Research Article

Comparative Analysis of Bacoside A Yield in Field Acclimatized and *in-vitro* Propagated *Bacopa monnieri*

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ABSTRACT

Bacopa monnieri is a nootropic plant is extensively explored for their therapeutic efficacy for various neurological disorders. The active phytopharmaceutical component bacoside A, which is a mixture of four triterpenoid glycoside saponins is majorly responsible for its therapeutic properties. In present study, variations in the yield of four different bacoside Acomponents *viz.*bacoside A_3, bacopaside II, jujubogenin isomer of bacopa saponin C and bacopa saponin C, with variation in culture conditions have been compared. In comparison to field acclimatized plant, the yield of total saponins varied differently among different germplasm of *Bacopa monnieri*. The specific growth rate of plant propagated in solidified MS (agar) media was ~ 12-15 folds slower than liquid media. Metabolic quotient (q) of MS liquid was ~15-30 folds higher in liquid media. A comparison of the growth rate and yield among MS agar & liquid revealed higher bacoside A yield among the plants propagated on MS agar but growth rate was significantly less than liquid conditions. No significant variations were observed in the total bacoside A content of BM-3 and BM-5, when propagated in MS liquid media (BMT-3 and BMT-5). However, insignificant reduction of total bacoside A was observed in BM6 and BMTL-6.Significantly high specific growth rate and metabolic quotient with comparable yield of bacoside A obtrude shoot culturing in liquid media as a suitable alternative strategy for large scale culture of this nootropic plant with insignificant variations due to environmental parameters.

Keywords: *Bacopa monnieri*, bacoside A, Yield, metabolic quotient, specific growth rate, HPLC.

INTRODUCTION

Paradigm shift toward wide acceptance of plant derived medicines for various diseases associated with minimal side effects has taken the world with stride and has provided an economic boost to the countries. Phytomedicines have been successfully used for various ailment and diseases; however its role in regulating neurological disorder is astonishing. This is because most of the neurological disorders find little or no efficient treatment with conventional medications and heed a suitable alternative in phytomedicines.[1]Among different plants, reported for alleviating neurological disorders, *Bacopa monnieri* (L.) Wettest (Family: Scrophulariaceae) is one of the most extensively documented perennial herb reported for its nootropic properties and successfully used as brain tonic and revitalizer[2]along with other therapeutic benefits *viz.,* diuretic, anti-inflammatory, analgesic, antipyretic and cardio-tonic properties.[3, 4]These therapeutic properties are attributed to different phytocompounds present in the plant *viz.,* glycosides, alkaloids (such as brahmine and herpestine), flavonoids, cucurbitacins, phenolics and saponins.[2,5,6]Nootropic properties of the plant are primarily attributed to the presence of two type of triterpenoid glycosides saponins called 'bacosides' namely bacoside A and bacoside B which were earlier considered to be isomers and later characterized to be a mixture of four triglycosidic and four diglycosidic saponins respectively.[7]

The cytoprotective properties of *Bacopa monnieri* extracts on stressed neuronal cell lines have been well studied and documented.[8] Studies have shown the active role of bacoside A in regulating various symptomatic features of neurological disorder.[9]The antioxidant properties of bacoside A are attributed to their ability to regulate the activities of antioxidant enzymes in the neuronal cells *viz.* superoxide dismutase, catalase and glutathione peroxidases.[10]

Bacopa monnieri has been extensively studied for its nootropic properties using both, *in-vivo* and *in-vitro* systems.[11]However, in one of the previous study by our group, comparative analysis of some herbal formulations of *Bacopa monnieri*, manufactured in India, has shown significant variations in phytocompounds yields.[12] This further elevates the concern of possible variations in their therapeutic potential and necessitates the need of standardization tests and their documentation in pharmacopeia and monographs.[13] Previous studies have shown the role of agro-climatic conditions (season, soil properties) and cultivation practices (stage of plant growth during harvest) as few significant reasons for variations in the yield of phytocompounds.[14,15]A strategic approach to overcome such variations could be by culturing plant under controlled culture conditions using *in-vitro* culture technique.[16]However role of *in-vitro* culture techniques on the yield of therapeutically important saponins have not been reported or compared. Current study is one of the few reports comparing four

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different bacoside A *viz.* (1) bacoside A_3, (2) bacopaside II, (3) jujubogenin isomer of bacopasaponin C and (4) bacopa saponin C, in field acclimatized *Bacopa monnieri,* procured from different locations within India and their comparison with plant propagated in MS agar and MS liquid medium. The study will further provide an impetus to explore a suitable culture conditions for large scale propagation of *Bacopa monnieri* plantlets for improving the production of commercially and therapeutically important bacoside A.

MATERIALS AND METHODS

Plant materials and their taxonomical identification

Bacopa monnieri procured from different research institutions in India *viz.* IIIM Jammu (BM-3), IHBT Palampur (BM-5) and CIMAP Lucknow (BM-6); were taxonomically identified and preserved in herbarium at Department of Botany, University of Delhi, and Delhi, India. No information on plants being hybrid or transgenic is available. All plants procured from different geographical locations were vegetatively propagated using seven stems, with eight nodes each excised out from parent plantlet and were maintained in a pot within campus premises in shade for 30 days. Plants were further propagated using *in-vitro* culture technique in MS agar and MS liquid and are henceforth designated with code BMTA-3, BMTA-5 and BMTA-6 for MS-agar and BMTL-3, BMTL-5 and BMTL-6 for MS-liquid respectively.

Reagents

Different bacoside A *viz.*bacoside A_3, bacopaside II, jujubogenin isomer of bacopasaponin C, bacopasaponin C and their mixture were purchased from Natural Remedies Pvt. Ltd. Bangalore, India. Acetonitrile (HPLC grade) was purchased from Merck India Pvt. Ltd. and orthophosphoric acid (AR grade) was purchased from CDH, India. Murashige & Skoogmedium was purchased from Himedia Laboratories Pvt. Ltd. India. All other reagents used in the study were of analytical grade.

Sub-culturing shoots of *Bacopa monnieri* on Solidified and liquid MS medium

Apices of field acclimatized plants were excised, washed in sterile milli- Q water and surface was sterilized with 0.1 % (w/v) mercuric chloride for 40 sec followed by 1% (v/v) sodium hypochlorite solution for 40 sec. Subsequently, explants were treated with 70 % (v/v) methanol for 30 sec, thoroughly washed with sterile milli-Q water and inoculated in Murashiage&Skoog (MS) medium containing (0.8 % w/v agar). After 30 days, *in-vitro* propagated plants (4 shoot with two nodes) were further sub-cultured in MS agar (0.8 % w/v agar) and MS liquid medium respectively. All cultures were maintained in plant growth chamber (Vista cell, India) at 25 ± 2 ºC with white light illumination of 2450 lux for 12 h followed by 12 h dark period. The cultures were harvested after 30 days and fresh weights of plants along with numbers of shoots were recorded.

Plant growth kinetic study

Growth of the *in-vitro* propagated plants was estimated using plant fresh weights, initially (time = 0 days) and after 30 days and was used to estimate specific growth rate of plant (Equation 1) as reported previously.[17]

$$\ln X_t - \ln X_o = \mu \cdot t \qquad (1)$$

where X_t and $X_o$are plant fresh weight after time (t) 30 days and initial fresh plant weight during inoculation respectively. Specific growth rate (μ) is inversely proportional to doubling time (generation time) of organism (Equation 2),

$$\mu = \frac{\ln 2}{t_d} \qquad (2)$$

Where μ is specific growth rate (day^{-1}) and t_d is doubling time or generation time.

Solvent extraction of phytochemicals using maceration technique and phytochemicals

Analysis

Phytocompounds from field acclimatized and *in-vitro* propagated (MS Agar and MS liquid media) plant were extracted in methanol using maceration technique as reported previously.[18] Briefly, dried plants were macerated in methanol for 3 days. After maceration, suspensions were centrifuged at 10,000 rpm for 15 min. Supernatants were collected, filtered through 0.45μm PTFE membranes and dried at room temperature. Crude dried extracts were weighted and dissolved in methanol. All stock solutions were stored in cryovials at -20 ºC for further analysis.

Quantification of bacosides using HPLC

Quantification of bacosides were performed using HPLC system equipped with a SPD-20A ultraviolet - visible photodiode array detector (PDA) and an LC-20 AD dual pump (Shimadzu, Japan). Analytical separations were carried out using AtlantisT3 RP-18 column (150 x 4.6 mm, 5 μm particle size), Waters India Pvt. Ltd. The HPLC method was validated for linearity, limit of detection (LOD), precision and accuracy.[19] Different ratios of solvents were studied to optimize the mobile phase for resolution of bacoside A (standard). All further HPLC analyses were performed using optimized mobile phase. Yield of bacoside A was estimated using Equation (3),

$$Y\ p/x = \frac{\Delta P}{\Delta X} \qquad (3)$$

Where ΔP and ΔX are change in product and dry weight after 30 days respectively, assuming insignificant initial dry weight and bacoside a concentration in the explants.

Calibration and Validation

The linearity of the method was evaluated in the 100 – 500 μg/ml range. Five concentrations of four standards

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(bacoside A_3, bacopaside II, isomer of bacopasaponin C and bacopasaponin C) were chosen for generating the calibration curves and 20 µl of each standard was loaded into the HPLC column. Two determinations (n =2) were carried out for each concentration of all standards. The correlation graphs were constructed by plotting mean peak area *versus* concentrations. The linearity was evaluated by linear regression analysis calculated by the least square regression method. Limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined based on SE of response and slope of each standard regression equation.[20]

Statistical Analysis

All experiments were performed in duplicate and results were reported as (mean ± standard error). Statistics was performed with Microsoft™Excel2007.

RESULTS AND DISCUSSION

Sub-culturing shoots of Bacopa monnieri in MS Agar and MS liquid medium

Micropropagation on agar medium is routinely and commonly performed to allow totipotent cells to proliferate into organized differentiated (plantlet) or unorganized undifferentiated (callus) cells.[21,22] The concept of plant propagation is not very new, however despite of significant effort for more than three decades now, only a few products produced by *in-vitro* plant culture has been commercialized.[23] Insufficient reports on bioprocess optimization and sustainability for large scale manufacturing of phytocompounds further affect commercialization of products. Recently, liquid cultures are efficiently used for *in-vitro* multiplication and for rapid growth of the plantlets.[24,25,26,27] Previous studies have reported the significance of plant propagation in liquid conditions as a cost-effective alternative to solid medium.[28,22]

Plant growth can be defined in a number of different ways *viz*., length of shoots, fresh and dry weight of shoots. In present study *in-vitro* propagated *Bacopa monnieri* shoots were further sub cultured in MS agar and MS liquid medium and number of shoots and fresh weight obtained after 30 days were compared (Table 1). Plant propagated in MS liquid showed higher number of shoots then MS agar propagated plant (Fig 1). Shoots induction per node explant (%) was estimated. Result showed higher shoot induction per node explant (%) in MS liquid then solid media suggesting better utilization of media component in liquid state.[29,30]

Table 1: Observation of *in-vitro* propagated *Bacopa monnieri* in MS agar and MS liquid

Observation	*In-vitro* propagated (MS-agar)			*In-vitro* propagated (MS-liquid)		
	BMTA-3	BMTA-5	BMTA-6	BMTL-3	BMTL-5	BMTL-6
Number of shoot	7±0.71	6±1.77	9±1.06	16±2.04	15±1.45	18±2.67
Initial fresh weight of plant (g)	0.141±0.03	0.130±0.01	0.178±0.04	0.196±0.02	0.133±0.03	0.183±0.01
Final Fresh weight of plant (g)	0.165±0.23	0.156±0.45	0.232±0.28	2.98±0.53	1.15±0.13	1.18±0.22

Figure 1: Induction of shoots of *in-vitro* propagated *Bacopa monnieri* on MS agar and MS liquid medium: (A-1) BMTA-3 ;(B-1) BMTA-5; (C-1) BMTA-6; (A-2) BMTL-3 ;(B-2) BMTL-5; (C-2) BMTL-6

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Plant growth kinetic study

Previous studies have shown the role of culture condition on the growth response estimated as doubling time (t_d).[28,31] Unlike previous studies, in present study specific growth rate (μ) of plant is inversely proportional to generation time (t_d), was estimated using change in fresh weight of plant in MS agar and MS liquid. Results revealed that specific growth rate (μ) of in-vitro propagated plants increased ~12-15 folds in MS liquid compared to MS agar. Concomitantly, significant variations were observed in generation time (t_d) among plants propagated in *in-vitro* conditions. Significant reductions in doubling time of plant propagated in MS liquid media were observed. Doubling time of agar propagated plants *viz.* BMTA-3, BMTA-5 and BMTA-6 decreased from 125.87, 152.23 and 105.32 day respectively to 9.23, 10.14, 8.31 day for BMTL-3, BMTL-5 and BMTL-6 respectively (Table 2). Previous studies by[31]on liquid culture of *Bacopa monnieri* for 20 days had shown reduction in doubling time in MS liquid medium by 8 fold compared to MS agar medium. Variations in the fold change of doubling time in our report from previous studies may be attributed to plant response to *in-vitro* culture conditions, analytical and kinetic methods of doubling time estimations and time of studies. However, results from our studies, in agreement with previous studies, suggest better growth of *Bacopa monnieri* in MS liquid medium. Significant larger doubling time of plant propagated in agar may be attributed to mass transfer limitations of dissolved oxygen and nutrient which are comparatively less in liquid static systems.[22] Sustainable development of a biomanufacturing process is depended on various metabolic properties and operational parameters. Yield coefficient ($Y_{P/X}$) is one such parameter which explains the metabolic response of in-vitro propagated living system.[32] In current study, yield coefficient ($Y_{P/X}$) of bacosides A of in-*vitro* propagated *Bacopa monnieri* in both MS agar and MS liquid conditions were compared. Specific growth rate and yield coefficient are also significantly important for analyzing the metabolic quotient (q) of living system (equation 4).[17]

$$q = \frac{\mu}{Y\,p/x} \qquad (4)$$

Equation is used to estimate the metabolic response of an organism to growth and non-growth associated metabolism.[17] Current study for the first time compared and reported the metabolic quotient of *Bacopa monnieri* propagated in MS agar and MS liquid media. Results showed that metabolic quotient of plants in MS liquid medium was ~ 15-30 folds higher compared to MS agar (Table 2). It is imperative to understand that cell metabolism is majorly dependent on substrate availability and its assimilation rate. Higher nutrient update, as discussed previously, may show better metabolic response, seen as change in phenotypic characteristics of plant including plant growth rate and weight and metabolite production. It was observed that increase in biomass of the plant showed proportionate increase in the yield

of product (bacosides A) under different culture conditions. However the studies become even more significant due to the non-availability of any previous study analyzing the metabolic quotient of *Bacopa monnieri* under *in-vitro* culture conditions. Analyses of metabolic quotient in liquid conditions further support the rationale of rapid growth in liquid media.

Table 2 Growth kinetic study of in-*vitro* propagated in MS agar and MS liquid *Bacopa monnieri*

Codes	Specific growth rate (Day^{-1})	Doubling time (Day)	(%) Shoot induction	Yield Coefficient (mg/g).	Metabolic quotient ($mg.mg^{-1}.day^{-1}$)
BMTA-3	0.006±0.001	125.87±4.60	43.75±6.07	12.04±1.67	0.249±0.0045
BMTA-5	0.005±0.002	152.23±5.55	46.87±3.69	18.21±0.26	0.251±0.0091
BMTA-6	0.007±0.004	105.32±4.57	53.12±9.61	13.16±0.91	0.502±0.0021
BMTL-3	0.082±0.001	9.23±0.79	122.92±8.87	11.35±0.55	7.179±0.833
BMTL-5	0.070±0.002	10.14±0.18	110.42±6.32	10.77±0.12	6.433±0.160
BMTL-6	0.082±0.004	8.31±0.17	114.58±11.59	09.22±0.20	8.858±0.344

Quantification of bacosides a components using HPLC

In our previous studies phytocompounds *viz.*saponin, flavonoids and phenolic in field acclimatized and *in-vitro* propagated methanolic extracts of *Bacopa monnieri* were compared (data not shown). On the basis of saponin yield equivalent to total bacosides a, quantification was performed for further bacosides profiling using HPLC.

Mobile phase for resolution of bacoside A mixture of four compounds, (1) bacoside A_3, (2) bacopaside II, (3) jujubogenin isomer of bacopasaponin C and (4) bacopa saponin C was optimized using isocratic elution with 0.05% orthophosphoric acid and acetonitrile (70:30 v/v) at the flow rate of 1.2 ml/min at 205nm wavelength. All further HPLC analysis was performed using optimized mobile phase. Analysis using isocratic elution minimizes the variations of the base line and provides precession and accuracy.[2,19,20] Calibration curves plotted by linear

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regression were based on area *verses* concentration of each compound at five different concentrations (100-500 µg/ml) of bacosides. The regression equation of these curves and there coefficients of regression (R^2) were confirming the linear relationship between peak area and concentration. The LOD and LOQ value for bacoside A components were estimated to be in the range of 0.001 - 0.030 and 0.004 - 0.076 respectively (Table 3). On the basis of these calibration data of bacoside A standards, *Bacopa monnieri* methanolic extracts of field acclimatized and *in-vitro* propagated (MS-agar and MS-liquid) plants were quantified using HPLC and there chromatogram are presented in Fig. 2, 3 and 4. Chromatogram showed four major peaks (numbered 1-4) corresponding to four bacoside A components *viz.* bacoside A_3 (retention time 32.4 min), bacopaside II (retention time 35 min), jujubogenin isomer of bacopasaponin C (retention time 42.4), and bacopasaponin C (retention time 47.5). The chromatogram of *Bacopa monnieri* extracts showed other undesirable peaks of other phytocompounds in the extracts. Results in figure 2 illustrate the HPLC chromatogram of field acclimatized *Bacopa monnieri* extracts for bacosides A components. A comparatively showed higher peak area was observed than *in-vitro* propagated (MS agar and MS liquid) plants (Fig. 3 & 4) due to higher extractable biomass of field acclimatized plant.

Previous studies have reported variations in phytocompounds yield among different field acclimatized *Bacopa monnieri* with variation in environmental conditions (season)[14]and age of harvest.[15]However no such comparative bacoside A components variations are reported among *Bacopa monnieri* from different locations of India. In current study, methanolic extracts of field acclimatized *Bacopa monnieri* of BM-3, BM-5 and BM-6 were analyzed for their bacoside A composition and variation in the yield coefficient of bacoside A components were compared with MS agar and MS liquid propagated plants (Table 4). Yields of total bacoside content within BM-3 and BM-5 were observed to increase in MS agar (BMTA-3 and BMTA-5) while no significant change in MS liquid media (BMTL-3 and BMTL-5) were observed. However higher specific growth rate and lower doubling time support the possible use of liquid culture technique for large scale cultivation of *Bacopa monnieri* over solid culture and field acclimatization, without any significant variation due to environmental conditions. In comparison, total bacoside A content of BM-6 increases in MS agar but decreases in MS liquid medium. No prior scientific information is available to support such a plant response; however it suggests that even under controlled culture conditions (*in-vitro*), *Bacopa monnieri* germplasm from different agro-climatic conditions does not show similar responses (total bacoside A yield). Moreover, role of culture conditions in modulating saponin biosynthetic pathway may not be ruled out. Comparative analysis of four different bacoside a components

viz. bacoside A_3, bacopaside II, jujubogenin isomer of bacopasaponin C and bacopa saponin C, showed variation among plants of different agro-climatic conditions (BM-3, BM-5 & BM-6) Moreover, different bacoside A components invariably varied with variation in culture conditions. The detailed analysis of different bacoside A components may play a pivotal role in affecting therapeutic potential of the extract. Previous study by[33]has explored the detailed saponin biosynthetic pathway of *Bacopa monnieri*. The limited information available about regulation of cytchrome P450 and glycosyl transferase[34]the final step in the bioconversion of β-amyrin to triterpenoid glycoside may be responsible for variations of different bacoside a components among different germplasm and under different culture conditions. Such studies are important from scientific and commercial perspective as they pave the way for successful designing of bio-manufacturing processes for large scale *in-vitro* propagation of *Bacopa monnieri* and for higher yield of therapeutically important phytocompounds, bacoside A.

Table 3: Calibration data of four bacosides standards using HPLC

Compound	Bacosides Standard	Linearity range	Regression equation	Correlation coefficient (R^2)	Limit of Detection (LOD)	Limit of quantification (LOQ)
1	Bacoside A 3	(100-500 µg/ml)	y = 6E+06x	0.991	0.030	0.090
2	Bacopaside II	(100-500 µg/ml)	y = 1E+07x	0.982	0.001	0.004
3	Jujubogenin Isomer of Bacopasaponin C	(100-500 µg/ml)	y = 5E+06x	0.995	0.025	0.076
4	Bacopasaponin C	(100-500 µg/ml)	y = 5E+06x	0.993	0.020	0.061

CONCLUSION

Bacoside A components, *viz.*bacoside A_3, bacopaside II, jujubogenin isomer of bacopasaponin C and bacopasaponin C, are cumulatively responsible for therapeutic properties of this saponin. Variation in their yield with agro-climatic conditions or *in-vitro* culture conditions may significantly affect their therapeutic potential and commercial acceptance. In the absence of any stringent guidelines for the validations of herbal medicines, it is even more important to explore the abiotic parameters regulating their biosynthesis.

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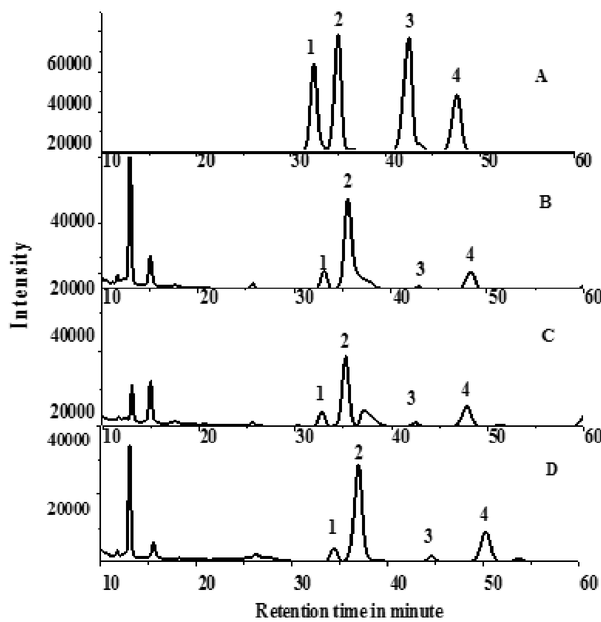

Figure 2: HPLC chromatogram of field acclimatized *Bacopa* monnieri extract: (A) Bacoside A Standard; (B) BM-3; (C) BM-5; (D) BM-6. Key to peak identities: (1) bacoside A3; (2) bacopaside II; (3) jujubogenin isomer of bacopasaponin C; (4) bacopasaponin

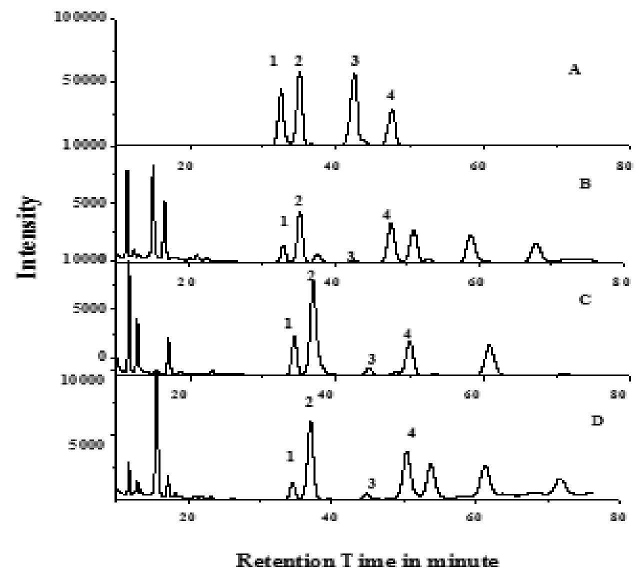

Figure 3: HPLC chromatogram of *in-vitro* propagated *Bacopa monnieri* extract in MS agar: (A) Bacoside A Standard; (B) BMTA-3; (C) BMTA-5; (D) BMTA-6. Key to peak identities: (1) bacoside A3; (2) bacopaside II; (3) jujubogenin isomer of bacopasaponin C; (4) bacopasaponin

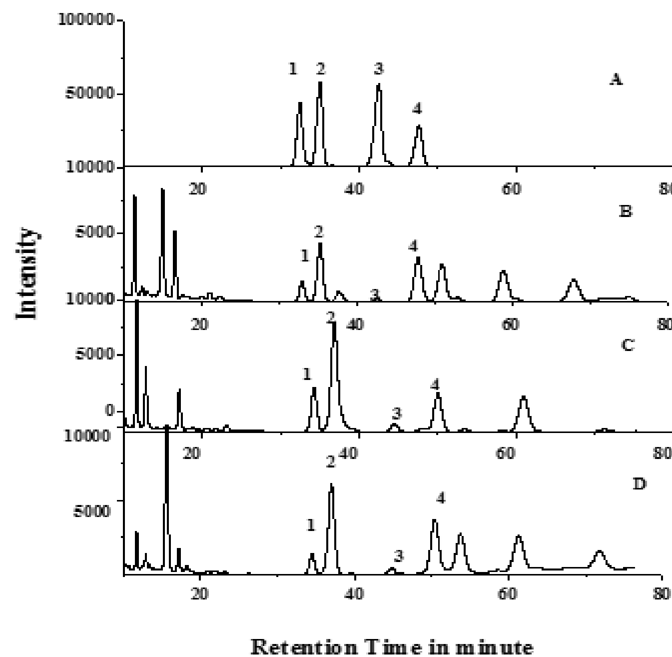

Figure 4: HPLC chromatogram of *in-vitro* propagated Bacopamonnieri extract in MS Liquid: (A) Bacoside A Standard; (B) BMTL-3; (C) BMTL-5; (D) BMTL-6. Key to peak identities: (1) bacoside A3; (2) bacopaside II; (3) jujubogenin isomer of bacopasaponin C; (4) bacopasaponin

Currently study had shown the variation in four different saponin among plants from different geographical and agro-climatic conditions. Invariably, their yield differs with variation in culture conditions. It was observed that the yield of bacoside A increases in MS agar but growth rate was significantly less than liquid conditions. No significant variations were observed in the total bacoside A content of BM-3 and BM-5, when propagated in MS liquid media (BMT-3 and BMT-5). However, insignificant reduction of total bacoside A was observed in BM6 and BMTL-6. A significant variation in the yield of individual bacoside A components were also observed among plant under

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different culture conditions. Results further strengthen the proposed rationale in the study on the modulating effect of environmental and culture conditions in regulation of saponins biosynthetic pathway. Such studies will further provide a support to optimization of bioprocess strategies of biomanufacturing of bacoside A.

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Table 4: Comparative analysis of total bacosides A in field acclimatized and *in-vitro* propagated *Bacopamonnieri* methanolic extracts

(Bacosides A mg/g dry weight of plant)	Field acclimatized			*In-vitro* propagated (MS-agar)			*In-vitro* propagated (MS-Liquid)		
	BM-3	*BM-5*	*BM-6*	*BMTA-3*	*BMTA-5*	*BMTA-6*	*BMTL-3*	*BMTL-5*	*BMTL-6*
Bacoside A3	3.12±1.30	1.59±0.13	1.10±0.35	1.70±0.20	4.15±0.28	1.57±0.16	2.10±0.07	2.14±0.10	1.16±0.03
Bacopaside II	4.32±0.70	3.11±0.41	4.66±0.04	3.52±0.66	6.71±0.27	4.28±0.14	3.63±0.12	3.94±0.08	3.36±0.12
Jujobogenin Isomer of bacopasaponin C	0.35±0.02	1.09±0.56	0.61±0.03	0.42±0.06	0.99±0.11	0.57±0.11	0.51±0.02	0.43±0.01	0.48±0.02
Bacopasaponin C	2.40±0.11	5.26±2.21	4.04±0.28	6.40±0.70	6.36±0.21	6.74±0.48	5.11±0.34	4.26±0.08	4.22±0.05
Total Bacoside A	10.19	11.05	10.41	12.04	18.21	13.16	11.35	*10.77*	9.22

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Conflict of Interest: None.

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