Phytochemical and Biological Investigations of *Bacopa monnieri* L.

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(Received: April 4, 2023; Accepted: June 15, 2023; Published (web): July 7, 2023)

ABSTRACT: *Bacopa monnieri* L. (Family: Scrophulariaceae), a medicinal herb of Bangladesh, has been used in traditional medicine to increase memory and brain power. The present research was aimed to investigate the phytochemical composition and biological activities of whole plant of *B. monnieri*. Through phytochemical analysis, two triterpenes, taraxerone and betulinic acid were obtained from *B. monnieri*. The crude methanol extract of the whole plant of *Bacopa monnieri* and its petroleum ether, dichloromethane, chloroform and aqueous fractions were screened for determination of several *in vitro* biological activities. In DPPH assay for antioxidant test, the dichloromethane fraction showed strong DPPH radical quenching activity (IC_{50} = 3.78 µg/ml). The chloroform fraction exhibited the maximum lethal concentration, LC_{50} value of 5.5 µg/ml against brine shrimp. In membrane stabilization assay, the crude extract and all solvent fractions revealed significant anti-inflammatory activity by inhibiting hemolysis of RBC induced by both hypotonic solution and heat. In thrombolytic assay, the petroleum ether fraction demonstrated highest thrombolytic activity (39.24%), when compared to the standard streptokinase (64.22%). In antimicrobial screening, the chloroform fraction displayed reasonable activity against most of the tested microorganisms. This study concludes that *B. monnieri* has potential antioxidant, anti-inflammatory and thrombolytic activities.

Key words: *Bacopa monnieri*, triterpene, antioxidant, cytotoxic, anti-inflammatory, thrombolytic, antimicrobial.

INTRODUCTION

Natural products have been documented as prominent sources of many important therapeutic agents for thousands of years. Based on their traditional uses, an inspiring number of clinically used pharmaceuticals including quinine, morphine, paclitaxel, vincristine, artemisinin etc. were derived from plant sources.[1,2] Recently, there has been rising interest in the chemical and biological properties of remedial plants so as to find out the bioactive lead molecules.[3,4] Consequently, in extension of the pharmacologic study on medicinal plants in Bangladesh[5-7] we have selected a traditionally important medicinal plant, *Bacopa monnieri* L., a memory enhancer drug of ayurveda[8,9] for phytochemical and biological investigations using different published methods.

B. monnieri (also known as Brahmi or water hyssop) is a nootropic herb which generally grows as weed in dump area and the rice fields. *B. monnieri* is familiar as a brain tonic to boost up memory development. It has been traditionally used in ayurvedic medicine for fever, inflammation, pain, asthma, epilepsy and cognitive dysfunction.[10,11] The plant is also reported to reduce dementia by free radical scavenging and concentration-dependent acetylcholine potentiation.[12] *B. monnieri* also demonstrates hepatoprotective and neuroprotective activities. The plant is used as a dietary antioxidant that can protect the brain against oxidative damage.[13] Phytochemical studies revealed the occurrence of

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Dhaka Univ. J. Pharm. Sci. **22**(2): 155-161, 2023 (December)
DOI: https://doi.org/10.3329/dujps.v22i2.67407

tetracyclic triterpenoid saponins, bacoside A and B, hersaponin, alkaloids and flavonoids.[14,15] Bacoside A and B are the main constituents accountable for learning and memory enhnacement.[16] The aim of this research was to explore *B. monnieri* for evaluation of its antioxidant, cytotoxic, anti-inflammatory, thrombolytic and antimicrobial activities. Attempts have been taken to isolate the pure molecules that may have some medicinal effect.

MATERIALS AND METHODS

Plant materials. Whole plant of *B. monnieri* was collected from Jashore in 2020. The plant sample was identified (Accession number: DACB-43597) in Bangladesh National Herbarium, Dhaka. Then the sample was sun dried and ground to a coarse powder. About 500 g of powder was macerated in methanol for several days. The crude extract of *B. monnieri* was then recovered by sieving the whole mixture through cotton plug and Whatman filter paper (No. 1). The filtrate thus obtained was subjected to a rotary evaporator to get a gummy mass of crude extract, designated as the methanol extract of *B. monnieri* (MEBM). The extract (5 g) was subjected for modified Kupchan partitioning[17] process to yield petroleum ether (PEF), dichloromethane (DMF), chloroform (CF) and aqueous (AQF) fractions.

An aliquot of the petroleum ether fraction (650 mg) was fractionated by column chromatography over silica gel. Total 110 test tubes were collected. The mixture of fraction from 45 to 80 was subjected to gel permeation chromatography over Sephadex (LH-20) using *n*-hexane: dichloromethane: methanol (2:5:1) solvent system. Preparative TLC of column fractions 14 and 15 gave compound **1** and **2**, respectively.

Taraxerone **(1):** ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.6 (1H, broad doublet, *J* = 6.0 Hz, H-15), 2.4 (*m*, H-2a), 2.25 (*m*, H-2b), 1.15 (3H, *s*), 1.13 (3H, *s*), 1.08 (3H, *s*), 1.03 (3H, *s*), 0.99 (3H, *s*), 0.97 (3H, *s*), 0.93 (3H, *s*), 0.84 (3H, *s*).

Betulinic acid (**2**): ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.71 (1H, br. *s*, H_b-29), 4.63 (1H, br. *s*, H_a-29), 3.27 (*m*, H-3), 2.99 (*m*, H-19), 1.72 (3H, *s*, H-30), 0.98, 0.94, 0.88, 0.80, 0.75 (5*s*, all tertiary -CH_3).

Antioxidant activity. Antioxidant activity was measured by DPPH assay method.[18] Briefly, DPPH solution (20 µg/ml) in methanol was mixed with test samples of *B. monnieri* dissolved in methanol. After 20 min incubation, the absorbance was measured at 517 nm. Here, tert-butyl-1-hydroxytoluene (BHT) and ascorbic acid (AA) were used as reference standards.

Cytotoxic activity. Brine shrimps (*Artemia salina* Leach) were used as test organisms to determine the cytotoxic effect of test materials.[19] Dimethyl sulfoxide (DMSO) was used to prepare target concentration of the test sample. Plant extracts were transferred to the vials containing nauplii in simulated sea water. The vials were scrutinized after 24 h and the quantity of viable nauplii was calculated. The percent of lethality of the nauplii for individual sample was calculated.

Membrane stabilizing activity. Membrane stabilization properties of plant samples were studied with heat-induced and hypotonicity-induced hemolysis assays.[20]

Thrombolytic activity. Thrombolytic assay of the extract was conducted following a conventional method[21] where streptokinase, SK (30,000 I.U.) and purified water were served as positive and negative thrombolytic control, respectively.

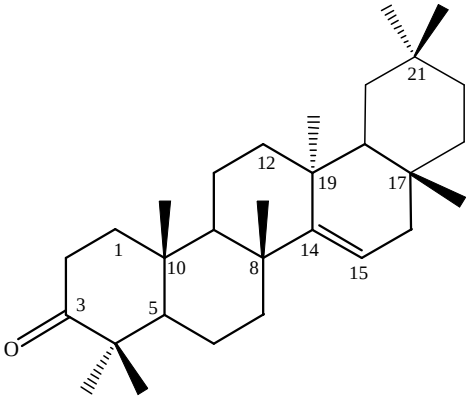
Antimicrobial activity. Antimicrobial activity was determined by disc diffusion method[22] using ciprofloxacin (30 µg) and ketoconazole (30 µg) as the control agents.

RESULTS AND DISCUSSION

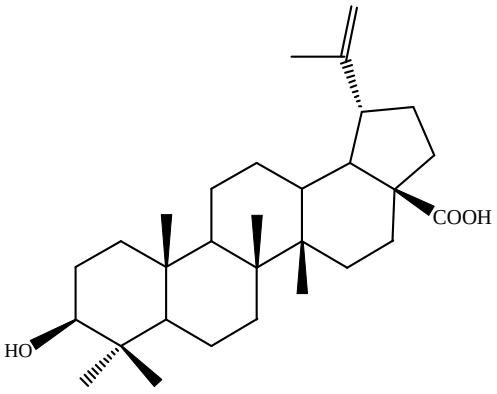
Two pentacyclic triterpenoids, taraxerone and betulinic acid were isolated from *B. monnieri* by chromatographic techniques. Their chemical structures were established by analysis of NMR spectral data and comparison with reported values.

The ^{1}H NMR spectrum of compound **1** exhibited a broad doublet of an olefinic proton at δ 5.6 (1H, broad doublet, *J* = 6.0 Hz, H-15) and two multiplets

at 2.4 and 2.25 due to methylene protons of H-2a and H-2b, respectively of a pentacyclic triterpene skeleton. The spectrum also showed signals for eight tertiary methyl groups at δ_H 1.15, 1.13, 1.08, 1.03, 0.99, 0.97, 0.93 and 0.84. The absence of oxymethine proton at C-3 suggested that C-3 was a ketonic functionality. Based on NMR data, compound **1** was established as taraxerone (Figure 1).[23]



Taraxerone (**1**)



Betulinic acid **(2)**

Figure 1. Compounds isolated from *B. monnieri*

The ^{1}H NMR spectrum of compound **2** displayed five signals at δ 0.98, 0.94, 0.88, 0.80, 0.75 for all tertiary methyl protons and a vinylic methyl proton signal at δ 1.72 (3H, s, H_3-30). The spectrum also showed two broad singlets at δ 4.63 and δ 4.71 which are assigned for two exomethylene protons. The appearance of two multiplet signals at δ 3.27 and 2.99 have been assigned to H-3 and H-19, respectively. Based on the published data[24], compound **2** was characterized as betulinic acid (Figure 1).

Polyphenolic compounds, also called plant secondary metabolites are well reported to ensure protective effects against oxidative stress which is linked to pathological conditions like cancer, atherosclerosis, diabetes, infections, inflammation and other chronic diseases. Different synthetic antioxidants are known to have carcinogenic effects in humans. Because of valuable effects on human health, the attention in natural antioxidant has significantly increased in current years.[25]

The antioxidant property of *B. monnieri* and the standards (BHT, ascorbic acid) was evaluated by DPPH assay and was presented by IC_{50} value (Half maximal inhibitory conc.). Here, the uppermost DPPH radical quenching potential was given by dichloromethane fraction (IC_{50} = 3.78 μg/ml) followed by the methanol extract (IC_{50} = 7.02 μg/ml), petroleum ether (IC_{50} =16.12 μg/ml), chloroform (IC_{50} = 25.53 μg/ml) and aqueous fraction (IC_{50} = 261.65 μg/ml) as compared to ascorbic acid (IC_{50} = 3.05 μg/ml) and BHT (IC_{50} = 21.20 μg/ml) (Table 1). Phenolic compounds show antioxidant properties because they are able to eliminate free radicals by donating hydrogen atom in biological systems.[25] Here, the tested samples of *B. monnieri* showed antioxidant potential by eliminating DPPH free radical. In a review article by Simpson *et al*.[13] *B. monnieri* was explained as a potential antioxidant which can lessen oxidative stress-induced change in brain, and thereby enhance the cognitive function.

The toxic action of the plant samples to brine shrimp was determined by standard protocol[26] and cytotoxicity was expressed as lethality concentration (LC_{50}). The crude extract and all the solvent fractions were found to possess significant cytotoxic principles. The LC_{50} were 5.5, 10.25 and 11.81 μg/ml for petroleum ether, chloroform and dichloromethane fraction, respectively (Table 1). Compared with the standard vincristine sulfate, VS (LC_{50} = 0.451 μg/ml), the lethal effect of the plant extractives may be due to the potent cytotoxic compounds present in *B. monnieri* which necessitates more investigation.

Several inflammatory diseases are becoming prevalent in elder people. Natural products are considered as substitute to the synthetic anti-inflammatory drugs. Thereby these natural compounds can serve as a basis in the development of bioactive lead compounds for treating inflammatory and other diseases.[27] Here, the anti-inflammatory potential of *B. monnieri* was determined by RBC membrane stabilization assay technique.[20] RBC membrane resembles to lysosomal membrane. Therefore, preserving the erythrocyte membrane integrity can be inferred to the stabilization of lysosomal membrane. Stressful conditions like heat, hypotonic solution, oxidant etc can damage the membrane integrity of RBC cell. When RBC is placed in hypotonic solution, excessive fluid is accumulated inside the RBC cell leading to hemolysis of RBC. So, the agents than can maintain the membrane integrity against stressful conditions can be considered to have anti-inflammatory potential.[28] Here, test samples of *B. monnieri* (2.0 mg/ml) can inhibit the hemolysis against heat and hypotonic solution (Table 2). In hypotonic solution induced condition, the crude extract revealed maximum 83.98% inhibition of RBC hemolysis as compared to aspirin (89.08%). During heat-induced hemolysis, the chloroform fraction showed 80.82% inhibition of RBC hemolysis. These outcomes deliver the proof of anti-inflammatory potential of the extract which showed a worthy protective effect of RBCs against traumatic conditions. Therefore, *B. monnieri* might be measured as a decent source of anti-inflammatory agents.

Due to high incidence of cardiovascular diseases, investigation has been carried out on novel thrombolytic agents with minimal side effects. *B. monnieri* was assessed for thrombolytic activity and the results are presented in figure 2. Here, the petroleum ether fraction exhibited highest thrombolytic activity (39.24%) as compared to the standard, streptokinase (64.22%). Negative control

Table 1. Antioxidant activity and cytotoxicity of the different fractions of *B. monnieri*.

Test sample	Antioxidant level (IC_{50} mg/ml)	Cytotoxicity (LC_{50} µg/ml)
BHT	21.20	--
AA	3.05	--
VS	--	0.451
MEBM	7.02	174.13
PEF	16.12	5.5
DMF	3.78	11.81
CF	25.53	10.25
AQF	261.65	272.40

Table 2. Membrane stabilizing effect of the different fractions of *B. monnieri*.

Sample	Concentration	% Inhiition of hemolysis	
		Hypotonic solution-induced hemolysis	Heat-induced hemolysis
MEBM	2 mg/ml	83.98	46.82
PEF	2 mg/ml	16.30	42.87
DMF	2 mg/ml	4.93	42.55
CF	2 mg/ml	44.07	80.82
AQF	2 mg/ml	37.80	10.44
Aspirin	0.10 mg/ml	89.08	42.12

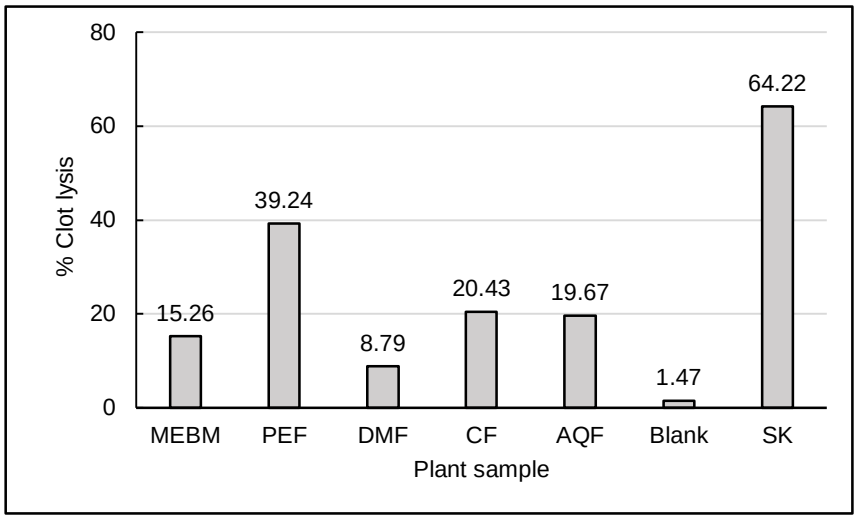


Figure 2. Thrombolytic activity of different fractions of *B. monnieri.*

Table 3. Antimicrobial activity of test samples of *B. monnieri.*

Microorganism	Zone of inhibition (mm)			
	MEBM	DMF	CF	Standard
Gram positive bacteria				Ciprofloxacin
Bacillus cereus	--	7.0	8.0	40.0
B. megaterium	8.0	--	--	36.0
B. subtilis	--	--	8.0	37.0
Sarcina lutea	9.0	9.0	--	46.0
Staphylococcus aureus	10.0	12.0	12.0	38.0
Gram negative bacteria				Ciprofloxacin
Escherichia coli	--	--	10.0	37.0
Pseudomonas aeruginosa	8.0	9.0	8.0	38.0
Salmonella Paratyphi	9.0	11.0	11.0	38.0
Salmonella Typhi	--	--	9.0	36.0
Shigella boydii	7.0	9.0	10.0	36.0
S. dysenteriae	--	--	9.0	38.0
Vibro mimicus	12.0	--	12.0	47.0
V. parahemolyticus	8.0	8.0	10.0	40.0
Fungi				Ketoconazole
Aspergillus niger	8.0	--	10.0	38.0
Candida albicans	10.0	11.0	10.0	42.0
Saccharomyces cerevisiae	9.0	--	10.0	37.0

(pure water) displayed little or no clot lysis activity. The chloroform and aqueous fractions showed 20.43% and 19.67% clot lysis activity, respectively. The observed thrombolytic potential of the test samples could be due to the existence of bioactive phytochemicals. More studies are thus required for development of new thrombolytic agents.

The research interest for novel antimicrobial agents from plant kingdom is rising day by day.[29] In order to find out the prospective antimicrobial

principles, *B. monnieri* was subjected for preliminary antimicrobial screening by disc diffusion method.[22] During antimicrobial test, the plant extract at concentration of 400 ug/disc showed mild to moderate activity against most of the test microorganisms (Table 3) and the outcomes were represented as diameter of the clear zone of growth inhibition. Among all, both *S. aureus* and *C. albicans* were sensitive to all test samples. The crude methanol extract exhibited moderate antimicrobial activity against *V. mimicus* (12.0 mm), *S. aureus* (10.0 mm) and *C. albicans* (10.0 mm). The dichloromethane fraction showed prominent inhibitory activity against *S. aureus* (12.0 mm), *Salmonella* Paratyphi (11.0 mm) and *C. albicans* (11.0 mm). The chloroform fraction revealed activity against most of the tested microbial species, notably with *S. aureus* (12.0 mm), *V. mimicus* (12.0 mm). The present results are in good agreement with the previous report that explained the antimicrobial property of *B. monnieri* leaf extract against several pathogens.[31] Therefore, *B. monnieri* can be considered as natural sources of antimicrobial agents. Antimicrobial properties of the plant extract may be due to the several phytochemicals (such as flavonoids, alkaloids, tannins, and terpenoids.) which are reported to inhibit the enzymes and proteins of the microbial cell membrane causing the cellular leakage which induces cell death.[30]

CONCLUSION

Phytochemical studies of *B. monnieri* led to the isolation of taraxerone and betulinic acid. The results of our bioassay suggest that *B. monnieri* possesses a variety of biological actions including antioxidant, cytotoxic, anti-inflammatory, thrombolytic and antimicrobial activities. However, more investigations are needed to separate and identify the active molecules accountable for these pharmacological actions.

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