Medicinal and Aromatic Plant Science and Biotechnology ©2008 Global Science Books

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

Anshu Srivastava • Neeta Shrivastava*

Department of Pharmacognosy and Phytochemistry, B. V. Patel Pharmaceutical Education and Research Development (PERD) Centre, Sarkhej-Gandhinagar Highway, Thaltej, Ahmedabad 380054, Gujarat, India

Corresponding author: * **neetashrivastava_perd@yahoo.co.in**

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 recent past, aseptically grown raw material and cell cultures have 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 planting material for standardized cultivation practice.

Keywords: antiradical, 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**, ethylenediamine tetraaceticacid 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 plant and plant extracts have been reported to possess a wide range of activities: anti-anxiety (Singh and Singh 1980), hepatoprotective (Sumathi *et al*. 2001), antioxidant (Tripathi *et al*. 1996), anti-ulcer (Sairam *et al*. 2001; Dorababu *et al*. 2004) and anticancer (Elangovan *et al*. 1995).

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 co-chromatography using 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

Received: 28 May, 2008. Accepted: 21 June, 2008.

Original Research Paper

et al. 2003; Srinivasa *et al*. 2004). 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 trichloro acetic acid (TCA) under ice-cold conditions. Total phenolics were extracted with 50% aqueous methanol at room temperature. 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-nitroblue 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.

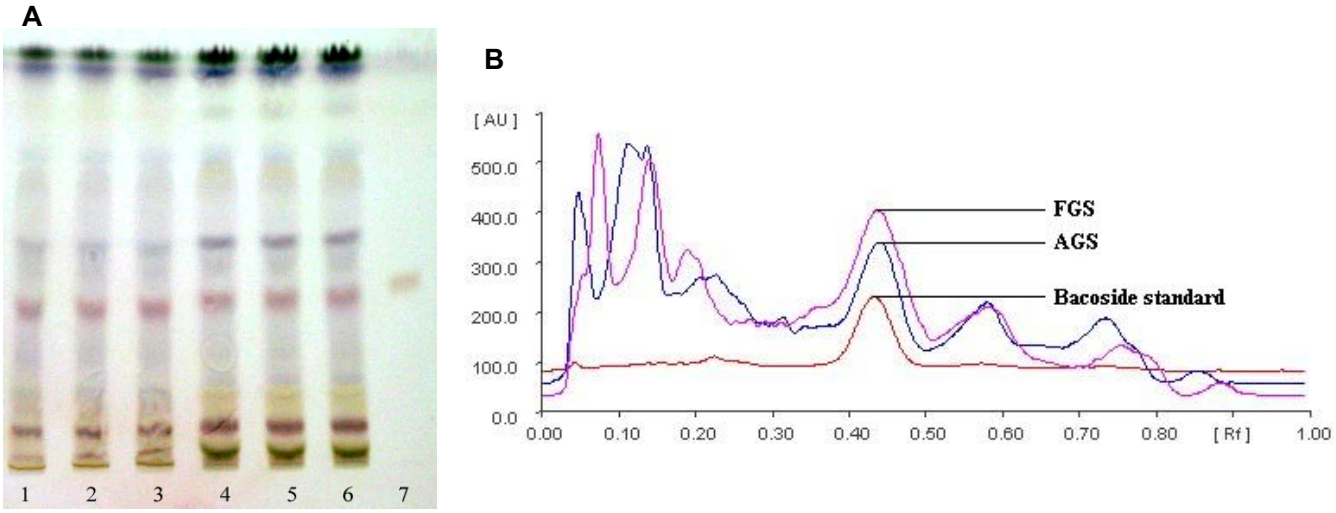

Fig. 1 (**A**) TLC fingerprint profile for bacoside A estimation in methanolic extract after derivatization with anisaldehyde sulphuric acid reagent. Lanes 1, 2, 3: Field grown shoots (FGS); Lanes 4, 5, 6: Aseptically grown shoots (AGS); Lane 7: Bacoside A standard. (**B**) TLC densitometric chromatogram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

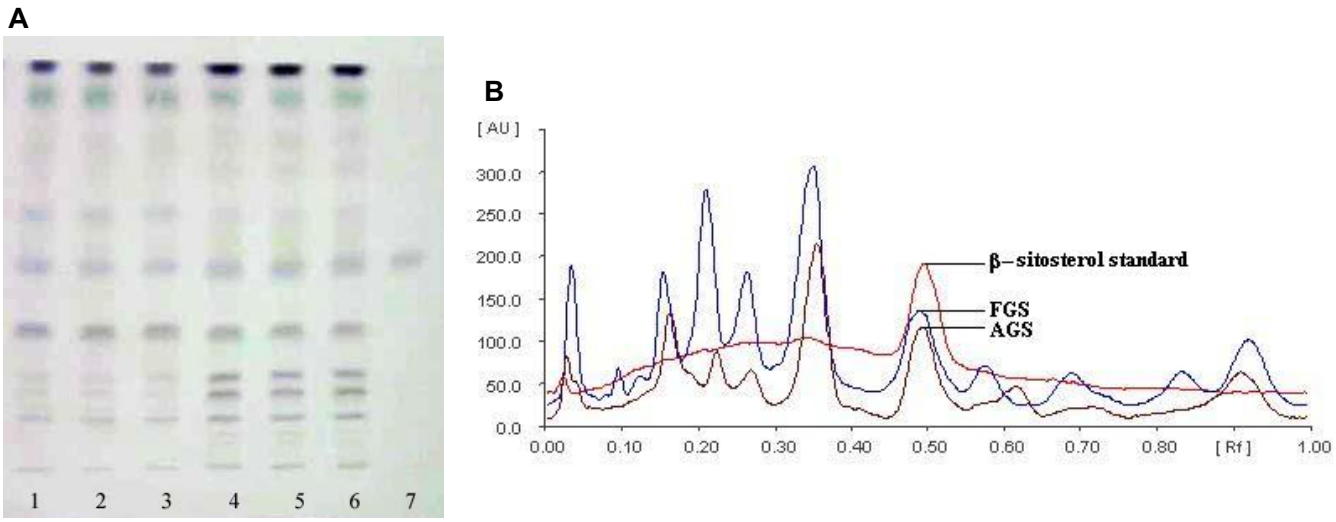

Fig. 2 (**A**) TLC fingerprint profile for *β*-sitosterol estimation in petroleum ether extract after derivatization with anisaldehyde sulphuric acid reagent. Lanes 1, 2, 3: Field grown shoots (FGS); Lanes 4, 5, 6: Aseptically grown shoots (AGS); Lane 7: *β*-sitosterol standard. (**B**) TLC densitometric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

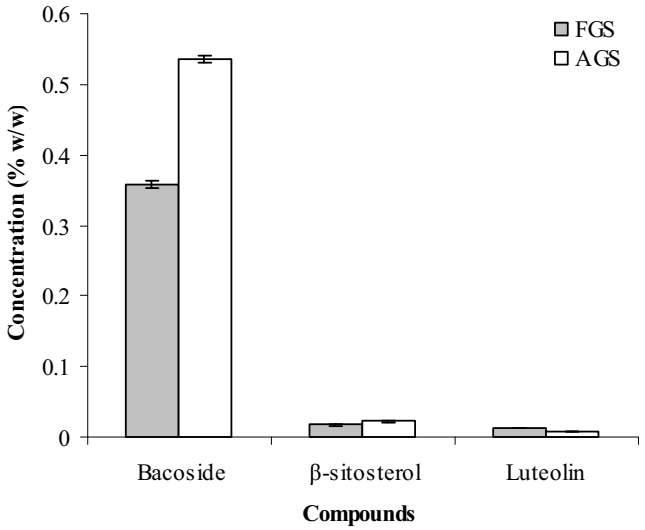

Fig. 3 Bacoside A, *β*-sitosterol and luteolin content in field grown shoots (FGS) and aseptically grown shoots (AGS). Results expressed as mean ± SD. Bacoside A ($p<0.001$), luteolin ($p<0.005$) and *β*-sitosterol ($p<0.05$).

The assay for superoxide scavenging activity was based on the capacity of the sample to inhibit the formation of blue formazan by scavenging the superoxide radicals generated in the riboflavin-NBT-Light system. The reaction mixture (3 ml) contained 50 mM phosphate buffer pH 7.6, 20 µg riboflavin, 12 mM ethylenediamine tetraaceticacid disodium salt (EDTA), 0.1 mg/ml NBT and different concentrations (100-1400 µg/ml) of plant extract, added in that sequence. The reaction was started by illuminating the reaction mixture for 5 min. Immediately after illumination, the absorbance was measured at 590 nm.

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* Leach. *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 (LC^{50}) values were determined.

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. 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.

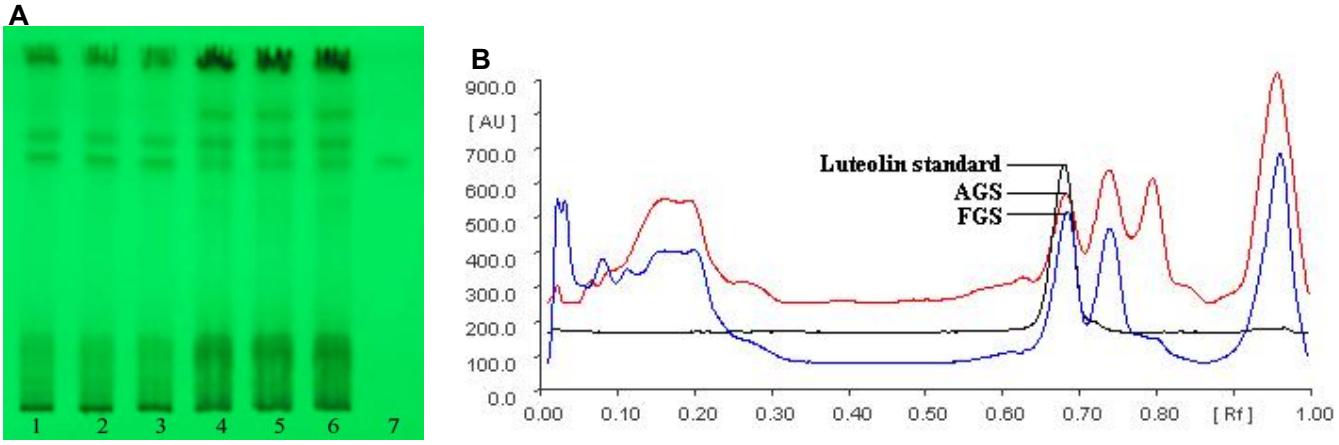

Fig. 4 (**A**) TLC fingerprint profile for luteolin estimation in methanolic extract at 254 nm. Lanes 1, 2, 3: Field grown shoots (FGS); Lanes 4, 5, 6: Aseptically grown shoots (AGS); Lane 7: Luteolin standard. (**B**) TLC densitometric chromatogram for luteolin estimation in field grown shoots (FGS)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

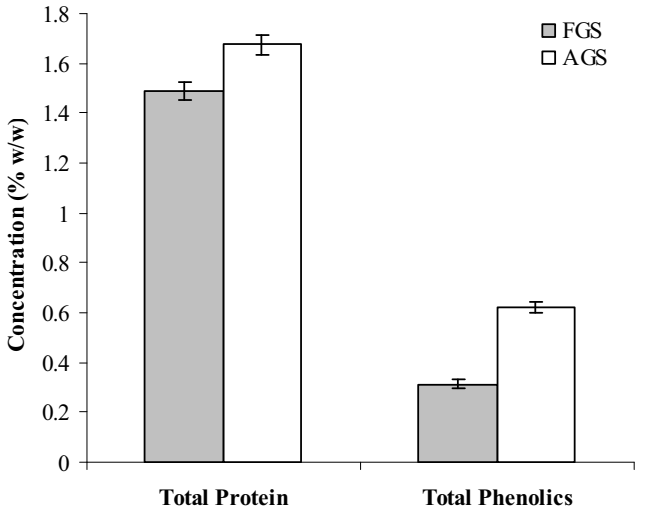

Fig. 5 Total protein and total phenolics content in field grown shoots (FGS) and aseptically grown shoots (AGS). Results expressed as mean ± SD. $p<0.05$.

Table 1 Antiradical activity of field grown shoots (FGS) and aseptically grown shoots (AGS) of *B. monnieri*.

Sample	Concentration (µg/ml)	% Inhibition*	IC^{50} (µg/ml)[a]
FGS	50	7.679 ± 0.123	
	100	13.447 ± 0.061	
	200	34.112 ± 0.062	292.61
	300	50.354 ± 0.061	
	400	64.366 ± 0.061	
AGS	50	15.518 ± 0.060	
	100	31.037 ± 0.061	
	200	60.090 ± 0.060	147.31
	300	72.582 ± 0.060	
	400	92.276 ± 0.000	

* Data represented as mean ± SD (n=3)
[a] $p<0.05$

Table 2 Superoxide scavenging activity of field grown shoots (FGS) and aseptically grown shoots (AGS) of *B. monnieri*.

Sample	Concentration (µg/ml)	% Inhibition*	IC^{50} (µg/ml)[a]
FGS	100	5.236 ± 0.061	
	200	21.270 ± 0.000	
	400	33.862 ± 0.000	
	600	40.635 ± 0.000	1047.09
	800	46.032 ± 0.000	
	1000	47.584 ± 0.061	
	1200	49.841 ± 0.000	
	1400	54.074 ± 0.000	
AGS	100	12.238 ± 0.000	
	200	21.573 ± 0.064	
	400	37.560 ± 0.064	
	600	49.252 ± 0.000	633.74
	800	57.369 ± 0.064	
	1000	61.373 ± 0.064	
	1200	65.059 ± 0.000	
	1400	70.003 ± 0.064	

* Data represented as mean ± SD (n=3)
[a] $p<0.05$

Preclinical *in vitro* assays showed very good antiradical (IC^{50}: 147.31 µg/ml) and superoxide scavenging activity (IC^{50}: 633.74 µg/ml) of AGS (**Tables 1, 2**). The brine shrimp lethality assay showed that AGS (LC^{50}: 637.01 µg/ml) also exhibited good cytotoxic activity, significantly better than FGS (LC^{50}: 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 *B. monnieri* 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 *B. monnieri* where whole plant is used as a drug.

ACKNOWLEDGEMENTS

The authors are thankful to Gujarat Council on Science and Technology (GUJCOST), Government of Gujarat, Gujarat, India and Industries Commissionerate, Government of Gujarat, Gujarat, India for financial support.

REFERENCES

Anonymous (1999) *The Ayurvedic Pharmacopoeia of India* (Vol II, Part I, 1st Edn), Government of India, Ministry of Health and Family Welfare, Department of Indian System of Medicine & Homoeopathy, New Delhi, India, 25 pp

Anonymous (2001) *The Wealth of India* (Vol VII), Council for Scientific and Industrial Research, New Delhi, India, 87 pp

Anonymous (2005) *Quality Standards of Indian Medicinal Plants* (Vol 3), Indian Council of Medical Research, New Delhi, India, 98, 236 pp

Beauchamp C, Fridovich I (1971) Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry* **44**, 276-287

Bourgaud F, Gravot A, Milesi S, Gontier E (2001) Production of plant secondary metabolites: a historical perspective. *Plant Science* **161**, 839-851

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254

Delporte C, Backhouse N, Erazo S, Negrete R, Vidal P, Silva X, López-Pérez JL, Feliciano AS, Muñoz O (2005) Analgesic-antiinflammatory properties of *Proustia pyrifolia*. *Journal of Ethnopharmacology* **99**, 119-124

Dhingra D, Parle M, Kulkarni SK (2003) Medicinal plants and memory. *Indian Drugs* **40**, 313-319

Dicosmo F, Misawa M (1995) Plant cell and tissue culture: Alternatives for metabolite production. *Biotechnology Advances* **13**, 425-453

Dorababu M, Prabha T, Priyambada S, Agarwal VK, Aryya NC, Goel R K (2004) Effect of *Bacopa monnieri* and *Azadiracta indica* on gastric ulceration and healing in experimental NIDDM rats. *Indian Journal of Experimental Biology* **42**, 389-397

Elangovan V, Govindasamy S, Ramamoorthy N, Balasubramaanian K (1995) *In vitro* studies on the anticancer activity of *Bacopa monnieri*. *Fitoterapia* **66**, 211-215

Gupta AP, Mathur S, Gupta MM, Kumar S (1998) Effect of the method of drying on the bacoside-A content of the harvested *Bacopa monniera* shoots revealed using a high performance thin layer chromatography method. *Journal of Medicinal and Aromatic Plant Sciences* **20**, 1052-1055

Hagimori M, Matsumoto T, Obi Y (1982) Studies on the production of *Digitalis* cardenolides by plant tissue culture. II. Effect of light and plant growth substances on digitoxin formation by undifferentiated cells and shoot-forming cultures of *Digitalis purpurea* L. grown in liquid media. *Plant Physiology* **69**, 653-656

Iwase A, Aoyagi H, Ohme-Takagi M, Tanaka H (2005) Development of a novel system for producing ajmalicine and serpentine using direct culture of leaves in *Catharanthus roseus* intact plant. *Journal of Bioscience and Bioengineering* **99**, 208-215

Mohapatra HP, Rath SP (2005) *In vitro* studies of *Bacopa monnieri* – An important medicinal plant with reference to its biochemical variations. *Indian Journal of Experimental Biology* **43**, 373-376

Namdeo AG (2007) Plant cell elicitation for production of secondary metabolites: A review. *Pharmacognosy Reviews* **1**, 69-79

Navarro CM, Montilla MP, Martin A, Jimenez J, Utrilla MP (1993) Free radical scavenger and antihepatotoxic activity of *Rosmarinus tomentosus*. *Planta Medica* **59**, 312-314

Ramachandra Rao S, Ravishankar GA (2002) Plant cell cultures: Chemical factories of secondary metabolites. *Biotechnology Advances* **20**, 101-153

Rani V, Raina SN (1998) Genetic analysis of enhanced-axillary-branching-derived *Eucalytus tereticornis* Smith and *E. camaldulensis* Dehn. plants. *Plant Cell Reports* **17**, 236-242

Sairam K, Rao ChV, Dorababu M, Goel RK (2001) Prophylactic and curative effects of *Bacopa monnieri* in gastric ulcer models. *Phytomedicine* **8**, 423-430

Sam TW (1993) Toxicity testing using the brine shrimp: *Artemia salina*. In: Colegate SM, Molyneux RJ (Eds) *Bioactive Natural Products*, CRC Press, Boca Raton, FL, pp 442-456

Satyavati GV, Raina MK, Sharma M (1976) *Indian Medicinal Plants* (Vol 1), Indian Council of Medical Research, New Delhi, India, 118 pp

Shim JY, Chang YJ, Kim SU (1998) Indigo and indirubin derivatives from indoles in *Polygonum tinctorium* tissue cultures *Biotechnology Letters* **20**, 1139-1143

Shrikumar S, Sandeep S, Ravi TK, Umamaheswari M (2003) A HPTLC determination and fingerprinting of bacoside A in *Bacopa monnieri* and its formulation. *Indian Journal of Pharmaceutical Sciences* **65**, 132-135

Shrivastava N, Rajani M (1999) Multiple shoot regeneration and tissue culture studies on *Bacopa monnieri* (L.) Pennell. *Plant Cell Reports* **18**, 919-923

Singh RH, Singh L (1980) Studies on the anti-anxiety effect of medhya rasayana drug, brahmi (*Bacopa monniera* Wettst.) – Part I. *Journal of Research in Ayurveda and Siddha (Quarterly)* **1**, 133-148

Singleton V, Rossi Jr. JA (1965) Colorimetry of total phenolics with phosphormolybdic-phosphotungtic acid reagents. *American Journal of Enology and Viticulture* **16**, 144-158

Srinivasa H, Bagul MS, Padh H, Rajani M (2004) A rapid densitometric method for the quantification of luteolin in medicinal plants using HPTLC. *Chromatographia* **60**, 131-134

Sumathi T, Susramanian S, Govindasamy S, Balakrishna K, Veluchamy G (2001) Protective role of *Bacopa monnieri* on morphine induced hepatotoxicity in rats. *Phytotherapy Research* **15**, 643-645

Tripathi YB, Chaurasia S, Tripathi E, Upadhyay A, Dubey GP (1996) *Bacopa monnieri* Linn. as an antioxidant: Mechanism of action. *Indian Journal of Experimental Biology* **34**, 523-526

Vivancos M, Moreno JJ (2005) β-sitosterol modulates antioxidant enzyme response in RAW 264.7 macrophages. *Free Radical Biology and Medicine* **39**, 91-97