Research Journal of Recent Sciences _____ ISSN 2277-2502
Vol. **2(1),** 13-16, January **(2013)** *Res.J.Recent Sci.*

Enhancement of Secondary Metabolite Biosynthesis in *Bacopa monnieri*: *An in vitro* Study

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Available online at: www.isca.in
Received 17th September 2012, revised 20th October 2012, accepted 5th November 2012

Abstract

Enhanced production of total saponins was obtained from 40 day old suspension cultures of Bacopa monnieri. The callus yielded not only a 166% increased concentration of saponins but also produced two novel prominent bands of saponins compared to the natural plant system. We report here for the first time an increased potential of cell lines of B. monnieri in suspension for production of saponins; the active constituents responsible for neuropharmacological and nootropic action of the plant. Our studies can be utilised in meeting the demand and supply ratio of the drug with constant and elevated production of saponins produced in short duration.

Keywords: Secondary metabolites, suspension culture, *Bacopa monnieri*, Triterpenoid saponins.

Introduction

Bacopa monnieri (Brahmi) is a well known memory booster[1] which propagates vegetatively and rarely by seeds. Its vegetative propagation is further hampered by specific habitat requirements[2]. The use of whole plant system for medicine, poor replenishment efforts and untrained plucking of the plant material leads this medicinal plant towards endangerment. The major chemical entity shown to be responsible for neuropharmacological effects and the nootropic action or antiamnestic effect of *Bacopa monnieri* is *Bacopa* saponins A, B, and C which are dammarane-type triterpenoid saponins[3]. Twelve such *Bacopa* saponins of pharmacological importance were reported by Murthy et al[4]. Since the supply is limited and faces constraints in meeting the increasing demand of these biochemicals, large scale use of plant suspension culture is the need of today. The technique is found to be an attractive alternative approach as it offers a controlled supply of biochemicals independent of season and availability of plants[5,6]. The wide variety of neuropharmacological actions of *Bacopa monnieri* opens up interesting avenues for further research and offers new perspectives in the treatment of related diseases. Present study was undertaken to appraise saponin production through cell suspension culture system compared to the natural plants of *Bacopa monnieri* to meet out the demand and supply ratio of the drug.

Material and Methods

Our previous studies exhibit superiority of Gamborg's B5 media over MS in producing enahanced proliferative callus from leaf explants of *B. monnieri* fortified with lower concentrations of 2,4-D (0.25 -0.5 mg. l^{-1})[7]. Hence the leaf explants were inoculated in Gamborg's B5 media supplemented with 2,4-D (0.5 mg. l^{-1}). After 20 days of incubation in the growth medium, the friable callus were transferred to second phase of culture with modified MS liquid medium for production of secondary metabolites following the procedure suggested by Rahman et al[8] . The medium was supplemented with α-naphthalene acetic acid (1 mg l^{-1}), kinetin (0.5 mg l^{-1}), casein hydrolysate (1 g l^{-1}) and sucrose (30 g l^{-1}). After 40 days the cell mass in suspension along with field grown natural plant system and plant parts (root and shoot) were dried separately in dark (for 4 days at 37°C) for extraction of saponins.

Extraction and estimation of total saponins: Methanolic extraction of total saponins was performed by the method developed by Murthy et al[4] and spectrophotometric estimation was done by the method developed by Ebrahimzadeh and Niknam [9] taking Saponin (Sigma) as standard.

Thin layer chromatography: For TLC, the solvent system consisted toluene: ethyl acetate: methanol: glacial acetic acid in the ratio of (3:4:3:1). For visualization of the bands, the plates were sprayed with freshly prepared anisaldehyde – sulphuric acid solution - (9ml of 98% Sulphuric acid, 85ml methanol, 10ml Glacial acetic acid, 0.5ml anisaldehyde). The plates were kept in hot air oven at 120°C for 10min for visualization of bands.

Results and Discussion

Cell suspension culture in synthetic media offers an alternative way for producing metabolites of interest to the traditional cultivation in fields or greenhouses. Discoveries of cell cultures capable of producing specific medicinal compounds at a rate similar or superior to that of intact plants have accelerated in the last few years[10] which include production of shikonin, anthocyanins, and ajmalicine and recently important anti-tumor agents like taxol, vinblastine and vincristine[11] in suspension has also been successfully established.

A seasonal variation in the saponin content was observed in the naturally growing plants of *B. monnieri*. Among all the seasons, the saponin content was found to be maximum in the plants harvested during Oct –Dec, shown in figure-1. Observing the seasonal variations and low yield of saponins in plants, we initiated our studies aiming at enhanced production of saponins in *in vitro* developed callus.

Suspension culture established from callus mass obtained from first phase of cultures proliferated rapidly in modified MS liquid medium for production of saponins. The extract obtained from 40 days old cell mass in suspension yielded a 166% increase in total saponin content as compared to the natural total plant system which is shown in figure-2. The cell lines were further proliferated and maintained under the similar conditions.

The extract obtained from callus when screened by thin layer chromatography (TLC) revealed bands comparable to and of high intensity than total plant, isolated root and shoot system. The chromatogram shown in figure-3 exhibit two unidentified novel bands (high intensity brown spot and another low intensity yellow spot) from callus which were not shown by the natural plant system, isolated roots or shoots in particular. Also it can be comprehended that shoot system which constitute the bulk of the total plant system contains more concentration of saponins as compared to the root system.

The first reports from Rahman *et al* [8] revealed the potential of the cell suspension cultures of *B. monnieri* for production of bacosides. However cell lines exhibited 20% decrease in bacoside content as compared to the glass house grown plants of *B. monnieri*, but potential of cell lines for producing secondary metabolites in artificial conditions could not be denied. Under similar conditions our studies on cell suspension cultures of *Brahmi* clearly demonstrated two novel bands of saponins along with a prominent 166% increase in the concentration of total saponins. The increase in saponins in our study may be attributed to the selection of different culture media for establishment of primary culture whereby it may be understand that the cell mass under favourable media conditions have accumulated enough metabolites which can be diverted to the secondary metabolite pathway in second phase of culture. Secondary metabolism in cultured cells and tissues is a dynamic process. The net accumulation of desired metabolites represents equilibrium between its biogenesis, storage and degradation within the cellular compartments or specialized tissues [12]. Cu^{2++}, K^{+}, PO_4^{3-} and total nitrogen concentration in the media have also been found to significantly affect the cell growth and saponin accumulation in cell cultures of various Panax species [13]. The types of tissue [14] and concentration of plant growth regulators in the medium were also found to influence the cell growth and ginsenoside; a triterpenoid saponin, production in the suspension cultures[15]. The other possible reason may be the genotype of the explant. Our previous work reveals the influence of media and genotype on *in vitro* growth of cultures where two accessions of *B.monnieri* exhibited a significant variation in regeneration *via* organogenesis and embryogenesis as well [7]. Hence, these two factors contribute significantly to the effective enhancement of saponins in our study.

Conclusion

The increase in the principle active component in cell suspension system established in the present study will facilitate in overcoming the problem of uncertainty and inadequacy in the supply of raw material from the natural sources through ensuring constant enhanced qualitative supply under controlled environment.

Future studies can be targeted on standardization of large scale production of *Bacopa* saponins in bioreactors and characterization of the novel compounds using NMR. The novel bands of saponins obtained from callus cultures also raise the possibility of their utility in therapeutic ailments. Genetic transformation of high yielding cell lines can also be explored. Exploitation of methodology developed in the present study may further improve the production of saponins to meet up the increasing demand for neuropharmacological drugs.

Acknowledgement

The authors are grateful to the management of Maharaja Ranjit Singh College of Professional Sciences, Indore, India for providing the necessary lab facilities and cooperation from Dr. Swarnjit Singh Cameotra, Institute of Microbial Technology, Chandigarh, India for TLC studies.

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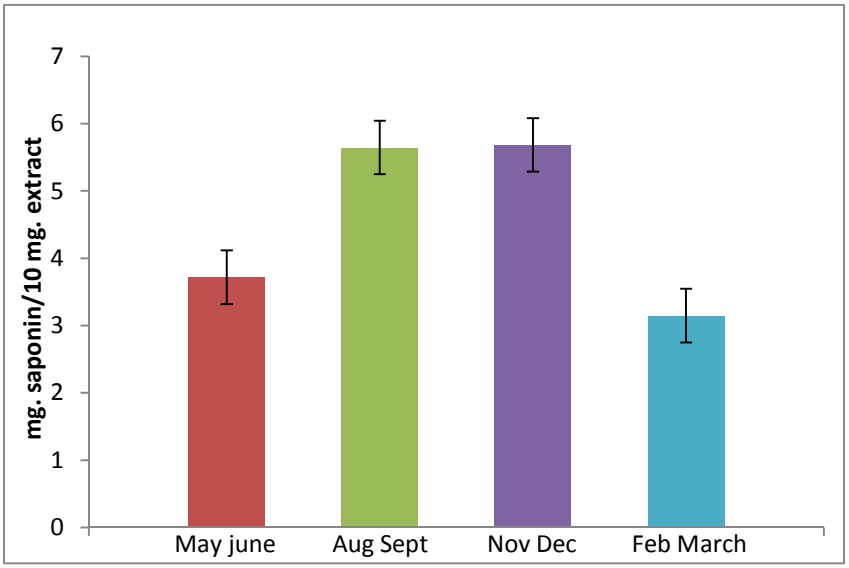

Figure-1
Seasonal variations in the saponin concentrations observed in naturally growing plants of *B. monnieri*

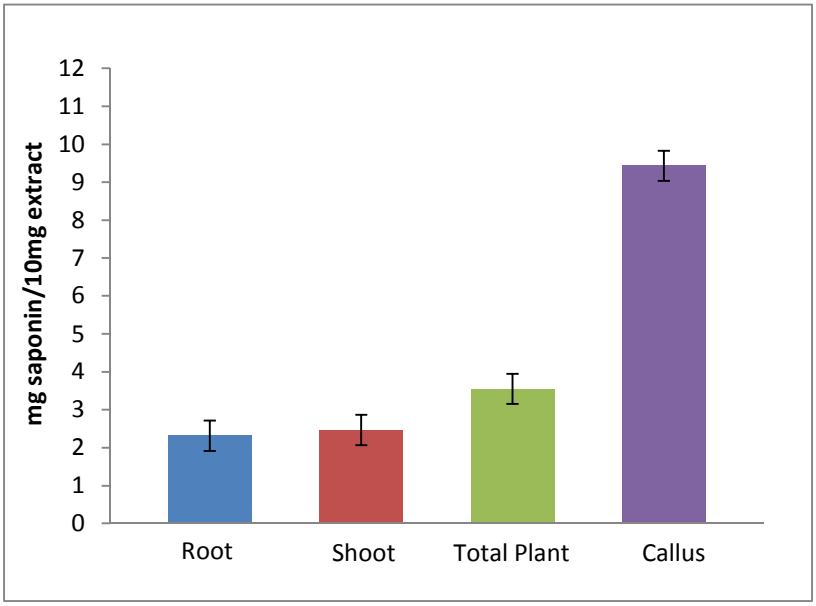

Figure-2
Increased production of saponins from callus as compared to the natural plant system of *B. monnieri*

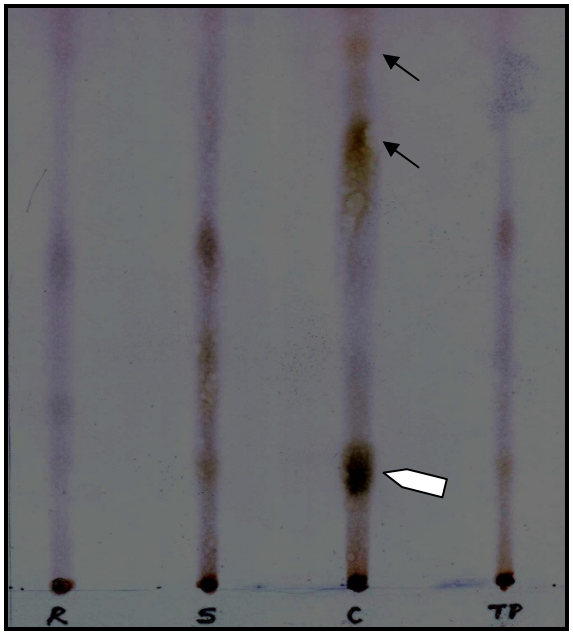

Figure-3

TLC plate exhibiting different bands of saponins obtained from Root system (R), Shoot system (S), Callus (C) and Total plant system (TP) of *B. monnieri*. The block arrow indicates the high intensity band as compared to the natural plant parts and total plant system, The novel bands are marked by single headed arrow in callus extracts