A study on *in-vitro* plant regeneration and screening of phytochemicals in *Bacopa monnieri* (L.) Pennell, a medicinally priced plant

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ABSTRACT

Bacopa monnieri (L.) Pennell or 'Brahmi' is a widely exploited plant due to its numerous medicinal values. The pharmacological properties of *Bacopa* are attributed mainly due to the presence of saponins called 'bacosides.' To meet the increasing pharmaceutical demands as well as to maintain genetic fidelity for quality control of the raw material, an efficient mass propagation technique is required. In this present study, we are reporting a direct organogenesis mediated multiple shoot regeneration protocol for mass propagation of *Bacopa* by altering the type and concentration of cytokinins. Furthermore, we have performed preliminary phytochemical studies of *in-vitro* regenerated plants. The analyses include qualitative biochemical tests, isolation of saponin and assay of antimicrobial activity.

Key words : Brahmi, saponin, bacosides, organogenesis

Introduction

Plant based drugs and formulations are showing a rising trend for the health care due to bio safety attributes they possess over modern medicines. *Bacopa monnieri* (L.) Pennell, (Family: Scrophulariaceae) used in traditional Indian medicine, the Ayurveda, for the improvement of intellect and memory for several centuries (Singh and Dhawan, 1997). The pharmacological properties of Bacopa were studied extensively and the activities were attributed mainly due to the presence of characteristic saponins called as "bacosides" (Deepak and Amit, 2004). Indian material medical cites the uses of this plant as a brain tonic, which is effective in maintaining the vigour and intellect. It is a reputed nerve tonic used for its ability to enhance memory, improve intellectual and cognitive functions, anti-inflammatory, analgesic, antipyretic, sedative and as an antiepileptic agent (Bhakuni et al., 1969; Sofowora, 1993; Zhou et al., 2007; Majumdar et al. , 2013).

Optimizing plant tissue culture protocol for mass multiplication of *Bacopa monnieri* may restrict overexploitation as well as conservation of this plant (Rathore and Singh, 2013) . Identification of plants based on DNA finger printing data associated with phytochemical markers may have relevance in quality control of raw materials. Preliminary phytochemical analysis by qualitative biochemical tests is a major concern for detection of active principles, present in it. Infectious diseases account for high proportion of health problems, due to a variety of bacterial etiological agents, such as, pathogenic *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* sp., *Bacillus* sp. Etc. The present study has been designed to determine antimicrobial activity of extracts of *Bacopa monnieri* (L.) Pennell against pathogenic *Bacillus* sp. and *Escherichia coli* by antimicrobial activity assay.

Materials and Methods

Collection and Sterilization of Explant

The disease-free, healthy, young leaves with petioles of *Bacopa monnieri* (L.) Pennell, collected from micropropagated plants in the lab. Explants were washed under running tap water for 2-3 times. Then were dipped in Tween 20 solution for 5 minutes and washed thoroughly with distilled water for 2-3 times. They were treated with 70% ethanol for 1min and were washed thoroughly with sterile distilled water under laminar air flow. Thereafter they were sterilized by dipping in 0.1% $HgCl_2$ soln. for 1min washed in sterile distilled water and were blotted dry. After soaking of surface water, leaf explants were cut horizontally and transferred aseptically into the media.

Transfer of Explants in Media

Leaf explants were aseptically implanted on MS medium (Murashige and Skoog, 1962). supplemented with specific concentrations of hormones [Kinetin (2.5-10 mg/L) and BAP (2.5-10 mg/L)**]** in 3 replica for each. The cultures were incubated under controlled condition of temperature (25±2!), light (2000 – 2500 lux for 16 h/d provided by fluorescent tubes) and 60-70% humidity.

Rooting and Hardening

Rooting of elongated shoots was attempted under in-vitro conditions. The plantlets were transferred to culture bottles containing rooting media- solidified with agar 0.8% containing ½ MS salts and 1% sucrose. After 7 weeks of growth the *in vitro* rooted plantlets were transferred to plastic glasses with sterile soil, under natural conditions for further growth.

Phytochemical Analysis by standard methods

The dried and powdered leaves were extracted with Methanol, Ethanol, Distilled water. The extracts were subjected to preliminary qualitative tests for identification of various phytochemicals (Patil et al., 2014; Jerusha et al., 2016).

Extraction of Total Bacoside

Fresh leaves of *Bacopa monnieri* (L.) Pennell, were dried in a hot air oven. 2.6gms of dried leaves was taken in a mortar and coarsely powdered. The coarse leaf powder was then extensively extracted with 12ml of methanol. This crude leaf extract was then poured into an oak ridge tube and kept for overnight with occasional shaking (Singh and Dhawan, 1997). Centrifuged at room temperature for 5 mins and the supernatant was taken in fresh tube. This would be treated as test sample- methanol leaf extract. A volume of 10ìl was applied on pre-coated TLC plate side by side with marker Bacoside-A (available at market as *Bacopa* mother tincture). The thin layer chromatography plate was run in a solvent (Toluene: ethyl acetate: methanol: formic acid = 3:3.5:2.5:1), air dried and observed with ultra-violet light exposure in transilluminator (Kumar et al., 2015).

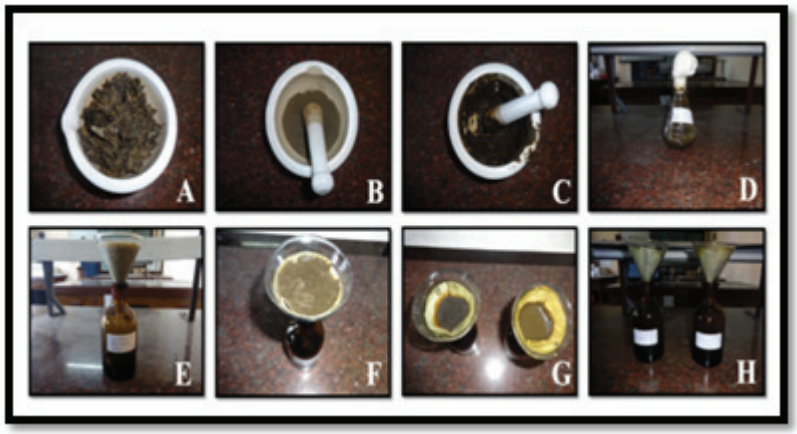

Fig. 1: A. Dried *Bacopa* leaves, B. Leaves were finely ground with pestle, C. Solvent (methanol/ethanol/distilled water) was added to make a fine paste, D. Mixture was poured in conical flask & placed in shaker at 25 ± 2! overnight, E. extract was filtered with glass funnel, whatman filtepaper no.1 in labeled amber colored bottle, F-G. Ongoing filtration process, filtrate being poured in the bottle & leaf residue was discarded, H. Prepared extracts (methanol, ethanol, aqueous) in bottles, stored at -4!.

Antimicrobial activity assay by Agar-well diffusion method

Antimicrobial activity of leaf extracts of *Bacopa monnieri* (L.) Pennell against clinical pathogens – *Bacillus* sp. and *Escherichia coli* was determined by using agar-well diffusion method (Mehta *et al.*, 2012). Then 0.1ml of the overnight grown nutrient broth containing bacterial cultures were spread over the respective LB agar plates using sterilized

spreader. Wells/Blocks of 3mm diameter were scooped out with sterilized cork borer. Wells were marked as 1, 2 and 3 in the lower side of each plate. It was followed by the addition of 0.1mL, each of 3 types of Bacopa leaf extracts (Me, Et and Aq extract in well no.1, 2 and 3 resp.) separately in 3 different wells. The plates were incubated overnight at 37!. After incubation the diameter of inhibition zones formed around each well was measured and expressed in millimeter to evaluate the antimicrobial activity. Inhibition zones of 8 to 10 mm were considered to be significant when testing plant extracts for antimicrobial activity.

Results

Effect of different hormones on direct shoot organogenesis from leaf explants

Induction of direct shoot organogenesis was observed at petiolar cut edges of leaf explants in MS basal medium supplemented with different concentrations of Kinetin (2.5-10 mg/L) and BAP (2.5-10 mg/L). Most responsive shoot bud regeneration was observed in MS+10 mg/L Kinetin **(Fig.2 and Table.1)**.

Phytochemical Characterization After extraction methanolic extract was found to be yellowish green, ethanolic extract green and aqueous extract was found to be dark brown in color. All phytochemical analysis were performed with these extracts **(Table.2)**.

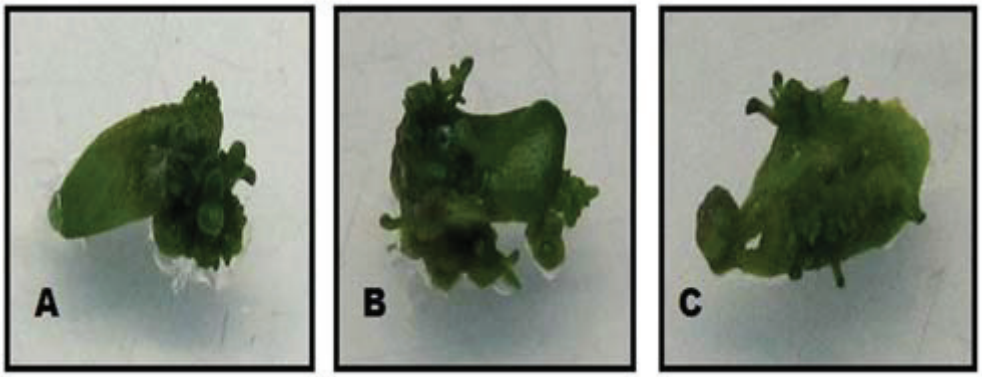

Fig.2.A-C. Shoot regeneration in leaf explants of *Bacopa monnieri* in MS medium supplemented with different cytokinins (BAP & Kinetin).

Table.1. Effect of different type of shooting media on shoot bud regeneration of *Bacopa monnieri.*

Sl No.	Treatments (MS Basal + PGR)	Conc. Of Hormone (mg/l)	Replica Set	Explant No.	No. of Days to organogenesis	Average No. of Days
1.	BAP	2.5	1.	1)	22	23.66±5.68
				2)	19	
				3)	30	
		5.0	2.	1)	29	29.33±0.57
				2)	30	
				3)	29	
		10.0	3.	1)	24	26±2
				2)	28	
				3)	26	
2.	Kinetin	2.5	1.	1)	22	22±5
				2)	27	
				3)	17	
		5.0	2.	1)	22	27±4.35
				2)	30	
				3)	29	
		10.0	3.	1)	19	24.33±5.50
				2)	24	
				3)	30	

Bacoside A was taken as a marker to detect the presence of bacoside, the triterpenoid saponin isolated from the methanolic extracts of *Bacopa monnieri* leaves using TLC. The marker bacoside A showed Rf value of 0.42cm and the methanol extract sample of *B. monnieri* leaves showed Rf value of 0.44cm **(Fig.3)**.

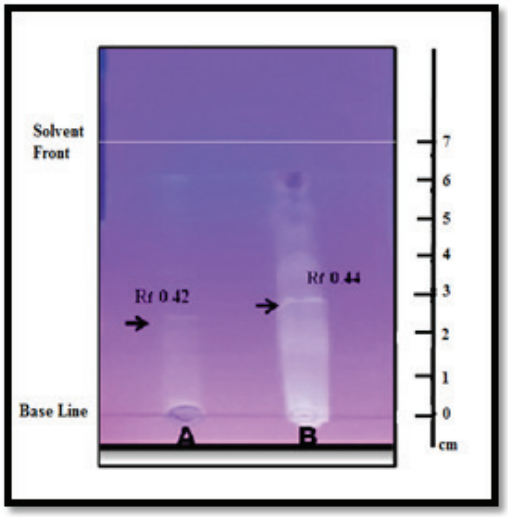

Table.2. Preliminary Phytochemical Analysis of *Bacopa monnieri* extracts

Solvent	Initial weight of leaves (gms)	Final weight of leaves (gms)	Color of extract
Methanol	50.7	4.08	Yellowish green
Ethanol	52.05	4.44	Green
Aqueous	53.48	4.49	Dark brown

Fig.3. TLC profile of the bacoside fraction(A.) Profile of Bacoside A (marker) with Rf value 0.42cm and (B.) Profile of methanol extract of *Bacopa monnieri* leaves, with Rf value 0.44cm

From the present study, it is evident that carbohydrates, flavonoids, terpenoids, alkaloids, quinones, coumarins are present in high amount in methanolic extract; tannins, terpenoids, phenols, coumarins, glycosides are present in high amount in ethanolic extract; and saponins, phlobatannin, phenols are present in high amount in aqueous extract. Cardiac glycosides, anthraquinone, steroids & phytosteroids are totally absent in any of the three extracts **(Fig.4, Table.3)**.

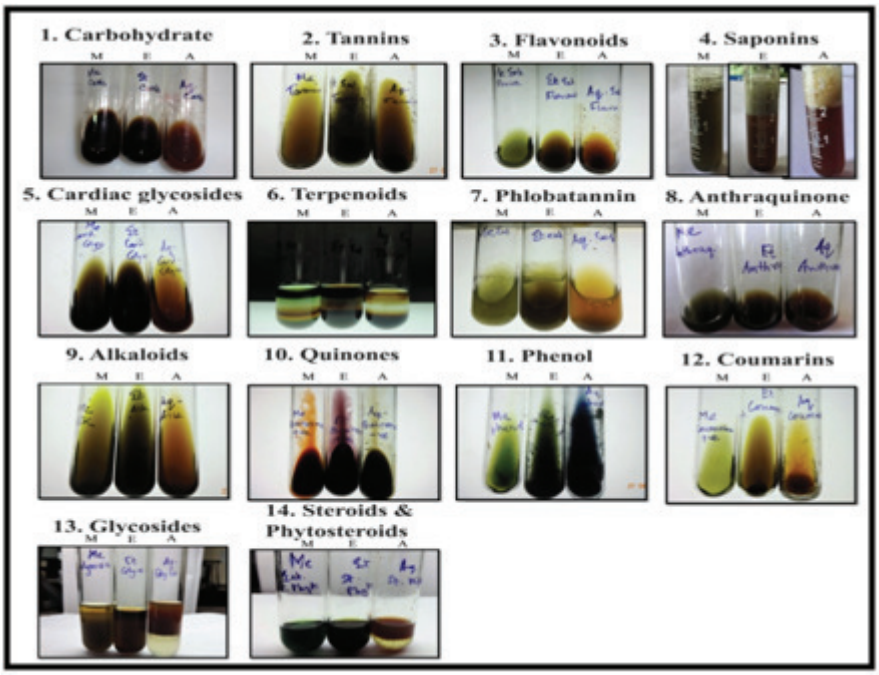

Fig.4. Phytochemical screening (Qualitative Analysis) of *Bacopa monnieri*. M : Methanolic; E:Ethanolic ; A: Aqueous Extracts.

Table.3. Qualitative Biochemical tests with observations . ++ = Highly positive, + = Weakly positive, -- = Highly negative, - = Weakly negative.

Sl No.	Test for	Me Extract	Et Extract	Aq Extract
1.	Carbohydrate	Purplish black ; ++	Greenish purple ;-	Reddish brown ;+
2.	Tannins	Brownish green ; --	Greenish black ; ++	Reddish brown ; -
3.	Saponins	Weakly +ve ;1.5 cm layer foam; foam persisted for only 3-4sec., during washing with water 8cm layer of persisting foam; +	Weakly +ve ; 1.5-1.8cm layer of foam; persisting longer period; during washing 0.5cm layer of foam; +	Highly +ve; 1.5cm layerof foam; Persisting longer period than Me extract ; ++
4.	Flavonoids	Brownish yellow ; +	Brown ; -	Reddish yellow ; -
5.	Cardiac glycosides	No ring formation light green soln. ; --	No ring formation dark brown soln. ; --	No ring formation light brown solution ; --
6.	Terpenoids	Formation of reddish brown colour ring at interface ; ++	Formation of reddish brown colour ring at interface ; ++	No reddish brown ring ; --
7.	Phlobatannin	Light green ; --	Brownish green ; -	Orange-brown ppt. ; +
8.	Anthraquinone	Deep green ; -	Brownish green ; -	Reddish brown ; -
9.	Alkaloids	Bottle green soln. ; ++	Brownish green ; -	Reddish brown ; -
10.	Quinones	Blood-red soln. ; ++	Purple red soln. ; +	Brownish black soln. ; -
11.	Phenols	Yellowish green soln., no ppt. (upper yellowish green & lower bottle green layer) ; +	Blackish green soln., lower layer of dense ppt. ; ++	Navy blue colour soln., preliminary appearance of 3.5cm of foam, persisting for 10mins.; no ppt. ; ++
12.	Coumarins	Lower layer of yellow soln. ; +	Yellowish brown soln. ; +	Yellowish orange soln. ; -
13.	Glycosides	Light green soln. (upper clear green lower opaque green soln.) ; -	Upper pink layer, lower brownish green soln. ; +	Upper broad brown layer, lower white opaque soln. ;-
14.	Steroids & phytosteroids	No ring formation, Bottle green soln. ; --	No ring formation, Brownish green ; --	No ring formation, 2 distinct layer – upper broad tamarind layer, lower transparent layer ; --

Study of antimicrobial activity

Antimicrobial activity of *Bacopa monnieri* was tested against Gram negative bacteria *Escherichia coli* and Gram positive bacteria *Bacillus* sp. Methanol extract exhibited maximum zone of inhibition (9 mm), ethanol extract exhibited minimum inhibition (3 mm) and aqueous extract exhibited moderate zone of inhibition (4 mm) against *Bacillus* sp. Whereas, in case of *Escherichia coli,* methanol extract exhibited maximum zone of inhibition (14 mm), ethanol extract exhibited moderate inhibition (8 mm) and aqueous extract exhibited minimum zone of inhibition (5 mm) (**Fig.5**).

From the above result of the susceptibility study, it is estimated that the test bacteria- *Bacillus* sp. and *Escherichia coli* are sensitive to these leaf extracts. The antimicrobial activity of *Bacopa monnieri* may be a result of individual or combination of the various bioactive compounds present in it. The demonstration of antimicrobial activity against both Gram negative and positive bacteria was an indication of its potential source for production of drugs with a broad spectrum of activity.

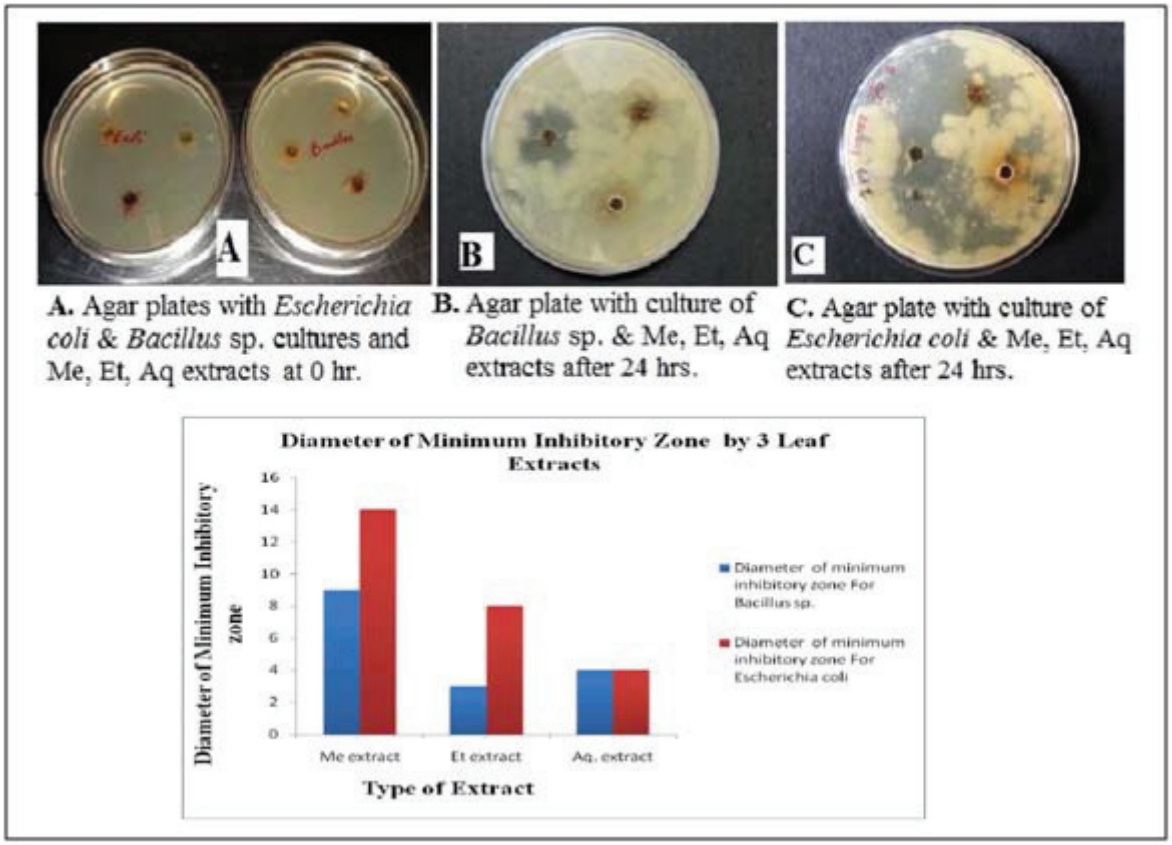

Fig. 5.Antimicrobial activity assay by Agar-well diffusion method. Me, Et and Aq extracts were added in well no. – 1, 2 and 3 respectively, in all plates.

Conclusion

According to National Medicinal Plant Board, Govt. Of India, annual demand of *Bacopa* during the year 2004-2005 was 662321.8 tons with an anticipated 7% annual growth rate. This requirement is rising enormously in view of the popularity of the *Bacopa* based drugs, like "Memory Plus" in the market, resulting in enormous overexploitation of the natural populations of this plant to meet the present requirement. The present study established an efficient

protocol for high frequency multiple shoot regeneration of *Bacopa* . The results of qualitative biochemical assay showed the presence of active phytochemicals like saponins, tannins, alkaloids, etc. that may lead to the future production of herbal medicines. . It was well documented from the present work that bacosideA, a bioactive triterpenoid saponin is a constituent of *B. monnieri* leaves and the validated TLC method can be acceptable for testing bacosideA in commercially available herbal medicines. Antimicrobial activity assay provided the scientific justification for the use of leaf extracts as antimicrobial agents and open the possibility of finding new effective pharmaceuticals, thereby decreasing the burden of drug resistance and side effects of synthetic medicines.

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