Phytochemical and Preclinical Screening of Aseptically Produced Herbal Raw Material: *Bacopa monnieri*

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### ABSTRACT

The demand for herbal raw material has increased tremendously due to renewed interest in plant-based medicines, especially in developed countries. This has led to the indiscriminate cutting and collection of medicinal plants from natural resources resulting in depletion of natural resources and in some cases extinction of species. In the present report, aseptically grown raw material and cell cultures have been received recognition as an alternative source for the production of herbal raw material and chemical constituents. A low concentration of therapeutically important chemical constituents, which is directly related to the efficacy of the plant, was the major limitation in the commercial application of aseptically grown raw material and cell cultures. Due to this limitation, the acceptance of aseptically regenerated plants in the world of herbal medicines and Ayurvedic products was not very encouraging. In the present investigation, we have not only established the protocol for aseptic culture of *Bacopa monnieri* (L.) Pennell, but also checked the aseptically grown material for its chemical constituents and efficacy through established bioassay models. Assessment of the presence of major phytochemicals and *in vitro* preclinical bioassays indicated that aseptically grown shoots can be a better source of raw material. This can stop further depletion of the species from nature and also provide a consistent quality raw material. Aseptically grown improved quality shoots can also be good quality plant material for standardized cultivation practice.

### Keywords:
anitiradical, bacoside A, brine shrimp lethality, luteolin, β-sitosterol

**Abbreviations:** AGS, aseptically grown shoots; BSA, bovine serum albumin; DPPH, 1, 1-di phenyl picryl hydrazine; EDTA, ethylene-diamine tetraacetic acid disodium salt; FGS, field grown shoots; HPTLC, high performance thin layer chromatography; NBT, nitroblue tetrazolium; TCA, trichloro acetic acid

### INTRODUCTION

Renewed interest in plant-based medicines has disturbed the demand and supply equation of raw material used for preparation of plant-based medicine. More demand and less supply has resulted in availability of adulterated/substituted or poor quality raw material for preparation of plant-based medicines which in turn affects efficacy. Aseptically grown, improved and consistent quality herbal raw material obtained by tissue culture can be a potential solution to this problem. However, this technique often faces setbacks because aseptically generated plant material does not thrive well in the medicinal market due to its low content of secondary metabolites, especially the active principles (Dicosmo and Misawa 1995; Ramachandra Rao and Ravishankar 2002; Namdeo 2007).

In the present study, we assessed the quality and efficacy of aseptically grown herbal raw material through various phytochemical and biochemical parameters and *in vitro* preclinical bioassays. The plant selected for the study is a well illustrated plant of the Indian System of Medicine (Ayurveda), *Bacopa monnieri* (L.) Pennell, Scrophulariaceae, commonly known as Brahmi. The entire plant is used as a drug and is well documented for improving intellect (Anonymous 2001). Brahmi is also documented in classical literature for its use in asthma, epilepsy, insanity and memory enhancement (Satyavati et al. 1976; Anonymous 1999).

The present investigation gives idea about the quality of the aseptically grown herbal raw material in terms of the presence of active constituents and other major metabolites and also about its therapeutic potential.

### MATERIALS AND METHODS

#### Plant materials

An authenticated *B. monnieri* plant growing in the botanical garden of our institute was used as the mother plant to aseptically regenerate shoots by a protocol established in our laboratory (Shrivastava and Rajani 1999). The leaves were collected when the plant was in a vegetative stage of development and used as explants. After a 10-week incubation period, the regenerated shoots were harvested and dried under shade until it dried completely and stored in an air-tight container at 4°C in a refrigerator. Field grown shoots (FGS) were also collected and processed as described above for *in vitro* material and stored. The dried plant materials were powdered in a grinder prior to the experiments as per the requirement.

#### Estimation of bacoside A, luteolin and β-sitosterol

The concentrations of bacoside A, β-sitosterol and luteolin in aseptically grown shoots (AGS) and FGS were estimated by high performance thin layer chromatography (HPTLC). Presence of the compounds was confirmed by comparing retention factor (Rf) and absorption maxima and also by spectral overlay of the bands of test sample with their respective standards. Methanolic extract of AGS and FGS were used for bacoside A and luteolin estimation (Gupta et al. 1998; Shrikumar...
Standards of bacoside A (80 μg/ml) and luteolin (40 μg/ml) were used to prepare their respective calibration curves. The mobile phases used for the preparation of a calibration curve and estimation of bacoside A, luteolin and β-sitosterol were ethyl acetate : methanol : water : butanol (8 : 1.25 : 0.8 : 0.25), n-propanol : ethyl acetate : water : glacial acetic acid (4 : 4 : 3 : 0.1) and toluene : methanol (9 : 1), respectively. The linearity range of the calibration curve of bacoside A was 400-800 ng with a 5.8411 slope, a 110.52 intercept and a correlation coefficient of 0.998. The calibration curve of luteolin was prepared in the linearity range of 80-240 ng having a 34.183 slope, a 2170.2 intercept and a 0.998 correlation coefficient. Petroleum ether extract of AGS and FGS were used for β-sitosterol estimation (Anonymous 2005). A calibration curve of standard β-sitosterol (40 μg/ml) was prepared, with a linearity range of 240-560 ng. The slope and intercept was calculated to be 6.1205 and 1324.71, respectively with a correlation coefficient of 0.999.

**Estimation of total protein and total phenolics**

Total protein and total phenolic content were estimated by the Bradford (Bradford 1976) and the Singleton and Rossi (Singleton and Rossi 1965) methods, respectively. Total protein was extracted from AGS and FGS with 80% ethanol followed by precipitation with 1 M trichloroacetic acid (TCA) under ice-cold conditions. Bovine serum albumin (BSA, 1 μg/ml) and gallic acid (100 μg/ml) was used as a standard for the estimation of total protein and total phenolics, respectively. The linearity range of the standard curve of BSA was 2-20 μg. The slope and intercept was calculated to be 0.0354 and 0.0275, respectively with a correlation coefficient of 0.998. The linearity range of the standard curve of gallic acid was 5-75 μg. The slope and intercept were calculated to be 0.0037 and 0.0015, respectively with a correlation coefficient of 0.999.

**Assay for antiradical and superoxide scavenging activity**

Antiradical and superoxide scavenging activities were measured spectrophotometrically by using the 1, 1-di phenyl picryl hydrazine (DPPH) model (Navarro et al. 1993) and the riboflavin-nitro-blue tetrazolium (NBT)-light system (Beauchamp and Fridovich 1971), respectively. A stock solution of DPPH (1.3 mg/ml) was prepared in methanol such that it showed an initial absorbance of 0.9 at 516 nm. Reduction in the absorbance, due to the methanolic extract of AGS and FGS, after 15 min incubation at room temperature was measured as antiradical activity. The reaction mixture (3 ml) contained methanol, DPPH and different concentrations (50-400 μg/ml) of plant extract.

**Fig. 1** (A) TLC fingerprint profile for bacoside A estimation in methanolic extract after derivatization with anisaldehyde sulphuric acid reagent. Lanes 1, 2, 3: Field grown shoots (FGS); Lanes 4, 5, 6: Aseptically grown shoots (AGS); Lane 7: Bacoside A standard. (B) TLC densitometric chromatogram for bacoside A estimation in field grown shoots (FGS) and aseptically grown shoots (AGS) at 525 nm.

**Fig. 2** (A) TLC fingerprint profile for β-sitosterol estimation in petroleum ether extract after derivatization with anisaldehyde sulphuric acid reagent. Lanes 1, 2, 3: Field grown shoots (FGS); Lanes 4, 5, 6: Aseptically grown shoots (AGS); Lane 7: β-sitosterol standard. (B) TLC densitometric chromatogram for β-sitosterol estimation in field grown shoots (FGS) and aseptically grown shoots (AGS) at 525 nm.
The reaction was started by illuminating the reaction mixture for 5 min. Immediately after illumination, the absorbance was measured at 590 nm.

Statistical analysis

All the experiments were repeated three times, each time in triplicate. The results are expressed in terms of percent dry weight basis (% dwb) as the mean ± SD. The results obtained from the study of different parameters in AGS were compared with FGS using a Student’s t-test. The difference between two samples was considered to be statistically significant when the value of p was less than 0.05.

RESULTS AND DISCUSSION

Quantitative assessment of bacoside A and β-sitosterol – the two major chemical constituents of the plant contributing to its medicinal properties (Dhingra et al. 2003; Shrikumar et al. 2003; Delporte et al. 2005; Vivancos and Moreno 2005) – revealed that these chemical constituents are not only present in AGS but their concentration is also significantly higher than FGS (bacoside A p<0.001, β-sitosterol p<0.05) (Figs. 1-3). Another minor chemical constituent, luteolin, was also found in AGS (0.007 % (w/w)) (Figs. 3, 4). The production of secondary metabolites is generally higher in differentiated tissues, so the shoots cultures are being used for the production of medicinally important compounds (Ramachandra Rao and Ravishankar 2002). There are a number of medicinal plants whose shoot cultures have been studied for the production of secondary metabolites (Hagimori et al. 1982; Shim et al. 1998; Iwase et al. 2005).

The higher concentration of metabolites in AGS could be explained by the fact that in uniform but simulated environmental conditions, aseptically grown plants experience a kind of stress i.e. culture stress (Rani and Raina 1998) and to adjust to this, they produce a higher amount of primary and secondary metabolites. Another, completely opposing reason could be that in culture conditions, the regenerated plants experience a steady environment like uniform light and moisture conditions, easily available nutrients, carbon source, etc., which enhances their growth and metabolic activity, which in turn is responsible for their higher primary and secondary metabolite concentration. Shoot cultures are raised through the simple use of appropriate hormonal balance and they exhibit genetic stability and good capacities for secondary metabolite production. Moreover, there also exists a link between growth and the production of secondary compounds in shoot cultures (Bourgaud et al. 2001).

The total protein and total phenolic content in AGS was estimated to be 1.640 % (w/w) and 0.623 % (w/w), respectively (Fig. 5). Earlier studies on metabolite concentrations of $B. \text{monnieri}$ also showed marked differences in the total sugar and protein content, both being present at higher concentrations in in vitro than in vivo shoots (Mohapatra and Rath 2005). Production and accumulation of a high amount of secondary metabolites, especially the active constituents, enhances the therapeutic value of the plant.

Brine shrimp lethality assay

Brine shrimp lethality assay was carried out to investigate cytotoxic activity of the plant extract (Sam 1993). The test is based on the ability of the extract to kill laboratory-cultured Artemia salina. nauplii brine shrimp. Various concentrations (100-3500 μg/ml) of ethanolic extract were tested for activity in 24-well plates. Each concentration contained 3 wells consisting of 20 nauplii in 2 ml of artificial sea water. Potassium dichromate (30 μg/ml) was used as a standard. After 24 h, the wells were observed for the number of survived shrimps in each well and the results were noted. From this data, the percentage mortality of brine shrimp was calculated for each concentration and the median lethal concentration (LC50) values were determined.

![Fig. 4](image-url)  
Fig. 4 (A) TLC fingerprint profile for luteolin estimation in methanolic extract at 254 nm. Lanes 1, 2, 3: Field grown shoots (FGS); Lanes 4, 5, 6: Aseptically grown shoots (AGS); Lane 7: Luteolin standard. (B) TLC densitometric chromatogram for luteolin estimation in field grown shoots (FGS) and aseptically grown shoots (AGS) at 355 nm.

![Fig. 3](image-url)  
Fig. 3 Bacoside A, β-sitosterol and luteolin content in field grown shoots (FGS) and aseptically grown shoots (AGS). Results expressed as mean ± SD. Bacoside A (p<0.001), luteolin (p<0.005) and β-sitosterol (p<0.05).
Preclinical in vitro assays showed very good antiradical (IC<sub>50</sub>: 147.31 μg/ml) and superoxide scavenging activity (IC<sub>50</sub>: 633.74 μg/ml) of AGS (Tables 1, 2). The brine shrimp lethality assay showed that AGS (LC<sub>50</sub>: 637.01 μg/ml) also exhibited good cytotoxic activity, significantly better than FGS (LC<sub>50</sub>: 758.69 μg/ml) (p<0.05). The brine shrimp lethality assay is considered a useful tool for preliminary assessment of toxicity and it has been used for the detection of fungal toxins, toxicity of plant extracts, heavy metals, pesticides and cytotoxicity (Sam 1993). The higher efficacy of AGS can be attributed to the higher concentrations of active constituents such as bacoside A, phenolic contents and other metabolites.

Results of the study indicate that AGS of <i>B. monnieri</i> contains high levels of secondary metabolites as estimated in terms of total phenolics and one of the pharmacologically active constituent, bacoside A along with minor ones.

**CONCLUDING REMARKS**

The presence of high levels of pharmacologically active constituent is a highly desirable feature for any medicinally important plant. This feature directly affects the efficacy of the plant. Hence, the study infers that aseptically grown plant material can be a better alternative of field grown plant material for the production of good and consistent quality raw material especially in case of small herbs like <i>B. monnieri</i> where whole plant is used as a drug.

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**Fig. 5** Total protein and total phenolics content in field grown shoots (FGS) and aseptically grown shoots (AGS). Results expressed as mean ± SD. p<0.05.
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