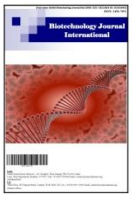

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In vitro Propagation of *Bacopa monnieri* (L.)

Poornima Raj[1], J. Anbumalarmathi[1*] and S. Aruna Sharmili[1]

[1]Department of Biotechnology, Stella Maris College (Autonomous), Chennai-600 086, India.

Authors' contributions

This work was carried out in collaboration among all authors. Author PR performed the statistical analysis and wrote the protocol. Author JA designed the study, managed the literature search and wrote the first draft of the manuscript. Authors JA and SAS managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

An experiment was conducted for standardization of *in vitro* propagation technique of *Bacopa monnieri* (L.), a medicinal herb of India. Healthy leaf segments of the herb were used as explants with basic Murashige and Skoog (MS) medium containing various combinations of different growth regulators for callus, shoot and root initiation. The best callus induction percentage (95.47%) was observed on MS + 0.5 mg/L NAA and 2.0 mg/L BAP (T_3). The maximum number of shoots (8), shoot length (9.30 cm) and shoot induction percentage (90.48%) was achieved on MS + 3.0 mg/L BAP and 1.0 mg/L Kn (ST_4). The maximum number of roots (8) and root length (7) was observed on MS + 1.5 mg/L IAA (RT_5). The rooted micro shoots were successfully hardened and acclimatized in green house and subsequently established in soil with survival rate of 90%.

Keywords: Bacopa monnieri L.; MS – Murashige and Skoog; growth regulators; in vitro; propagation; rooting; hardening.

1. INTRODUCTION

Bacopa monnieri (L.) Wettst. (Family - Scorphulariaceae) is a creeping glabrous, succulent herb, rooting at nodes whose habitat includes wetlands and muddy shores [1]. It is commonly known as Water hyssop, Brahmi, Jalbrahmi and Nir – brahmi. It is native to the

**Corresponding author: E-mail: amalarmathi@gmail.com;*

wetlands of Southern and Eastern India, Australia, Europe, Asia, and North and South America [2].

The plant has been extensively used in the traditional system of medicine for centuries including Ayurveda. It was earlier used as a brain tonic to enhance memory development, learning and concentration to provide relief to patients with anxiety or epileptic disorders. In India, it has been used for treating dermatosis, anaemia, diabetes, cancer and infertility. It contains alkaloids – Brahmin, nicotine, and herpestine. Des – saponin glycosides – terpenoid and saponins (*Bacosides A & B*) [3].

Bacopa monnieri has become endangered due to many reasons. *In vitro* regeneration holds tremendous potential for the production of high-quality plant-based medicine. Therefore, there is the need to encourage *in vitro* plant propagation which is considered one of the important strategies for ex-situ biodiversity conservation. The best commercial application of tissue culture technique is the production of true to type plants at a very rapid rate compared to conventional methods. Tissue culture plants are reported to grow faster and mature earlier than their seed propagated progenies [4]. Multiplication of plants of tissue culture can occur through the enhanced formation of axillary shoots and production of adventitious shoots either directly from the explant or through the intermediate stage of callus followed by rooting of individual shoots and also by somatic cell embryogenesis [5,6].

Plant tissue culture technique is an integral part of the genetic transformation process [7,8], production of secondary metabolites, important bioactive [9] compounds and production of somaclonal variants by callus culture [10]. The present investigation has been carried out for developing a micropropagation protocol after assessing the response of leaf explants to find out a suitable media with supplementation of phytohormones which would facilitate rapid multiplication.

HPLC is gaining interest in the analysis of plant extracts. The separations by HPLC are more rapid and accurate than classical methods and provide high resolution and sensitivity [11]. Saponins are terpenoids, glycosides distributed widely in the plant kingdom. Medicinally important species are rich in these compounds. Saponins are usually difficult to detect by HPLC-UV since most lack a strong UV chromophore. Generally, they are monitored at lower UV wavelengths ranging from 200 to 210 nm, and for these reasons acetonitrile-water gradients have been the mobile phase of choice using reversed-phase C-18 columns since acetonitrile absorbs less at this wavelength compared to methanol (Geoffrey et al. 2007).

2. MATERIALS AND METHODS

2.1 Collection of Explants

Bacopa monnieri L. plant was collected from Purapuzha, at Malappuram district of Kerala. The collected plants were further grown in a pond of Stella Maris College (Autonomous), for fresh sample collection. The present experiment was conducted in Stella Maris College (Autonomous), Chennai in November 2018.

2.2 Culture Media

The basal culture medium used in the present study was Murashige and Skoog culture medium (MS medium) [12], supplemented with 3% sucrose and 0.8% agar and different growth hormones. The MS medium was supplemented with auxin or its combinations with cytokinin for the initiation of callus and different combinations of cytokinin and auxins for *in vitro* shoot and root regeneration respectively.

2.3 Preparation of Explants

Stem segments with leaves of approximately 25 – 30 cm with 10 - 12 internodes were carefully excised using sterilized surgical blade or scalpel and brought to the laboratory. The plant material was washed with running tap water by continuous shaking to remove traces of dust and mud followed by distilled water [13].

2.4 Surface Sterilization

Surface sterilization of leaves explants was done under aseptic condition in laminar airflow. The leaves were cut into 1 – 1.5 cm and were washed with sterilized distilled water 3 - 4 times, for 10 min. This was followed by washing with surfactant (Tween-20: 2 - 3 drops/100 ml water) for 5 min and rinsed with sterilized distilled water. Explants were treated with 70% ethanol (2 - 3 min), 0.1% $HgCl_2$ (1 - 2 min) followed by rinsing with 70% ethanol and thrice with sterilized distilled water. Explants were kept in a Petri plate with Whatman filter paper to drain out the water

and leaves were ready for inoculation [14]. The culture was inoculated containing one explant per test tube and three explants per culture bottle. Then for each treatments, 12 test tubes and five culture bottles were maintained.

2.5 Callus Induction

For callus initiation, leaves were inoculated on MS medium supplemented with different concentrations of auxins and cytokinins : T_1 : MS + 0.5 mg/L IAA + 2.0 mg/L BAP,T_2 : MS + 0.5 mg/L NAA, T_3 : MS + 0.5 mg/L NAA + 2.0 mg/L BAP, T_4 : MS + 0.5 mg/L Kn, T_5 : MS + 0.5 mg/L NAA + 1.0 mg/L BAP, T_6 : MS + +0.5 mg/L NAA + 1.0 mg/L BAP + 0.5 mg/L Kn, T_7 : MS + 1.0mg/L 2,4-D + 0.5 mg/L BAP, T_8 : MS + 1.5 mg/L NAA, T_9 : MS + 0.5 mg/L IAA + 1.5 mg/L BAP and T_{10} : MS 1.0mg/L IAA+ 1.0 mg/L BAP are used. The cultures were incubated at 25 ± 2°C under 16/8 hours light regime provided by cool white fluorescent lamp [15]. Characteristics such as number of days to callus induction, callus induction percentage (%) and callus weight (g) were observed.

2.6 *In vitro* Shoot Regeneration

Leaf derived calli were transferred to shoot regeneration media with different concentrations of cytokinins: ST_1: MS + 1.0 mg/L BAP, ST_2: MS + 2.0 mg/L BAP + 0.5 mg/L Kn, ST_3: MS + 2.0mg/L BAP, ST4: MS + 3.0 mg/L BAP + 1.0 mg/L Kn, ST5: 1.5 mg/L Kn and ST_6: MS + 2.0 mg/L Kn. The culture bottles were incubated at 25±2°C [15]. Characteristics such as number of shoots, shoot length (cm), percentage of response (%) were observed.

2.7 *In vitro* Root Regeneration

The regenerated shoots are transferred to root regeneration media with different combinations of auxins with MS basal media. The concentrations were: RT1: MS + 0.5 mg/L, IAA + 1.0 mg/L NAA, RT2: MS + 1.5 mg/L NAA, RT3: MS + 1.0 mg/L IAA + 0.5 mg/L NAA, RT4: MS + 1.0 mg/L IAA + 2.0 mg/L NAA, RT5: MS + 1.5 mg/L IAA and RT6: 2.5 mg/L NAA. The cultures were maintained in culture room at 25±2°C for 8-6 h of day and night under the cool white fluorescent light [15]. Characteristics such as number of roots, root length (cm) were observed.

2.8 Hardening

In vitro rooted plantlets were taken out from the test tubes and culture bottles and washed with sterilized distilled water to remove the excess medium. They were transferred to polythene bags containing a mixture of sterile soil: sand: manure (1:1:1) and were transferred to pots after 2 weeks and were periodically watered.

2.9 High-Performance Liquid Chromatography (HPLC)

2.9.1 Sample preparation

The dried material of *Bacopa* was soaked in water for 24 hours and squeezed to discard water before 4 hour percolation with 7 ml of 95% ethanol per 1g of dried plants and callus for 3 times. The methanolic extract was filtered and dried under reduced pressure. Each extract (10 mg) was dissolved in 10 ml methanol and filtered through a 0.45 µm nylon filter before injection into the chromatographic system [16].

2.9.2 HPLC condition

Chromatographic separation was carried out with Shimadzu prominence, equipped with binary gradient pump (SPD – 20 A & LC – 20AD – UFLC). The mobile phase comprising of Acetonitrile and water in volume ratio of (40:60), was used for the analysis. The system was run isocratically over 25 min with a flow rate of 1 ml/min, 20 µL of the sample was injected in the chromatographic system and the detection was done at 205 nm.

3. RESULTS AND DISCUSSION

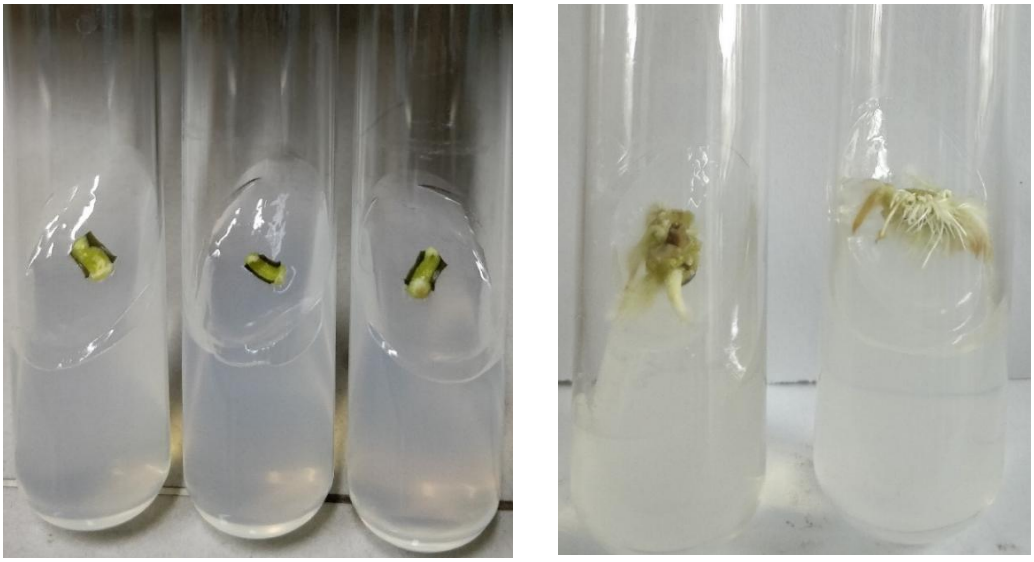
3.1 Callus Induction

In vitro callus was induced from leaf explants of *B. monnieri.* The leaves were cultured on MS media amended with various growth regulators. After 5 days, the callus growth from leaf explants on T_3 (MS + 0.5 mg/L NAA + 2.0 mg/L BAP) recorded the maximum callusing rate (95.47%) and callus weight (1.05 g). Similarly, Priya et al. 2015, reported that callusing rate of 71 % was observed on MS medium supplemented with 0.5 mg/L NAA and 2.0 mg/L BAP (Table 1) (Fig. 1).

Table 1. Effects of different growth regulators on callus formation in *Bacopa monnieri* L.

Treatments	Concentration of growth regulators (mg/L)					Number of days to callus induction	Callus induction percentage (%)	Callus weight (g)
	2,4-D	NAA	IAA	BAP	Kn			
T_1	-	-	0.5	2.0	-	10	67.45	0.42
T_2	-	0.5	-	-	-	7	75.28	0.58
T_3	-	0.5	-	2.0	-	5	95.47	1.05
T_4	-	-	-	-	0.5	11	84.17	0.47
T_5	-	0.5	-	1.0	-	7	63.15	0.33
T_6	-	0.5	-	1.0	0.5	5	82.6	0.63
T_7	1.0	-	-	0.5	-	8	70	0.78
T_8	-	1.5	-	-	-	8	83.48	0.81
T_9	-	-	0.5	1.5	-	10	72.39	0.75
T_{10}	-	-	1.0	1.0	-	11	90.18	0.91

2,4-D - 2,4-dicholrophenoxy acetic acid, NAA - 1-naphthalene acetic acid, IAA – 3 - Indole acetic acid, BAP - 6-benzylaminopurine, Kn – Kinetin



a. Callus initiation **b. Callus proliferation**

Fig. 1. Callus induction from leaf explants of *Bacopa monnieri* (L.) on MS medium supplemented with 0.5 mg/L NAA and 2.0 mg/L BAP (T_3)

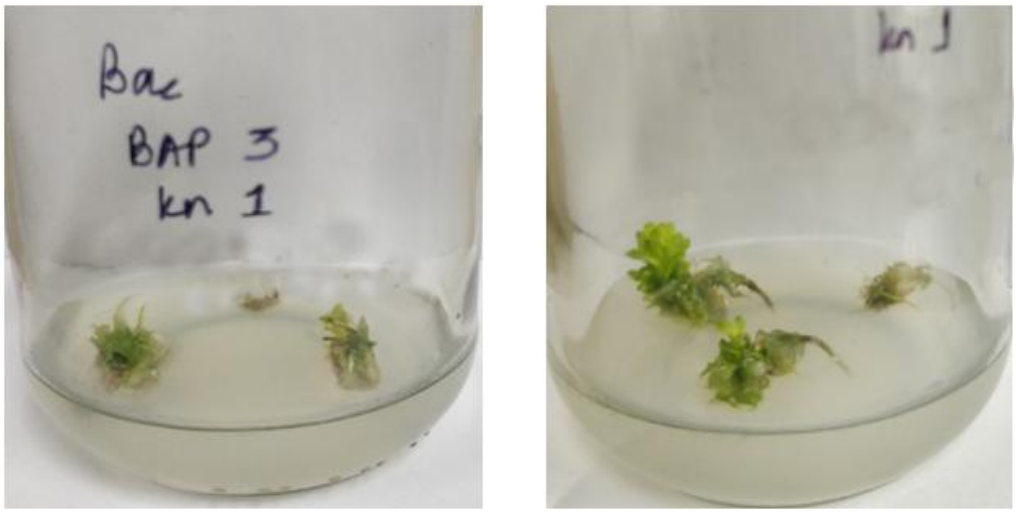
3.2 *In vitro* Shoot Regeneration

In vitro shoot regeneration depends upon a different type of concentration of cytokinins. In the present study, ST_4 (MS + 3.0 mg/L BAP + 1.0 mg/L Kn) showed maximum number of shoots (8), maximum shoot length of about (9.30 cm) and maximum percentage of response of about (90.48 %) (Table 2) (Fig. 2). This is in a contradiction with earlier reports by [17] who found that maximum numbers of plants were obtained on medium containing Kn/2 i.p. (0.1 mg/L) and Kn (1 mg/L) in shoot tip. [18] reported that BAP was found to be suitable than Kn as BAP resulted in quicker and better response than the later. Direct regeneration of shoots and roots occurred in nodal explants in Bacopa on MS medium containing NAA 0.1 mg/L and BAP 0.5 mg/L. [19] also reported that the addition of BAP increased in number of shoots, mean shoot length and number of roots/explants.

Table 2. Effects of different concentrations of BAP and Kn for shooting response in *Bacopa monnieri* L.

Treatments	Concentration of growth regulators (mg/L)		Number of Shoots	Shoot Length (cm)	Percentage of response (%)
	BAP	Kn			
ST_1	1.0	-	5.00±0.31*	8.56±0.04*	71.20
ST_2	2.0	0.5	5.94±0.28*	4.04±0.04*	80.14
ST_3	2.0	-	3.24±0.13*	3.53±0.09*	40.01
ST_4	3.0	1.0	8.45±0.07*	9.30±0.14*	90.48
ST_5	-	1.5	4.0±0.37*	6.70±0.09*	60.27
ST_6	-	2.0	4.0±0.27*	6.00±0.24*	50

BAP - 6-benzylaminopurine, Kn – Kinetin.
**Mean±SD*



a. Shoot initiation **b. Shoot proliferation**

Fig. 2. Shoot formation and regeneration in MS media supplemented with 3.0 mg/L BAP and 1.0 mg/L Kn (ST_4)

3.3 *In vitro* Root Regeneration

Once the healthy shoots were generated, various combinations of auxins were tested for rooting. In the present study RT_5; MS + IAA 1.5 mg/L recorded maximum number roots (8) and root length (7.00 cm) (Table 3) (Fig. 3). Similar results also reported by [19] where the role of auxin is well established for enhancing rooting. The present study is in contradiction with report of [17] who found that MS + 1.5 mg/L Kn has given root length (2.60 cm) with number of roots (10).

3.4 Hardening

In vitro plants are very delicate and prone to sudden environment changes that may damage the plants unless it is adapted to the new environment. Hence, these rooted plantlets were then gently removed from culture bottles, washed initially to removed adhered agar and traces of the medium to avoid contamination and washed with distilled water. It was shifted to pots and kept in polyhouse with survival rate of 90% (Fig. 4).

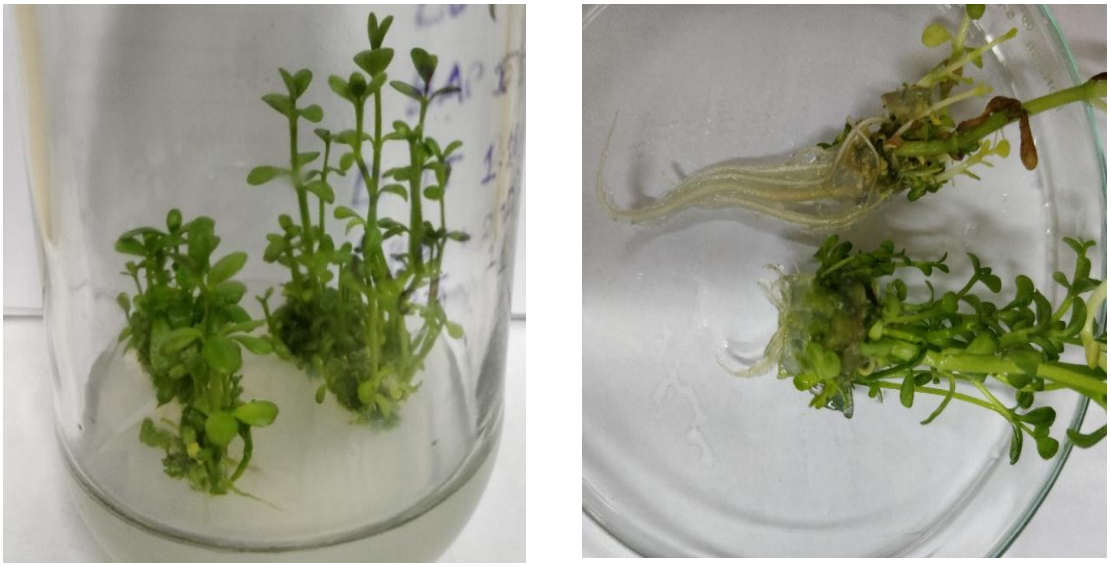
3.5 HPLC

The identity of the band of saponin, the plant and callus sample extract was confirmed by overlaying the UV absorption spectra of plant and callus sample at 205 nm which showed retention time at 23.538 min and 23.622 min respectively (Fig. 5 and Fig. 6).

Table 3. Effects of different concentrations IAA and NAA on *in vitro* rooting in *Bacopa monnieri* L.

Treatments	Concentration of growth regulators (mg/L)		Number of roots	Root length (cm)
	IAA	NAA		
RT_1	0.5	1.0	5.80±0.96*	6.21±0.89*
RT_2	-	1.5	6.40±0.96*	2.10±1.37*
RT_3	1.0	0.5	4.12±0.26*	1.60±0.51*
RT_4	1.0	2.0	5.01±0.26*	6.99±0.22*
RT_5	1.5	-	8.60±0.96*	7.00±0.96*
RT_6	-	2.5	8.00±0.96*	4.01±0.96*

NAA - 1-naphthalene acetic acid, IAA – 3 - Indole acetic acid.
**Mean±SD*



a. Root formation and regeneration of plant **b. Proliferated roots**

Fig. 3. Root formation and regeneration in MS media supplemented with 1.5 mg/L IAA (RT_5)

Fig. 4. Hardening of *Bacopa monnieri*

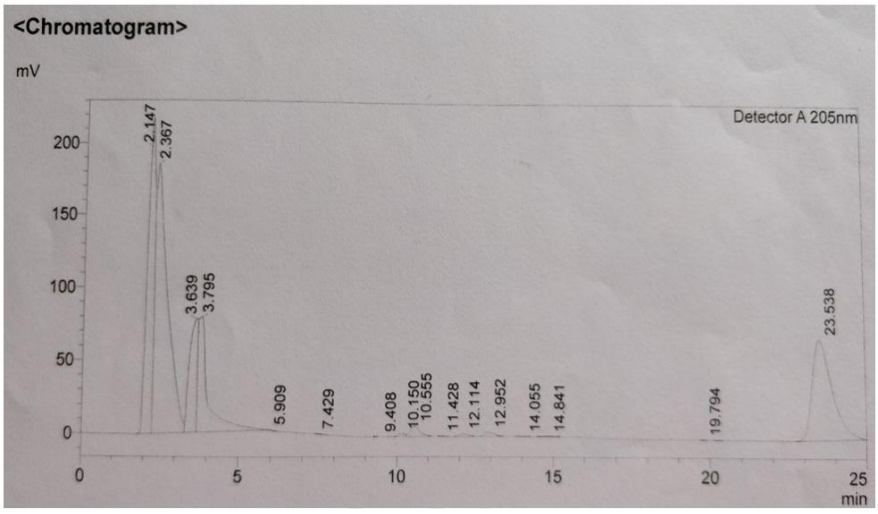


Fig. 5. Saponin peak at retention time of 23.538 minutes in *Bacopa monnieri* plant

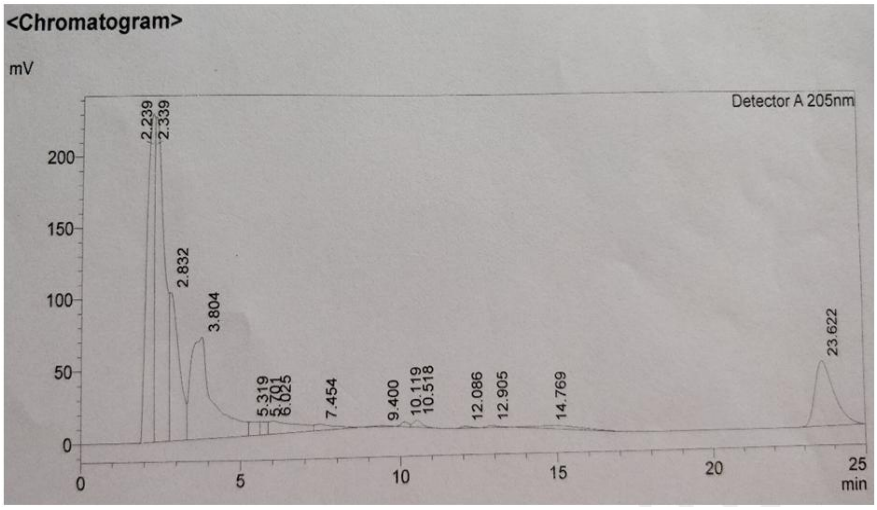


Fig. 6. Saponin peak at retention time of 23.622 minutes in *Bacopa monnieri* callus

4. CONCLUSION

Bacopa monnieri is a medicinal plant for treating dermatosis, anaemia, diabetes, cancer and infertility. The objective of the present study was to develop a micropropagation protocol resulting in the production of large number of healthy plantlets. Besides, the multitude of disease-free plants produced open the scope for utilization of plant material for antimicrobial testing and suitable pharmaceutical preparations. Callus tissue has its significance as it can be exploited for the production of soma-clonal variants, synthetic seeds production as well as for the production of secondary metabolites and certain bioactive compounds. HPLC was found to be simple, precise, sensitive and accurate for the quantification of saponin in *Bacopa monnieri*.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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