



ISSN No: 0975-7384
CODEN(USA): JCPRC5

J. Chem. Pharm. Res., 2010, 2(6):191-198

Pharmacological investigation of *Bacopa monnieri* on the basis of antioxidant, antimicrobial and anti-inflammatory properties

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ABSTRACT

Bacopa monniera, also referred to as *Bacopa monnieri*, *Herpestis monniera*, water hyssop, and “Brahmi,” has been used in the Ayurvedic system of medicine for centuries. The whole plant extracts of the plant were evaluated for antioxidant, antimicrobial and anti-inflammatory properties. The methanol extracts of the plant were found to have potent antioxidant, antimicrobial and anti-inflammatory properties. The aqueous extracts of the plant were found to have fewer activities in comparison to methanol extracts. Petroleum ether and hexane extracts showed negligible activities in comparison to the above extracts. These active crude methanol extracts were also assayed for cellular toxicity to fresh sheep erythrocytes and found to have no cellular toxicity.

Keywords: *Bacopa monnieri*, antimicrobial activity, antioxidant activity, anti-inflammatory activity, methanol extracts.

INTRODUCTION

Bacopa monniera, a member of the Scrophulariaceae family, is a small, creeping herb with numerous branches, small oblong leaves, and light purple flowers. In India and the tropics it grows naturally in wet soil, shallow water, and marshes. The herb can be found at elevations from sea level to altitudes of 4,400 feet, and is easily cultivated if adequate water is available.

Flowers and fruit appear in summer and the entire plant is used medicinally [1-3]. Compounds responsible for the pharmacological effects of Bacopa include alkaloids, saponins, and sterols. Many active constituents – the alkaloids Brahmine and herpestine, saponins d-mannitol and hersaponin, acid A, and monnierin – were isolated in India over 40 years ago. Other active constituents have since been identified, including betulic acid, stigmastanol, beta-sitosterol, as well as numerous bacosides and bacopasaponins. The constituents responsible for Bacopa's cognitive effects are bacosides [4, 5]. Wound healing property of Bacoside –A isolated from the plant was observed [6]. Free radical scavenging activity and protective effect on DNA damage of plant were observed [7]. Anti-inflammatory of plant extracts were observed in rodents [8]. *In vitro* antioxidant and phytochemical activities of methanolic extracts of the plants were studied [9]. Antimicrobial fractions of various aerial parts of the plant were studied [10].

EXPERIMENTAL SECTION

All the chemicals and reagents used were from C.D.H and Ranchem. Glass wares used were from Borosil. The media and broth used for microbial culture were from Hi-Media Pvt. Limited, Bombay, India.

Plant material

The authenticated sample was collected from Teen Dhara region of Srinagar region of Garhwal (U.K), India and was further confirmed in Botanical Survey of India (BSI), Dehradun. Voucher specimens have been deposited in BSI, Dehradun, India.

Preparation of plant extracts

The method [11] was adopted for preparation of plant extracts with little modifications. Briefly four 20 g portions of the powdered plant material were soaked separately in 100 ml of water, hexane, methanol and petroleum ether for 72 h. Each mixture was stirred after every 24 h using a sterile glass rod. At the end of extraction, each extract was passed through Whatman filter paper no1 (Whatman, England). The filtrate obtained were concentrated *in vacuo* using rotary evaporator at 30°C.

Determination of Antibacterial and Antifungal activity

Culture Media

The media used for antibacterial test was soyabean casein digest agar/broth and Sabouraud's dextrose agar/broth of Hi media Pvt. Bombay, India.

Inoculum

The bacteria were inoculated into soyabean casein digest agar\broth and incubated at 37⁰ C for 4 h and the suspension were checked to provide approximately 10⁵ CFU/ml. Similar procedure is done for fungal strains by inoculating in Sabouraud's dextrose broth for 6 h.

Microorganisms used

The test organisms (*Bacillus subtilis* ATCC6051, *Proteus vulgaris* ATCC 6380, *Salmonella typhimurium* ATCC 23564, *Pseudomonas aeruginosa* ATCC 25619, *Escherichia coli* K-12 and *Staphylococcus aureus* were the bacterial strains obtained from institute of Microbial Technology (IMTECH) Chandigarh, India. The fungal test organisms used for study are *Candida*

albicans, *Aspergillus niger*, *Saccharomyces cerevisiae* and *Penicillium notatum* obtained from pure lab cultures of Dept. of Microbiology, Sai Institute of Paramedical & Allied Sciences (SIPAS) Dehradun, India.

Determination of antibacterial and antifungal activity

The agar well diffusion method [12] was modified. Soyabean casein digest agar (SCDA) was used for bacterial cultures. The culture medium was inoculated with the microorganism separately suspended in soyabean casein digest broth. Sabouraud's dextrose agar (SDA) was used for fungal cultures. The culture medium was inoculated with the fungal strains separately suspended in Sabouraud's dextrose broth. A total of 8 mm diameter wells were punched into the agar and filled with plant extracts and solvent blanks (distilled water, hexane, methanol and petroleum ether as the case may be). Standard antibiotic (Chloramphenicol, concentration 1mg/ml) was simultaneously used as positive control. The bacterial plates were then incubated at 37°C for 18 h. The antibacterial activity was evaluated by measuring the diameter of zone of inhibition observed. The same procedure was done for determining antifungal activity but in this case standard antibiotic (Fucanazole, concentration 1 mg/ml) was used as positive control and fungal plates were incubated at 37°C for 72 h. Here also the diameter of zone of inhibition observed was measured.

Determination of MIC and MBC

The antibacterial and antifungal plant extracts were then after evaluated to determine MIC and MBC values. The serial dilution technique by using N-saline for diluting the plant extract was adopted and serially diluted plant extract tubes were incubated for 48 h. The minimum dilution of the plant extract that kills the bacterial and fungal growth was taken as MLC (Minimum lethal count) while the minimum dilution of plant extract that inhibits the growth of the organism was taken as MIC.

Determination of cellular toxicity using sheep erythrocytes

The method [13] was employed to study cellular toxicity. Briefly 10 fold serial dilution of the extract were made in phosphate buffered saline. A total volume of 0.8ml for each dilution was placed in an ependroff tube. A negative control tube (containing saline only) and a positive control tube (containing tap water) were also included in the analysis. Fresh sheep erythrocytes were added to each tube, to give a final volume of 1 ml. Solutions were incubated at 37°C for 30 minutes and all tubes were centrifuged for 5 minutes and then observed for hemolysis.

Determination of *in vitro* antioxidant activity

Determination of Antioxidant Activity by DPPH Radical Scavenging Method

The extract solution for the DPPH test [14] was prepared by re-dissolving 0.2 g of each dried extract in 10 ml methanol. The concentration of DPPH solution was 0.025 g in 1000 ml of methanol. Two ml of the DPPH solution was mixed with 10, 20 and 40 µl of the plant extract/methanol solution and transferred to a cuvette. The reaction solution was monitored at 515 nm, after an incubation period of 30 minutes at room temperature, using a UV-Visible Systronics spectrophotometer.

The inhibition percentage of the absorbance of DPPH solution was calculated using the following equation:

Inhibition% = (Abst=0 min - Abst=30 min) / Abst=0 min × 100

Where Abst=0 min was the absorbance of DPPH at zero time and Abst=30 min was the absorbance of DPPH after 30 minutes of incubation. Ascorbic acid (0.5 mM) was dissolved in methanol and used as a standard to convert the inhibition capability of plant extract solution to the Ascorbic acid equivalent. IC₅₀ is the concentration of the sample required to scavenge 50% of DPPH free radicals.

Determination of Superoxide Anion Radical Scavenging Activity

Superoxide Anion Radical scavenging Activity was measured [15] with some modifications. The various fractions of plants extracts were mixed with 3 ml of reaction buffer solution (pH 7.4) containing 1.3 μM riboflavin, 0.02 M methionine and 5.1 μM NBT. The reaction solution was illuminated by exposure to 30W fluorescent lamps for 20 minutes and the absorbance was measured at 560 nm using Systronics UV-VIS spectrophotometer. Ascorbic acid (0.5 mM) was dissolved in methanol and used as a standard to convert the inhibition capability of plant extract solution to the Ascorbic acid equivalent. The reaction mixture without any sample was used as negative control.

The Superoxide anion radical scavenging activity (%) was calculated as:

$$\frac{A_o - A_s}{A_o} \times 100$$

Where, A_o = absorbance of positive control

A_s = absorbance of sample

Determination of *in vivo* anti-inflammatory activity

Animals

Male albino rats (180–200 g) were used taking into account international principles and local regulations concerning the care and use of laboratory animals [16]. The animals had free access to a standard commercial diet and water *ad libitum* and were kept in rooms maintained at 22 ± 1°C with a 12-h light/dark cycle. The institutional animal ethical committee has approved the protocol of the study.

Carrageenan-induced edema in rats

6 Groups of five animals each were used. Paw swelling was induced by sub-plantar injection of 0.1 ml 1% sterile carrageenan in saline into the right hind paw. The solvent extracts of *B. monnieri* at dose of 100, 200 and 400 mg/kg were administered orally 60 minutes before carrageenan injection. Aspirin (10 mg/kg) was used as reference drug. Control group received the vehicle only (10 ml/kg). The inflammation was quantified by measuring the volume displaced by the paw, using a plethysmometer at time 0, 1, 2, 3, and 4 h after carrageenan injection. The difference between the left and the right paw volumes (indicating the degree of inflammation) was determined and the percent inhibition of edema was calculated in comparison to the control animals.

Statistical analysis

The results were expressed as mean \pm S.D. Statistical significance was determined by analysis of variance and subsequently followed by Turkey's tests. P values less than 0.05 were considered as indicative of significance. The analysis was performed using INSTAT statistical software.

RESULTS AND DISCUSSION**Determination of antimicrobial activity**

The antibacterial activity was determined by measuring the diameter of zone of inhibition recorded. Methanol extracts were found to be most potent antimicrobial agent in comparison to other extracts. Aqueous extracts showed no activity against any of the microorganisms. Hexane and petroleum ether extracts showed similar antimicrobial activity but less significant in comparison to methanol extracts. The results are in accordance with the previous studies done on this aspect. The MIC of the methanol extracts was found to be lowest i.e 0.039 mg/ml for *E.coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Saccharomyces cerevisiae*. The experiments were performed in triplicates and the mean of diameter of zone of inhibition were observed. The results are reported in **Figure 1** and **Table 1**.

Table 1: Determination of Antibacterial activity by well-diffusion method

Microorganisms	Diameter of zone of inhibition (mm)				Chloramphenicol (1mg/ml)	Fucanazole (1mg/ml)	M	W	P	H
	M	W	P	H						
<i>E.coli</i>	25	NA	16	07	28	---	NA	NA	NA	NA
<i>Salmonella typhimurium</i>	26	NA	17	06	27	---	NA	NA	NA	NA
<i>Staphylococcus aureus</i>	25	NA	07	05	28	----	NA	NA	NA	NA
<i>Bacillus subtilis</i>	18	NA	08	07	30	----	NA	NA	NA	NA
<i>Proteus vulgaris</i>	17	NA	16	08	25	----	NA	NA	NA	NA
<i>Pseudomonas aeruginosa</i>	16	NA	07	07	27	----	NA	NA	NA	NA
<i>Candida albicans</i>	18	NA	08	06	---	28	NA	NA	NA	NA
<i>Saccharomyces cerevisiae</i>	25	NA	19	07	---	27	NA	NA	NA	NA
<i>Aspergillus niger</i>	16	NA	18	08	----	29	NA	NA	NA	NA
<i>Penicillium notatum</i>	17	NA	18	09	----	28	NA	NA	NA	NA

M, Methanol; W, Aqueous; P, Petroleum ether; H, Hexane; NA, No Activity.

Determination of Antioxidant Activity by DPPH Radical Scavenging Method

DPPH Radical scavenging activity was determined of each of the fractions of the plants extracts. The experiments were performed in triplicates and mean values of Antioxidant activity of each of the fraction of the plant extract were determined. The four extracts of *Bacopa monnieri* (whole plant) tested for antioxidant activity using DPPH radical scavenging was determined, the methanol and aqueous successive extracts showed the maximum antioxidant activity with IC₅₀ values of 46.00 μ g/ml and 43.10 μ g/ml, respectively. The petroleum ether and hexane extracts also showed antioxidant activity with IC₅₀ values of 52.18 and 50.07 μ g/ml. The known antioxidant ascorbic acid exhibited IC₅₀ value of 78.17 μ g/ml as shown in **Figure 2** and **Table 2**.

Figure 1: Antimicrobial activity of plant extracts of *Bacopa monnieri*

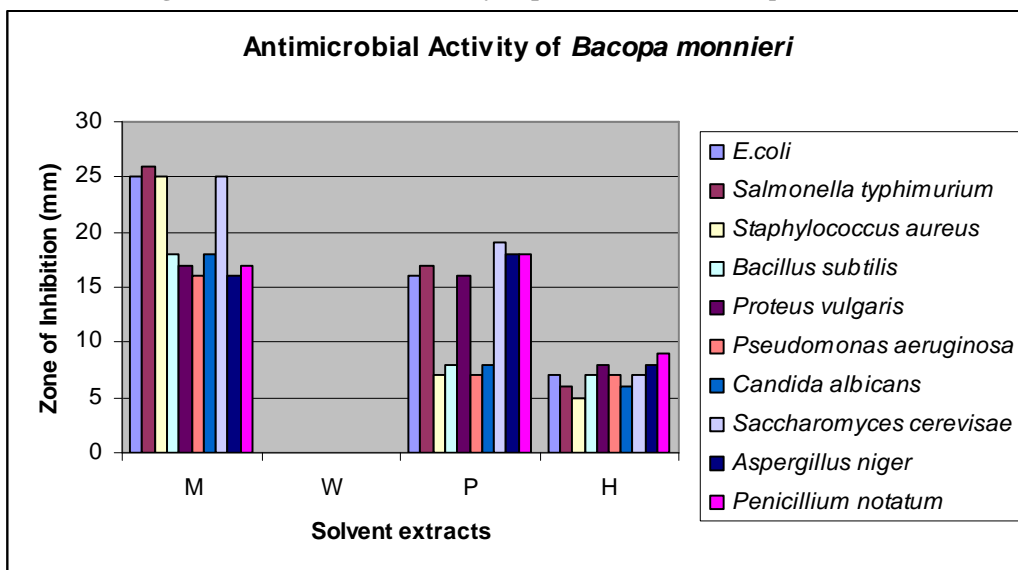
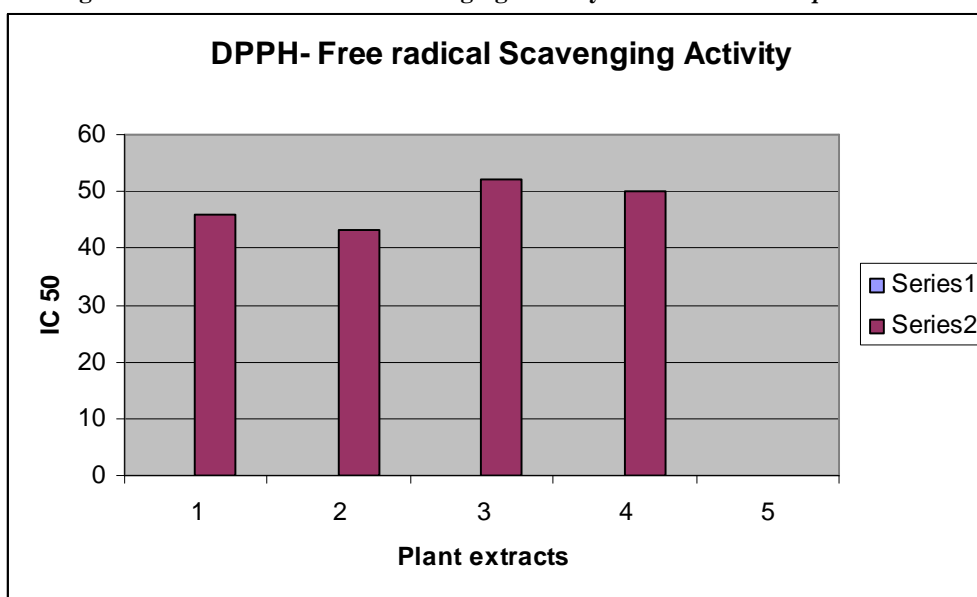


Figure 2: DPPH Free radical Scavenging Activity of extracts of *Bacopa monnieri*



1, Methanol extracts; 2, Aqueous extracts; 3, Petroleum ether extracts; 4, Hexane extracts

Table 2: Antioxidant Activity by DPPH Radical Scavenging Method

Plant	DPPH-Radical Scavenging Method (IC50); $\mu\text{g/ml}$			
	M	W	P	H
<i>Bacopa monnieri</i>	46.00	43.10	52.18	50.07

Ascorbic acid = 78.17 $\mu\text{g/ml}$

The results are the average of three determinations for extracts.

Determination of Antioxidant activity by Superoxide Anion Radical Scavenging Activity

Superoxide anion radical scavenging was determined of each of the fractions of the plant extracts. The experiments were performed in triplicates and mean values of antioxidant activity of each of the fraction of the plant extract were determined. The values of antioxidant activity determination by Superoxide Anion Radical Scavenging method follow the same order as that of DPPH assay.

Among the four extracts of *Bacopa monnieri* (whole plant) tested for antioxidant activity using Superoxide Anion radical scavenging method, the methanol and aqueous successive extracts showed the maximum antioxidant activity with 65.68 % and 62.34 % inhibition. The petroleum ether and hexane extracts also showed antioxidant activity with 56.67% and 54.18% inhibition. The known antioxidant ascorbic acid exhibited 87.8% inhibition as shown in **Table 3**.

Table 3 Antioxidant Activity by Superoxide Anion Radical Scavenging Method

Plant	Superoxide Anion Radical Scavenging Method (%)			
	M	W	P	H
<i>Bacopa monnieri</i>	65.68	62.34	56.67	54.18

Ascorbic acid = 87.80%

The results are the average of three determinations for each of the extracts.

Determination of Anti-inflammatory activity**Carrageenan-induced edema in rats**

The anti-inflammatory effects of the solvent extracts of *Bacopa monnieri* on carrageenan-induced edema in rat's hind paws are presented in **Table 4**. The anti-inflammatory activities of extracts were found to have effect in dose-dependent manner. There was a gradual increase in edema paw volume of rats in the control group. However, in the test groups, methanol extract and aqueous fractions (100 mg/kg) showed a significant reduction in the edema paw volume. There was no reduction in inflammation found in case of rats treated with petroleum ether and hexane extracts.

Table 4: Anti-inflammatory activities of different extracts of *Bacopa monnieri*

Paw volume (ml) \pm SD

Experiment	Control	Aspirin (25mg/kg orally)	Methanol extract (100 mg/kg)	Aqueous extract (100mg/kg)	Petroleum ether (100mg/kg)	Hexane (100mg/kg)
1h after treatment	0.25 \pm 0.003	0.21 \pm 0.003	0.23 \pm 0.003	0.28 \pm 0.003	0.20 \pm 0.003	0.34 \pm 0.003
2h after treatment	0.25 \pm 0.003	0.18 \pm 0.003	0.20 \pm 0.003	0.24 \pm 0.003	0.15 \pm 0.003	0.34 \pm 0.003
4h after treatment	0.25 \pm 0.003	0.16 \pm 0.002	0.10 \pm 0.002	0.14 \pm 0.002	0.30 \pm 0.002	0.34 \pm 0.002

\pm , S.D, Standard Deviation

The results showed that methanol fractions of the leaves causes significant reduction in inflammation i.e 92 % (100 mg/kg) followed by crude aqueous extract i.e 85 % (100 mg/kg) compared to standard anti-inflammatory drug aspirin i.e 68.62% (25 mg/kg). The values of reduction in paw volume, 0.10 \pm 0.002, 0.14 \pm 0.002 and 0.16 \pm 0.002 were found significantly of methanol extract, aqueous extract and aspirin, respectively at 4 h after carrageenan administration. The present study provides evidence that the methanol fraction and aqueous

extract of *Bacopa monnieri* acts as potent anti-inflammatory agent in rats in acute inflammation model. Further studies are needed to isolate and identify some active compounds which might be responsible for potent antimicrobial, antioxidant and anti-inflammatory activity.

Acknowledgement

Thanks for the cooperation to research staff of National Institute of Malaria Research, Hardwar (U.K) for carrying out the research. Sincere thanks for the cooperation to Mr. Himanshu Gupta, Mr. S.N Uniyal for the technical support.

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