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Research Article

Comparative Qualitative and Quantitative Analysis of Phytochemicals in Five Different Herbal Formulations of *Bacopa monnieri*

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ABSTRACT

Current study highlights the qualitative and quantitative analysis of different therapeutically important phytochemicals in five different herbal formulations of *Bacopa monnieri*. Crude extraction of phytochemicals were optimized using two different methods and solvents *viz.* conventional solvent extraction (CSE) method and maceration method, with one of the herbal powder formulation, HF-1. Based on higher cumulative phytochemicals yield in macerated methanolic extract, all further extractions from different herbal formulations were performed using maceration in methanol. Spectrophotometric analysis of total phenolic content (TPC), total flavonoids content (TFC) and saponins yield showed variations among different formulations. Like TPC, TFC was also comparatively higher in macerated methanolic extract of HF-3 than other formulation while saponins were comparatively higher in HF-4. Also, thin layer chromatographic (TLC) analysis of all five formulations showed variations in the saponins among different formulation. Saponins being the major component showing nootropic properties of the plant extract, suggesting possible variations in therapeutic potential.

Keywords: Nootropic, *Bacopa monnieri*, saponins, phenolics, flavonoids, TLC

INTRODUCTION

Neurological disorders have taken the world with stride and are one of the major reasons for reducing the economic growth of any country. According to a WHO report, the neurological disorders constituted around 6.3% of the global burden of disease in 2005 and is expected to increase to 12% by 2030 with Alzheimer and other dementias, projected to show a 66% increase from 2005 to 2030[1]. The existing medications have adverse side effect and they do not modify the disease progression in long run[2]. Plant derivatives in herbs, medicinal plant extracts and other such botanicals have been used by population for their documented therapeutic potentials in neurological disorders[3,4]. *Bacopa monnieri* (family: Scrophulariaceae) is one such medicinal herb that has been mentioned in ancient Ayurvedic treaties (An ancient Indian Literature) as a nootropic plant. It has been used by Ayurvedic medical practioners for almost 3000 years and is reported to play a pivotal role in alleviating symptoms of neurological disorder[5]. Moreover, the extract has also been reported for anti-inflammatory, antipyretic, antidepressant, antimicrobial, anticancer and antioxidant properties[5-10]. These therapeutic properties of the plant are attributed to the presence of different phytocompounds with saponins being the most important, due to its significant role in neuroprotection by modulation of antioxidant enzymes (namely superoxide dismutase and catalase) in stressed neuronal cells[11]. Previous studies have also shown the variation in bacosides content in the cultivated plant with change in time (annual analysis), seasons, and different growth stages and in different plant part[12,13]. Many different Ayurvedic formulations of *Bacopa monnieri* are available, however one major concern is lack of well defined standard identification tests and their documentation in pharmacopoeias and monographs[14]. Current study evaluates and compares the phytoconstitutents of *Bacopa monnieri* formulations procured from different manufacturers in India. The study will provide an impetus on the need of standardization methods that may play a pivotal role in overcoming variations in the therapeutic potential of commercially available *Bacopa monnieri* formulations.

MATERIALS AND METHODS

Ayurvedic formulations

Five ayurvedic formulations of *Bacopa monnieri*, manufactured in India, were used for this study (Table 1).

Chemicals

All chemicals used were of analytical grade, unless specified. Bacoside A, bacoside I, bacosides A_3, bacopasaponin C, isomer of bacopasaponin C, bacoside II, bacosine and luteolin were purchased from Natural Remedies Pvt Ltd Bangalore (India). 1.1- Diphenyl, 2 picrylhydrazyl (DPPH) and vanillin were purchased from Himedia Laboratories Pvt Ltd (India). HPLC grade methanol, acetonitrile, glacial acetic acid, n-butanol and ethyl acetate were purchased from Qualigen (India).

Preparation of Extracts

Extracts were prepared using two different method *viz*

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Table 1: Details of herbal formulations from different manufactures

S. No	Name of Herbal formulation	Code	Batch No.	Manufacture date	Company	Net content of *Bacopa monnieri*	Saponin/Bacoside Content
1	Brahmi (powder)	HF-1	015	August-2013	Sri Jain Ayurvedic Pharmacy, Hyderabad, India	Plant powder	No information
2	Brahmi (Tablet)	HF-2	0915	September-2015	Nirogrm India Pvt,Ltd., Haryana, India.	375mg	No information
3	Brahmi (Capsule)	HF-3	22200230G	July-2012	Himalya Drug Company, Bangalore, India.	250mg	No information
4	Brahmihills (Capsule)	HF-4	AC131	September-2015	Isha Agro Developers Pvt. Ltd., Maharashtra, India.	300mg	Saponin- 27% Bacosides-25%
5	Brahmivit (Capsule)	HF-5	WF15179	September-2015	WEST-COST Pharmaceutical Works Ltd., Ahemdabad, Gujrat, India.	250mg	No information

Table 2: Phytochemical analysis of HF-1 herbal formulation extracted using conventional solvent extraction method (CSE)*

S. No.	Solvent	Extraction Yield (%) ((mg dried extract/mg dried formulation) × 100)	TPC** (mg Gallic acid equivalent/g Formulation)	TFC*** (µg Luteolin equivalent/mg formulation)	Saponin (mg Bacoside A equivalent/g formulation)
1.	Ethyl acetate	4.76±0.77	0.81±0.02	0.52±0.05	13.10±0.02
2.	Hexane	1.92±0.82	0.26±0.01	0.42±0.02	2.41±0.01
3.	Methanol	2.84±0.62	1.91±0.01	0.46±0.02	8.48±0.03
4.	Water	1.61±0.41	1.71±0.01	2.41±0.01	2.58±0.01
5.	Acetonitrile	3.38±0.25	0.92±0.01	2.58±0.01	6.51±0.01

*CSE- Conventional solvent extraction
** TPC- Total phenolic content
*** TFC- Total flavonoid content

conventional solvent extractions (CSE) and maceration, as reported previously, with some modification[15,16]. All initial studies on optimization of extraction procedures were performed using one of the powdered formulation, HF-1. For conventional solvent extraction (CSE) method, 0.2g of HF-1 powder was extracted in 20 ml different solvents, *viz.* methanol, water, acetonitrile, ethyl acetate, hexane. Suspensions were incubated at room temperature (25± 1 °C) for 20 min followed by incubated at 50 °C for 60 min. The suspensions were rapidly cooled in ice bath followed by centrifugation at 10000 rpm for 15 min at room temperature (25± 1 °C). Supernatants were filtered using 0.45 µm PVDF / PTFE membranes and allowed to dry at room temperature. Crude dried extracts were weighted and dissolved in methanol. All stock solutions were stored in cryovials and kept at -20 ºC till further analysis. For maceration, 1 gm HF-1 powder was macerated with 10 ml of different solvents (methanol, 50% (v/v) aqueous methanol, ethanol, 50% aqueous ethanol, acetone and 50% (v/v) aqueous acetone) for 24 h at room temperature (25 ºC ± 1). The suspension was centrifuged at 10000rpm for 15 min. Supernatants were filtered using 0.45µm PVDF / PTFE membranes and allowed to dry at room temperature. Crude dried extract were weighted, dissolved in methanol. All stock solutions were stored in cryovials and kept at -20 ºC till further analysis.

Determination of phytochemicals compounds in formulations

Determination of Total phenolic content (TPC)

Total phenolic content was evaluated using Folin Ciocalteau method as described previously with some modifications [17]. For preparation of standard plot, 25 µl varying concentration of gallic acid (0.20, 0.40, 0.60, 0.80, 1.0 mg / ml) were mixed with 1.25 ml Folin Ciocalteu reagent (0.2 N) and 1ml 7.5% (w/v) sodium

Table 3: Phytochemical estimation of HF -1 herbal formulation extracted using maceration method

S. No.	Solvent	Extraction Yield (%) ((mg dried extract/ g dried formulation)×100)	TPC* (mg gallic acid equivalent/g Formulation)	TFC** (µg Luteolin equivalent/mg formulation)	Saponin (mg Bacoside A equivalent/g formulation)
1.	Methanol	3.77±0.09	3.29± 0.08	1.12±0.10	22.31±0.07
2.	Methanol 50% (v/v) aqueous	2.78±0.30	2.58±0.064	0.21±0.02	4.67±0.04
3.	Ethanol	2.86±0.16	1.96±0.02	0.47±0.01	7.78±0.04
4.	Ethanol 50% (v/v) aqueous	4.34±0.23	3.18±0.009	0.25±0.01	2.07±0.06
5.	Acetone	2.66±0.36	1.85±0.05	0.56±0.01	7.16±0.10
6.	Acetone 50 % (v/v) Aqueous	2.79±0.37	4.29±0.05	0.65±0.03	6.45±0.08

* TPC- Total phenolic content
** TFC- Total flavonoids content

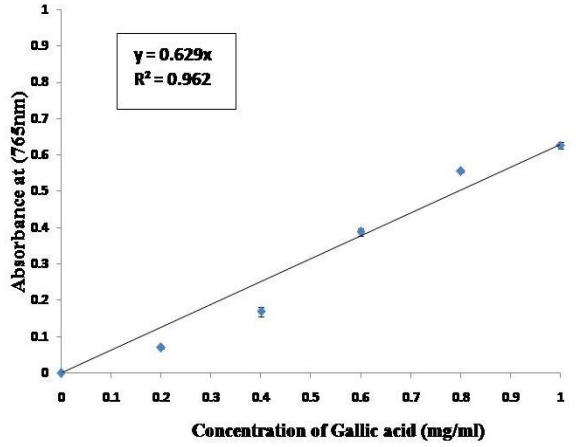

Figure 1: Calibration curve of Gallic acid for estimation of Total Phenolic Content

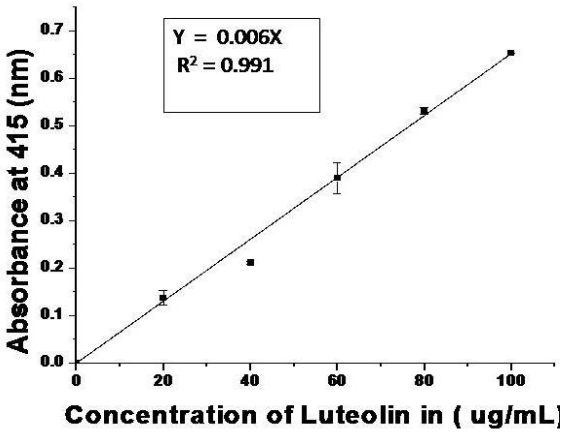

Figure 2: Calibration curve of Luteolin for estimation of total flavonoids content

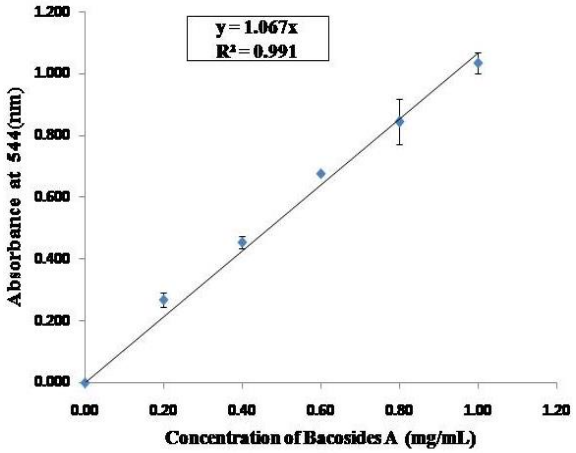

Figure 3: Calibration graph of Bacoside A

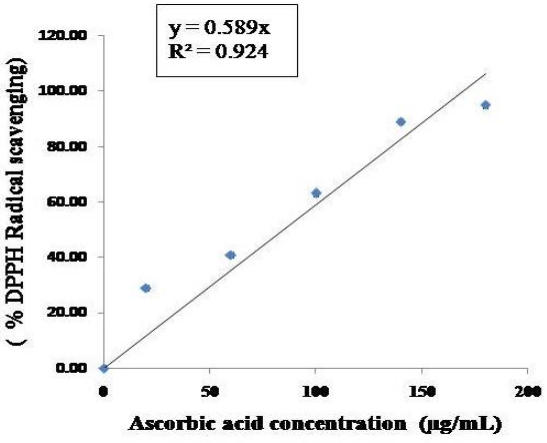

Figure 4: DPPH radical scavenging capacity of ascorbic acid

carbonate. The solution was incubated for 3 h at room temperature. Absorbance was measured at 765nm and calibration curve was plotted. TPC of herbal formulation extracts were analyzed using similar procedure and results were expressed as mg gallic acid equivalent (GAE) /g herbal formulation.

Determination of Total Flavonoid content (TFC)

Total flavonoid content was evaluated using aluminium chloride method, as described previously, with some

Table 4: Phytochemical estimation of different herbal formulations, methanolic extract Obtained by maceration method

S. No.	Herbal formulation	Extraction Yield ((%) (mg dried extract/mg dried formulation)×) 100)	TPC* (mg Gallic acid equivalent/g Formulation)	TFC **(µg Luteolin equivalent/mg formulation)	Saponin (mg Bacoside A equivalent/g formulation)
1.	HF-1	2.89	8.04±0.01	1.6±0.01	25.53±0.01
2.	HF-2	6.089	4.99±0.01	0.81±0.01	26.54±0.01
3.	HF-3	20.72	38.73±0.01	5.27±0.01	128.71±0.04
4.	HF-4	29.48	37.97±0.01	4.34±0.01	139.47±0.04
5.	HF-5	21.28	22.77±0.01	4.19±0.02	62.50±0.02

* TPC- Total phenolic content
** TFC- Total flavonoid content

Table 5: DPPH radical scavenging capacity of Bacosides standard

Bacosides	DPPH radical scavenging capacity (%)	Ascorbic acid equivalent µg/mg powder
Bacoside A	2± 0.01	1.13±0.01
Bacopasade I	2±0.01	1.13±0.01
Isomer of Bacopasaponin C	1±0.02	0.57±0.02
Bacoside II	1.13±0.01	0.64±0.01
Bacoside A3	1.0±0.03	0.58±0.03

Table 6: DPPH radical scavenging capacity of herbal formulations

Herbal formulation	DPPH (%) radical scavenging capacity	Ascorbic acid equivalent µg/mg Herbal formulations
HF-1	15.62±0.01	2.12 ± 0.01
HF-2	32.08±0.01	4.36 ±0.01
HF-3	80.79±0.03	10.97±0.03
HF-4	90.16±0.01	12.25±0.01
HF-5	84.05±0.01	11.42±0.01

Table 7: TLC analysis of Bacosides standards

S. No.	Bacosides standards	R_f value
1.	Bacosides A	0.61
2.	Bacosides II	0.61
3.	Bacoside A3	0.62
4.	Bacopasaponin C	0.58
5.	Isomer of bacopasaponin C	0.58
6.	Bacosides I	0.39
7.	Bacosine	0.96

modifications[18]. For preparation of standard plot, 50 µl varying concentration of luteolin (20, 40, 60, 80, 100 µg / ml) were mixed with 95% (v/v) ethanol, 10 µl 10% (w/v) aluminium chloride, 10 µl of 1M potassium acetate and 280 µl of distilled water. The solution was incubated for 30 min at room temperature. Absorbance was measured at 415 nm and calibration curve was plotted. TFC of herbal formulation was estimated using similar procedure and results were expressed as µg luteolin equivalent / mg herbal formulation.

Saponins content

Saponins content was evaluated using, vanillin – sulphuric acid method as described previously, with some modification[19]. For preparation of standard plot, 0.125 ml varying concentration of bacosides A mixture (0.2, 0.4, 0.6, 0.8, 1 mg / ml) was mixed with 0.125 ml 8% (w/v) vanillin (in ethanol) and 1.25ml 72% (v/v) sulphuric acid. The mixture was incubated at 60 ^{0}C for 10 min, and then cooled in ice bath. Absorbance was measured at 544 nm and calibration curve of concentration of bacoside A *versus* absorbance (544 nm) was plotted. Saponins content in herbal formulations were quantified using the same procedure. Results were expressed as mg bacosides A equivalent / g herbal formulation.

Extraction & analysis of phytocompounds in different herbal formulations

Based on saponins, total flavonoids content and total phenolics content, further extraction of all commercial *Bacopa monnieri* formulation were performed in methanol using maceration method. All formulations were analyzed for saponins, TFC and TPC. All stock solution were stored in cryovial and kept at -20 ºC till further analysis.

DPPH radical scavenging capacity (DRSC)

Extraction of all *Bacopa monnieri* formulations, for determination of radical scavenging activity, was performed with 25 mg formulation macerated with 25 ml methanol. The suspension was centrifuged and filtered as reported earlier (Material and Methods). Weighed amount of dried extracts were further solubilised in DMSO. DRSC of herbal formulation extracts were measured based on the method, with some modification[20]. The reaction mixture containing 950 µl of DPPH methanolic solution (absorbance at 517 nm of 0.9 ± 0.02) was mixed with 50 µl extract and the solution was incubated at 37 ºC for 30 min. The reduction in absorbance at 517 nm was estimated after incubation, using UV-Visible spectrophotometer. Percent radical scavenging activity of the extracts was determined by comparison with a methanol treated control (equation 1). All experiments were performed in triplicates. Ascorbic acid was used as positive control. Results were further expressed as µg ascorbic acid equivalents / mg herbal formulation. In addition, the DPPH activities of different bacosides standards (3mg/ml in methanol) were also compared.

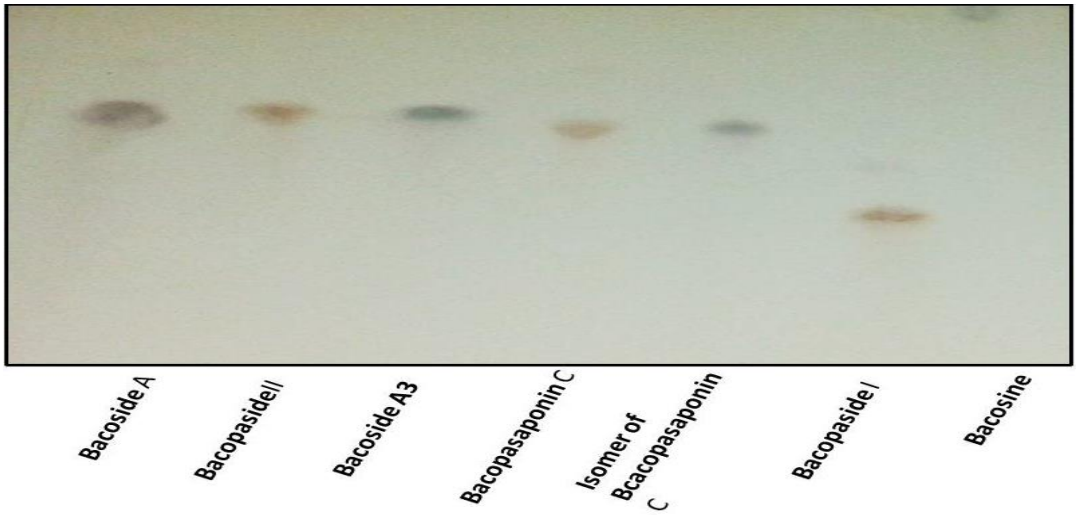

Figure 5: TLC analysis of Bacosides Standards

$$\text{DPPH Radical scavenging (\%)} = \left(\frac{A_c - A_s}{A_c}\right) \times 100$$

where A_c is the absorbance of control and A_s is the absorbance of antioxidant of crude extract and standards.

Thin Layer Chromatography

Saponin being the major therapeutically important constituent of *Bacopa monnieri* was analyzed using TLC based on method previously reported, with some modification[14]. Methanolic bacoside A mixture solutions (bacoside II, bacoside A3, bacopasaponin C, isomer of bacopasaponin C), baccoside I and bacosine used as a standard for all further analysis. Briefly 10 µl standard solutions and methanolic extract of herbal formulations were loaded on precoated TLC silica gel GFR $_{254}$ plates. The bands were developed under chamber saturation condition (saturation for 30 min). The plates were air dried for 30 min, derivatized with vanillin – sulphuric acid (Godin reagent) solution and heated at ambient temperature for 15 min in hot air oven, gives a blue or purple spots of triterpene saponin. R_f value of bacosides standard and herbal formulation was calculated using equation 2:

$$R_f = \frac{\text{Distance travelelled by sample}}{\text{Distance travelled by mobile phase}}$$

RESULTS AND DISCUSSION

Bacopa monnieri, a well documented nootropic herb is widely distributed in the warmer regions of the world including southern states of USA and South Asian subcontinent[5]. Previous studies on germplasms, collected from different regions of India, have shown low level of genetic diversity, which may be attributed to vegetative propagation of the plant species[21,22]. Morphological and phytochemicals analysis of the field grown *Bacopa monnieri* has shown variations in different seasons, plant parts and growth stages, and this may be due to variations in environmental parameters[13]. Phytochemicals variations are also evident in the formulations used in this study. Comparative evaluation of standard formulations, manufactured in India, has been scarcely reported. Studies performed by Saini et al, (2012) emphasize the variation in bacosides among different batches of the same manufacturers[23]. Current study differs from previous existing studies in highlighting the variations in type of bacosides and antioxidant potential of five different formulations, manufactured in India.

Extraction and analysis of phytochemicals

In this study, phytochemicals extraction from one of the herbal formulation HF-1, used as control, has been optimized using two different methods, *viz.* conventional solvent extraction method and maceration method. Results showed variation in the amount of dried extracts and phytochemicals in different extracts (Table 2 & 3). Variations in the results may be due to variation in the solubility of phytochemicals in different solvents and on the choice of extraction methods[15,16]. While conventional extraction method showed comparatively higher yield of extract with ethyl acetate (4.76±0.77 % w/w), the yield of dried extract by maceration was comparatively higher with 50% (v/v) ethanol (4.34±0.23 % w/w). However, comparison of cumulative total phenolic content (TPC), total flavonoid content (TFC) and saponins were highest in methanol when extracted using maceration method. This may be attributed to solvent properties and solubility of phytochemicals. Previous studies have shown that the polar, protic solvents extracts significantly greater amount of phytochemicals then aprotic and nonpolar solvents[15]. Better efficiency of methanol as a solvent may be related to its intermediate polarity.

Total phenolic content (TPC)

Existing studies suggest that often phenolic content of plant material can be related to their antioxidant activity[24]. In present study, methanolic extracts of all different herbal formulations were evaluated for their total phenolic content (TPC), based on calibration curve, prepared using Gallic acid (GAE) as standard, with regression equitation, Y = 0.629 x (R^2= 0.962) (Fig.1). Results were expressed as 'mg GAE equivalent /g dried herbal formulations'. Comparative analysis showed that TPC was highest in the methanolic extract of HF- 3 and decreased in the following order: HF-3> HF-4> HF-5> HF-1> HF-2 (Table-4). Variations of TPC in different

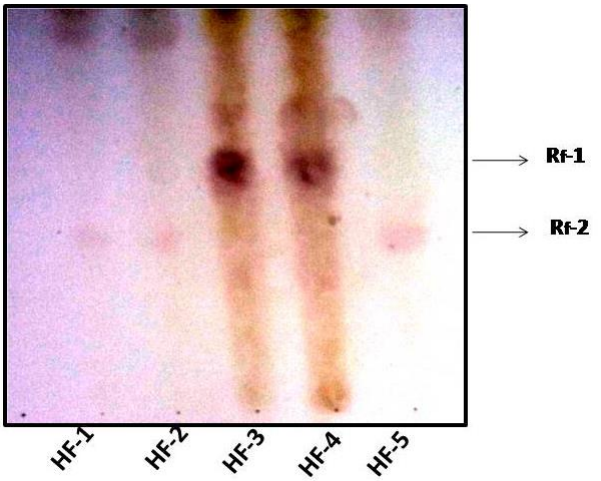

Figure 6: TLC analysis of herbal formulations

Table 8: TLC analysis of saponins in different herbal formulations

S. No.	Herbal formulations	R_f -1	R_f -2
1.	HF-1	ND*	0.38
2.	HF-2	ND*	0.38
3.	HF-3	0.57	ND*
4.	HF-4	0.57	ND*
5.	HF-5	ND*	0.38*

* ND: Not detected

formulations may be largely attributed to various morphological, genetic and environmental parameters affecting plant cultivation.

Total flavonoids content (TFC)

Methanolic extracts of herbal formulation were compared for their total flavonoid content (TFC) from calibration curve, prepared using luteolin as standard, with regression equation Y= 0.006 x (R^2 = 0.991) (Fig.2). Results were expressed as µg Luteolin equivalent /mg of dried herbal formulations'. Like TPC analysis, comparative analysis showed that TFC was also highest in the methanolic extract of HF- 3 and decreased in the following order: HF-3> HF-4> HF-5> HF-1> HF-2 (Table-4).

Saponins Content

Vanillin and sulphuric acid method was used for estimation of total saponin content in different herbal formulation[19]. Methanolic extracts of different herbal formulation were compared for their total saponins content. Analysis was performed based on calibration curve, plotted using bacoside A mixture as standard with regression equation, Y= 1.067 x (R^2 =0.991) (Fig.3). Results were expressed as 'mg bacoside A equivalent / g dried herbal formulations. Unlike TPC and TFC, herbal formulation HF- 4 was found to contain the highest amount of saponin (bacoside A) and were found to decrease in the following order: HF-4> HF-3> HF-5> HF-2> HF-1. Results also indicated no correlation between TPC, TFC and saponins among different formulations. While the former two were comparatively higher in HF-3, saponins were higher in HF-4 (Table 4)

DPPH Radical scavenging capacity (DRSC)

DPPH radical scavenging activity is widely measured to evaluate the free radical scavenging ability of various samples. This method is based on the reduction of DPPH in the presence of a radical scavenger due to the formation of non radical form of DPPH-H[25]. In the following study, DRSC of different herbal formulations were compared using ascorbic acid as standard and calibration graph was plotted (Fig. 4). Results were expressed as µg ascorbic acid equivalent / mg dried herbal formulations (Table 5 & 6). Results indicate no significant DRSC of bacosides standards. Thus the significant role of bacosides in antioxidant potential of herbal formulation can be ruled out. Also, comparatively higher DRSC of herbal formulations suggest role of other phytochemicals that may differ in different source plants used in formulations.

Qualitative analysis of bacosides using Thin Layer Chromatography

Thin layer chromatographic analysis of all bacoside standards and methanolic herbal formulations was performed using n-Butanol: Acetic acid: water (36: 6: 8 v/v) as mobile phase. Results showed variations in the characteristics band patterns of saponins, among different herbal formulations. TLC of standards showed unresolved band of bacoside A mixture (R_f value = 0.61), bacoside II (R_f value 0.62), bacoside A_3, bacopasaponin C and isomer of bacopasaponin C (R_f value 0.58) while distinct band were observed for bacoside I (R_f value = 0.38) and bacosine (R_f value = 0.96) (Fig. 5 & table 7). Out of five herbal formulations, HF-3 and HF-4 showed characteristics band corresponding to standards bacoside A mixture, and bacoside I while HF-1, HF-2, and HF-5 showed bands corresponding to bacoside I only (Fig. 6 & table 8). Variations in TLC band profile among different herbal formulation may be attributed to the variations in phytochemicals among the source plants. Different parameters that may affect the yield of phytochemicals amongst plant species could be cultivation practices and conditions, environmental parameters, season and plant growth stage[26]. However, the role of extraction solvents and method in variations among phytochemcals in herbal formulations may not be ruled out.

CONCLUSION

Current study highlights the variation in phytochemicals and antioxidant potential of different herbal formulations of *Bacopa monnieri*. The plant is known to be a rich source of triterpenoid saponins which are extensively documented for its nootropic properties. However little is known about their variation among plant species. Current study highlighted variation in the phytochemicals among different herbal formulation. Also antioxidant potential differs among different formulations. Such results can be justified based on possible variations in plant cultivation practices. Also the role of extraction methods & solvents in affecting cumulative yield of phytochemicals cannot be ruled out. Results also highlight the need of standardization methods of formulation to improve the efficacy of many such Ayurvedic herbal formulations.

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