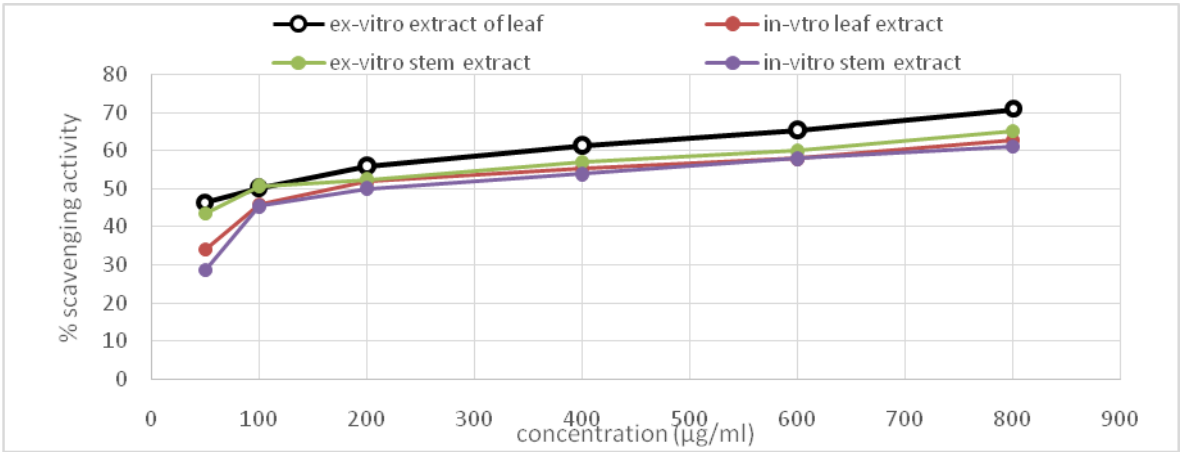


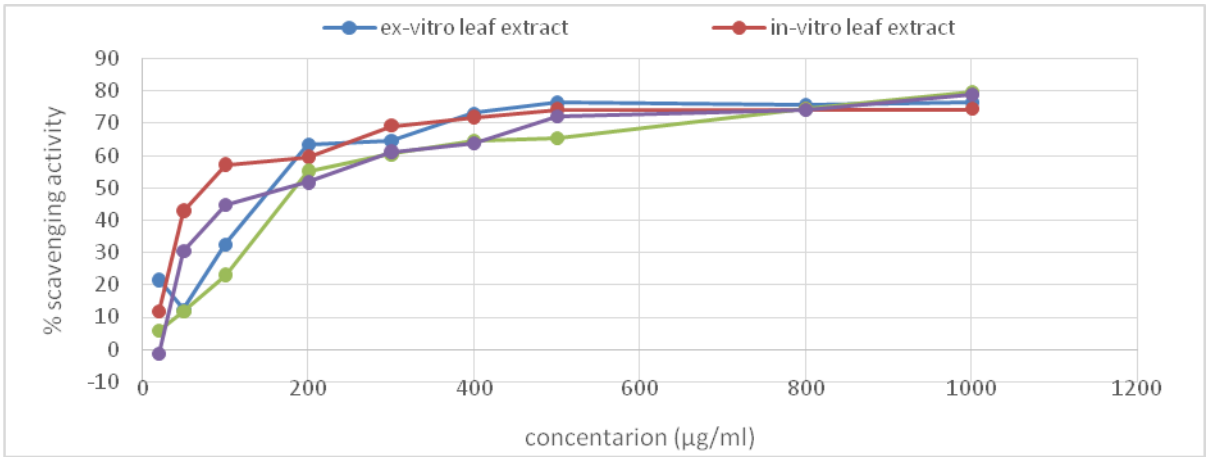
Figure shows the reductive capability of alcoholic extract of BM compared to ascorbic acid. We observed a direct correlation between antioxidant activity and reducing power of BM extract. Like the

antioxidant activity, reducing power of BM extract increases with increasing amount of sample; 62.82421 (In vivo leaf) 70.89337 (In vivo stem) inhibition was observed and 65.12968 (In vitro leaf) 61.0951 (In vitro stem) at 800 µg/ml. Percentage comparative reduction is shown in the figure. (Fig. 7)

Superoxide dismutase (SOD)

The superoxide radical scavenging activity was based on the capacity of the herbal preparation to inhibit blue formazan formation by scavenging the superoxide radicals generated in riboflavin-light-nitro blue tetrazolium system. Riboflavin is taken as standard and absorbance is taken at 560nm. Percentage inhibition was 76.4935182421 (In vivo leaf) 74.28571 (In vivo stem) 79.61039 (In vitro leaf) 78.83117 (In vitro stem) found comparable to the standard at 1000 µg/ml. The comparative study is shown in the figure. (Fig. 8).

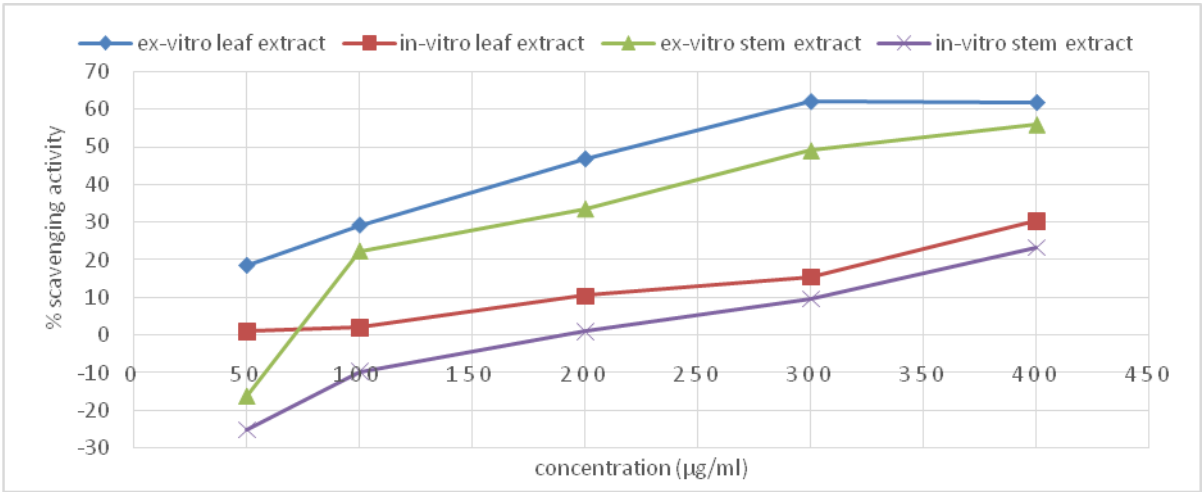
Fig. 8: % inhibition of *in-vivo* and *in-vitro* plant extract of BM.



Lipid peroxidation

The protective effects of the examined BM extracts on peroxidation of lipids have been evaluated by the TBA assay using two systems of induction, Fe^{2+}/ascorbate and Fe^{2+}/H_2O_2. The inhibition of LPO was determined by measuring the formation of MDA, using liposomes as an oxidizable substrate. However, the TBA test is non-specific for MDA and therefore non-lipid substances present in plant extracts, together with peroxidation products other than malondialdehyde, can react positively with TBA. Absorbance is taken at 532nm. Percentage inhibition was 30.40 (In vivo leaf) 74.28571 (In vivo stem) 55.81 (In vitro leaf) 23.27791 (In vitro stem) found comparable to the standard at 400 µg/ml. The comparative study is shown in the fig. 9.

Fig. 9: % inhibition of *in-vivo* and *in-vitro* plant extract of BM.



CONCLUSION

Bacopa monnieri has always been a topic of interest to a myriad of researchers. From tissue culture point of view several studies have been performed to propagate the plant *in vitro.* The objective of the present study was to develop a micropropagation protocol resulting not only in production of large number of healthy plantlets but is also suitable for long term maintenance of short cultures of Brahmi. Use of low concentration of plant growth regulators and minimization of time required for field transfer of tissue culture raised plantlets are the highlights of the study. Besides, the multitude of disease - free plants produced open the scope for utilization of plant material for antimicrobial testing and suitable pharmaceutical preparations. The anti oxidant potential of *In vitro* (leaf and stem) is slightly less as compared to the *In vivo* (leaf and stem) BM plant extract but is comparable. The reduction may be due to the less maturation of the *In vitro* plant material. The phytochemical study of the plant In *vitro* and *In vitro* reveals the presence of various phytoconstituents (Phynolics and flavonoids) responsible for the antioxidant effect.

ACKNOWLEDGEMENT

We are thankful to Prof. K. N. Tiwari, Professor of Botany, MMV, BHU, Varanasi for providing facility for the tissue culture work.

REFERENCES

[1] Braca A, Tommasi ND, Bari LD, Pizza C, Politi M, and Morelli I (2001). Antioxidant principles from Bauhinia terapotensis. Journal of Natural Products 64: 892–895.

[2] Ceasar SA, Maxwell SL, Prasad KB, Karthigan M, Ignacimuthu S. Highly efficient shoot regeneration of *Bacopa monnieri* (L.) using a two-stage culture procedure and assessment of genetic integrity of micropropagated plants by RAPD. Acta Physiol Plant. 2010; 32:443- 452.

[3] Hagerman A., Harvey-Muller, I. Makkar, H.P.S., Quantification of tannins in tree foliage – good laboratory manual, Vienna: FAO/IAEA, 2000, PP 4-7.

[4] Harborne, J.B., 1975, Biochemical systeamatics of flavonoids. In the Flavonoids, J.B. Harborne, H. Mabry, (Eds). Academic Press: New York, San Fransisco, pp 1056-1095.

[5] Khandelwal KR. Practical pharmacognosy: Techniques and Experiments. 17th ed. Pune India: Nirali Prakashan; 2007. 13.

[6] Kokate CK. Practical pharmacognosy 1st ed. New Delhi, India: Vallabh Prakashan; 1986. p. 15-30.

[7] Kumaran A., Karunakaran J. , *In-vitro* antioxidant activities of methanol extract of five *Phyllathus* species from India, LWT, 40, PP 344-352.

[8] Lamaison, J.L and A. Carnt, 1990. Teneurs en acid resmarinique en derivers hydroxycinnamiquest totaud et activities antioxidets chez les Apiacees, les Borrabinacees et less Lamiacees medicinal. Phaemaceutica, Acta Helvetiae, 65:315-320.

[9] Meyer AS, Isaksen A (1995). Application of enzymes as food antioxidants. Trends Food Sci. Technol., 6: 300-304.

[10] Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth of bioassays with tobacco cultures. Physiol. Plant.15:473-497.

[11] Nishikimi M, Appaji N, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun 1972;46(2):849-54.

[12] Oyaizu, M. 1986 Studies on product of browning reaction prepared from glucose amine. Japanese Journal of Nutrition 44: 307-315,

[13] Price, M.L., A.D. Hagerman and L.G. Bilture, 1980.Tannin content of cow peas, chick peas, pigeon peas and mung beans. J Agriculture and food Chemistry, 28: 459-461.

[14] Rathore S, Singh N. *In vitro* conservation of *Bacopa monnieri*- an endangered medicinal plant. Global J Bio-Sci Biotechnol. 2013; 2(2):187-192.

[15] Richa Jain, Bheem Prasad and Manju Jain, In- vitro regeneration of Bacopa monnieri (L.): A highly valuable medicinal plant, Int.J.Curr.Microbiol.App.Sci (2013) 2(12): 198-205.

[16] ROBAK, J. & GRYGLEWSKI, R.J. (1988). Flavonoids are scavengers of superoxide anion. Biochem. Pharmacol., 37, 837-841.

[17] Shah JD. Studies in growth and ecology of *Bacopa monnieri* (L.) Pennell.-a medicinal herb. Ph.D. Thesis, Banaras Hindu University, Varanasi, India; 1965.

[18] Sharma N. Micropropagation of *Bacopa monneiri* L. Penn.- an important medicinal plant. M.Phil. Thesis. Thapar Institute of Engineering and Technology Patiala, India; 2005.
[19] Sharma S, Kamal B, Rathi N, Chauhan S, Jadon V, Vats N, Gehlot A, Arya S. *In vitro* rapid and mass multiplication of highly valuable medicinal plant *Bacopa monnieri* (L.) Wettst. Afr J Biotechnol. 2010; 9(49):8318-8322.
[20] Shrivastava N, Rajani M. Multiple shoot regeneration and tissue culture studies on *Bacopa monnieri* (L.) Pennell. Plant Cell Rep. 1999; 18(11):919-923.
[21] Shruti Kaushal, Arun Karnwal, Yuvneet Rai, Potential Plant Growth-Promoting Activity of Rhizobacteria Pseudomonas sp in Oryza sativa, J. Nat. Prod. Plant Resour., 2013, 3 (4):38-50.
[22] Sinha S. and Saxena R. (2006), Effect of iron on lipid peroxidation, and enzymatic and non-enzymatic antioxidants and bacoside-A content in medicinal plant Bacopa monnieri L. Chemosphere 62, 1340Ð1350.
[23] Tiwari V, Singh BD, Tiwari KN. Shoot regeneration and somatic embryogenesis from different explants of Brahmi [*Bacopa monniera* (L.) Wettst]. Plant Cell Rep. 1998; 17:538-543.
[24] Tiwari V, Tiwari KN, Singh BD. Comparative studies of cytokinins on *in vitro* propagation of *Bacopa monniera*. Plant Cell Tiss Org Cult. 2001; 66:9–16.
[25] Ved DK, Goraya GS. Demand and Supply of Medicinal Plants in India. NMPB, New Delhi and FRLHT, Bangalore, India; 2007.