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# *In Vitro* and *In Vivo* Anticancer Activity of Bacoside A from Whole Plant of *Bacopa Monnieiri* (Linn)

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**Abstract: Problem statement:** Natural products have long been a fertile source of cure for cancer, which is projected to become the major cause of death in this century. Major classes of anticancer compounds include alkaloids, terpenoids, flavonoids and lignans. We have chosen terpenoids (bacosides) because terpenoids like taxol are currently being used in cancer chemotherapy. The anticancer activity of Bacoside A (containing Bacoside A3) isolated from the whole plant of plant *Bacopa monnieiri* (Linn.) was evaluated in *in vitro* and *in vivo* experimental models. **Approach:** The Bacoside A was isolated from ethyl acetate insoluble fraction (BM-2B) of chloroform**:** Methanol (2: 1) (BM-2) extract of B. monnieiri whole plant. The cytotoxicity (*in vitro*) of Bacoside A (BM2BF$_8$-BSD) was carried out by means of MTT assay using MCF-7 (Human breast cancer), HT-29 (Human colon adeno carcinoma) and A-498 (Human kidney carcinoma) cell lines. The *in vivo* anticancer activity of Bacoside A was evaluated against Ehrlich ascites carcinoma (EAC) tumor bearing mice. **Results:** The Bacoside A (31.38 %) rich fraction, coded BM-2B containing Bacoside A3 (8.09 %) was showing potent cytotoxicity. Oral administration of BM-2B to tumor bearing mice at the dose of 250 and 500 mg kg$^{-1}$ body weight for 10 days, showed significant reduction in percent increase in body weight, tumor volume, packed cell volume, viable tumor cell count and increased non-viable cell count when compared to the untreated mice of the EAC control group. The restoration of hematological parameters towards normalcy was also observed. **Conclusion:** The results suggests that the Bacoside A (31.38 % Bacoside A containing 8.09 % Bacoside A3) rich fraction (BM-2B) exhibits significant anticancer activity in both *in vitro* and *in vivo* experimental models.

**Key words:** Ehrlich ascites carcinoma, cytotoxicity assay, percent increase, experimental models, viable tumor, bearing mice, control group**,** insoluble fraction, *bacopa monnieiri,* tumor bearing mice

## INTRODUCTION

Natural products have long been a fertile source of cure for cancer, which is projected to become the major cause of death in this century. There are at least 2, 50,000 species of plants out of which more than one thousand plants have been found to possess significant anticancer properties. While many molecules obtained from nature have shown wonders, there are huge number of molecules that still either remains untapped or studied in detail by the medicinal chemists (Mukherjee *et al*., 2001). Out of 121 prescription drugs in use today for cancer treatment, 90 are derived from plant species. Almost 74% of these were discovered from folklore claim. In the period between 1981 and 2002, 48 of 65 drugs approved for the therapy of cancer were based on natural products or mimicked natural products in one form or another (Shishodia and Aggarwal, 2004).

Many herbs have been evaluated in clinical studies and are currently being investigated phytochemically to understand their tumoricidal actions against various cancers. Major classes of anticancer compounds include alkaloids, terpenoids, flavonoids and lignans. We have chosen terpenoids from *Bacopa monnieiri* whole plant, because terpenoids like taxol are currently being widely used in cancer chemotherapy (Spiridon, 2006; Kim and Park, 2002).

The *Bacopa monnieiri* (Scrophulariaceae) whole plant constitutes the well known drug brahmi.

It is astringent, laxative, carminative, digestive, depurative, cardiotonic, diuretic, bronchodilatory, emmenogogue, febrifuge and tonic. It is also known to have anti-inflammatory, anticonvulsant and antiulcer properties. It is used in the indigenous system of medicine for the treatment of asthma, hoarseness, insanity, epilepsy and as a potent nerve

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tonic (Warrier *et al.*, 1994; The Wealth of India, 1988). The drug forms an important ingredient of a number of Ayurvedic preparations such as Brahmighritam, Brahmirasayanam. The juice of leaves is given to children for relief in bronchitis and diarrhea. The paste of leaves is used as remedy for rheumatism (The Wealth of India, 1988).

The plant *Bacopa monnieiri* has been reported to contain steroidal and triterpenoidal saponins as major constituents along with alkaloids (brahmin, herpestine, nicotine), sterols (ß sitosterol, stigmasterol and stigmasterol esters), triterpenes (betulic acid and betulinic acid), fatty hydrocarbons (dotriacontane, triacontane, heptacosane, nonacosane and hentriacontane) glycosides (phenyl glycosides, mannitol) and aminoacids (aspartic acid, alpha-alanine, glutamic acid and serine) as minor constituents (Rastogi and Mehtrotra, 1995; Rastogi and Mehrotra, 1991; Rastogi and Mehrotra, 1993).

The steroidal saponins, Bacoside A and Bacoside B are optical isomers and Bacoside B may be an artifact forming during the isolation of Bacoside A. The other bacosides are Bacoside A1 and Bacoside A3 (Rastogi and Mehrotra, 1998). The other bacosides have been identified as dammarane type triterpenoid saponins known as bacopasaponin A-G and bacopasides I-V. The bacopasaponins A-G and bacosides I-V, contain either jujubogenin or psedojujubogenin as aglycone part and L-arabinopyranosyl, D-glucopyranosyl as glycone moieties (Chakravarty *et al.*, 2001; Mahato *et al.*, 2000; Garai *et al.*, 1996a; Garai *et al.*, 1996b; Chakravarty *et al.*, 2003). The triterpenoids reported from this plant *Bacopa monnieiri* are bacogenin A1, bacogenin A2 (an isomer of A1), bacogenin A3 and bacogenin A4 (embelin lactone) and known betulic acid and betulinic acid (Rastogi and Mehrotra, 1993).

The standardized methanolic extract of this plant containing 38% of Bacoside A was reported to show antiulcerogenic activity when administered at the doses of 10-50 mg $kg^{-1}$ b.wt. twice daily for 5 days in different gastric ulcer models (Sairam *et al.*, 2001).

Hence, in the present study, we investigated the anticancer properties of major terpenoids Bacoside A (containing Bacoside A3) isolated from the whole plant of *Bacopa monnieiri* (Linn.) against Ehrlich ascites carcinoma (EAC) tumor bearing mice.

## MATERIALS AND METHODS

**Plant materials:** *Bacoapa mannieiri* whole plant (BM) collected from Ramanagaram district, Karnataka, India. The plant was authenticated by Dr.Kannan, Botanist, R and D Center, The Himalaya Drug Company, Bangalore, Karnataka, India.

**Chemicals and reagents:** 3-(4, 5-dimethyl thiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide (MTT), Dulbecco's modified eagles medium (DMEM), Fetal Bovine Serum (FBS) from Sigma Aldrich, Dimethyl Sulfoxide (DMSO) from E-Merck, Ethylenediamine Tetra Acetic Acid (EDTA) from Hi-media, trichloroacetic acid from SD fine chemicals, 5-flurouracil (5-FU) was purchased from Sigma. The standard Bacoside A (Bacoside A saponins including Bacoside A3 (12.5 %), bacopaside II, jujubogenin (an isomer of bacopasaponin C) and bacopasaponin C) Lot no.: 00002005-T9K of purity 96.2% was purchased from Chromadex. All other chemicals and solvents used in this study were of analytical grade.

**Instruments:** Linomat V, Reprostar 3 from CAMAG, Switzerland, HPLC system from Shimadzu, (Model**:** Prominence).

**Extraction:** The air-dried powdered material (7 kg) was subjected to repeated extraction by maceration at room temperature with various solvents of different polarity such as chloroform (20 L), chloroform**:** Methanol (2:1) (20 L) and methanol (20 L) successively for 7 days with each solvent. Each extract was filtered and concentrated and dried at reduced pressure and controlled temperature (40-60°C) in a rotary evaporator, which yielded 110 g of dark greenish colored chloroform extract (BM-1), 673 g greenish brown colored chloroform: Methanol (2: 1) extract (BM-2) and 520 g of brown colored methanol extract (BM-3).

**Fractionation:** The dried extracts of BM-1 (105 g), BM-2 (570 g) and BM-3 (500 g) were once again subjected to exhaustive fractionation by maceration at room temperature. The BM-1 extract was fractionated with hexane (4 L) to separate hexane soluble (BM-1A) and insoluble (BM-1B) fractions, BM-2 extract was fractioned with ethyl acetate (12.5 L) to separate ethyl acetate soluble (BM-2A) and insoluble (BM-2B) fractions and BM-3 extract was fractioned with n-butanol (7.5 L) to separate n-butanol soluble (BM-3A) and insoluble (BM-3B) fractions. Each fraction was then filtered, concentrated and dried at reduced pressure and temperature using rotary evaporator, which yielded 75 g dark green colored BM-1A, 30 g green colored BM-1B, 115 g greenish brown BM-2A, 410 g brown colored BM-2B , 157 g brown colored BM-3A and 240 g colored brown BM-3B.

**Identification and estimation of major terpenoids or Bacosides (Bacoside A):**

**Thin Layer Chromatography (TLC):** All the BM extracts (BM-1, BM-2 and BM-3) and its fractions (BM-1A, BM-1B, BM-2A, BM-2B, BM-3A and BM-3B) were subjected to identification of major terpenoids (Bacosides or Bacoside A) by TLC method (Waganer and Bladt, 1996).

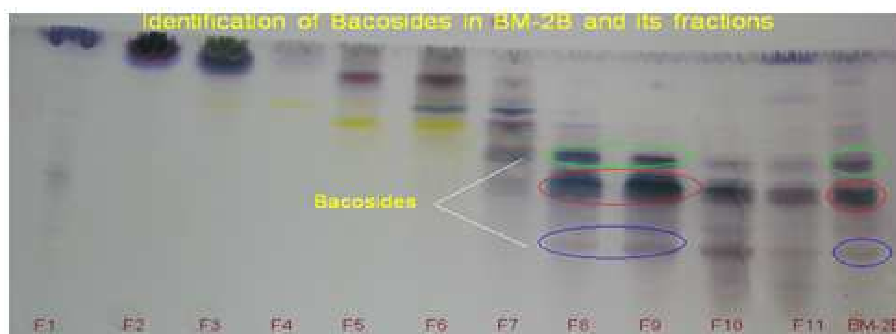


Fig. 1: Identification of Bacosides in BM-2B and its fractions by TLC

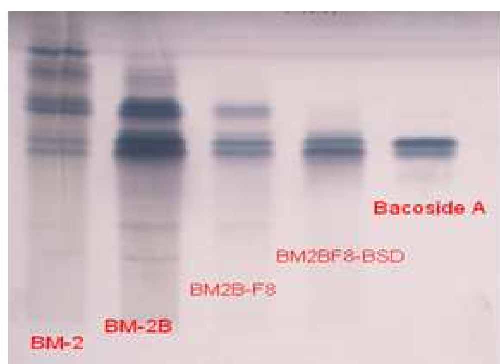


Fig. 2: Identification of Bacoside A in BM-2, BM-2B, BM2B-F8, BM2BF8-BSD by TLC

The extracts and fractions were dissolved with respective soluble solvent at a concentration 100 mg $mL^{-1}$ and filtered through Whatmann no 1 filter paper. 10 µL of extract/fraction was spotted on CAMAG Linomat V as 10 mm band width on a precoated Silica gel 60 $F_{254}$ plate of uniform thickness (0.2 mm). Develop the plate in the solvent system chloroform: methanol: water 70: 30: 04 to a distance of 8.5 cm. Identify the bands of Bacosides by dipping the plate with vanillin sulphuric acid reagent and heating at 110°C for 5-10 min.

**High Performance Liquid Chromatography (HPLC):** The extracts BM-1, BM-2 and BM-3 and its fractions BM-1A, BM-1B, BM-2A, BM-2B, BM-3A and BM-3B were also subjected to HPLC to estimate Bacoside A. One gram extract or fraction was dissolved by methanol sonication and made up to 100 mL with same solvent and filtered through 0.22 µ syringe filter. Bacosides A (containing Bacoside A3) from Chromadex at 1 mg $mL^{-1}$ concentration was used as standard.

**Solvent system:** Water**:** Acetonitrile (67: 33) HPLC, Make-Shimadzu, Model**:** Prominence, LC-20 AD Pump, SIL-20 AC HT auto sampler, SPD-20 A UV-Vis detector, Column-C18/Thermo 5µ (Size 250×4.6 mm), flow rate-1.6 mL $min^{-1}$ and detection at UV 205 nm wave length.

**Isolation and characterization of major terpenoids or Bacosides (Bacoside A):** As per HPLC analysis, fraction BM-2B was rich in Bacoside A (31.38 %) (containing 8.09 % Bacoside A3) was further subjected to column chromatography to separate Bacoside A. BM-2B (400 g) subjected to separation by column chromatography on Silica gel (60-120) by elution with chloroform (1 L), chloroform**:** Ethyl acetate mixture (12.5 L), ethyl acetate (3 L), ethyl acetate: Methanol mixture (7.5 L) with increasing polarity up to methanol (3 L) led to separation of 11 fractions designated as BM2B-$F_1$ to $F_{11}$. Each fraction was subjected for the identification of terpenoids or Bacosides by TLC (Fig. 1) (Waganer and Bladt, 1996).

The green mixed creamish crystalline powder fraction BM2B-$F_8$ fraction (20 g) having Bacoside A rich content by TLC and HPLC, subjected to repeated column chromatography on silica gel with chloroform: Ethyl acetate mixture solvent system. The Bacoside A rich fraction was recrystallized with methanol until a principal spot is obtained by TLC (Fig. 2) and peaks by HPLC (Fig. 3), which is designated as BM2B$F_8$-BSD (Bacoside A). The remaining fractions BM2B-$F_1$ to $F_7$ and $F_9$ to $F_{11}$ were not significant enough to be considered for further purification.

The isolated compound BM2B$F_8$-BSD (Bacoside A) was co-chromatographed with standard of Bacoside A by TLC (Fig. 2) and HPLC (Fig. 3). The mass spectra of isolated compound were matched with standard Bacoside A.

**Cell culture:** Human breast cancer (MCF-7), Human colon adeno carcinoma (HT-29), Human kidney carcinoma (A-498) and Ehrlich ascites carcinoma (EAC) tumor cells were obtained from National Centre for Cell Sciences (Pune, India). The cultures were maintained in Dulbecco's Modified Eagles Medium (DMEM) containing 10% heat inactivated Fetal Bovine Serum (FBS), penicillin (100 units $mL^{-1}$) and streptomycin (100 µg $mL^{-1}$) at 37°C in 5% $CO_2$. Cells were grown in 25 $cm^2$ tissue cultures flask until confluent and used for cytotoxicity assays.

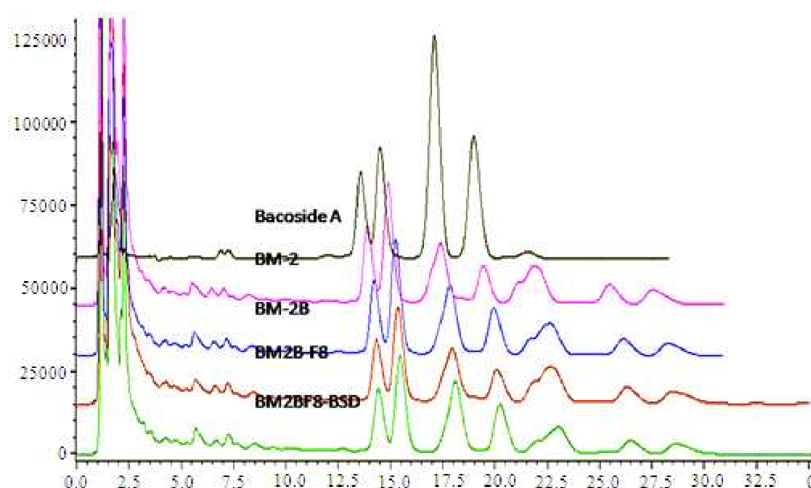


Fig. 3: Identification of Bacoside A in BM-2, BM-2B, BM2B-F8 and BM2BF8-BSD by HPLC

Ehrlich ascites carcinoma (EAC) tumor cells were maintained *in vivo* in Swiss albino mice, by i.p transplantation. Tumor cells aspirated from the peritoneal cavity of mice were washed with saline and were administered I.P. to induce ascites tumor.

***In vitro* cytotoxicity of Bacosides:**

**Preparation of sample suspension and solution:** Stock solution: Since the extract BM-2 and its fraction BM-2B was rich in Bacoside A (25.97 % and 31.38 % respectively) was subjected to cytotoxicity along with isolated compound $BM2BF_8$-BSD (Bacoside A). BM-2 extract, fraction BM-2B and isolated compound $BM2BF_8$-BSD were separately dissolved in DMSO and volume was made up with MEM/DMEM supplemented with 2% inactivated FBS/NBCS to obtain a stock solution of 5 mg $mL^{-1}$ concentration, sterilized by filtration and stored at -20°C. Serial working solutions were prepared from stock solution at concentrations of 200, 100, 50, 25 and 12.5µg $mL^{-1}$ with MEM/DMEM medium.

Stock cells of MCF-7, HT-29 and A-498 cell lines were cultured in RPMI-1640 and DMEM supplemented with 10% sheep serum, penicillin (100 IU $mL^{-1}$) and streptomycin (100 µg $mL^{-1}$) in a humidified atmosphere of 5% $CO_2$ at $37^0$C until confluent. The cells were dissociated with 0.2 % trypsin, 0.02 % EDTA in PBS. The cytotoxicity assay was carried out by adding 0.1 mL of cell suspension containing 10,000 cells to each well of a 96 well microtitre plate and fresh medium containing different concentrations of the extract, fraction and isolated compound were added at 24 h after seeding. Control cells were incubated without the test item and with DMSO (solvent). The very small percentage of DMSO present in the wells (maximum 0.2%) was proved not to affect the experiment. The microtitre plates were incubated at 37°C in a humidified incubator with 5 % $CO_2$ for a period of 3 days. The assay was done in triplicates. Cells were observed at different time intervals during incubation in the presence of the test item. Cellular viability was determined by the standard MTT assay method (Vijayan *et al*., 2003; Suthindhiran, 2009; Syamsudin *et al*., 2009; Chick, 2010).

***In vivo* anticancer activity:**

**Animals:** The ascitic antitumor studies induced by Ehrlich ascites carcinoma (EAC) tumor cells were carried out by using healthy adult Swiss albino mice weighing 20-25 g. They were obtained from the animal house facility of The Himalaya Drug Company, Bangalore. The Ehrlich ascites carcinoma (EAC) tumor cell induced study was carried out by using inbred fresh female BALB/c mice weighing 20-25 g. All the animals were kept under standard laboratory conditions with a 12 h light-dark cycle. Animals were provided with commercial pelleted diet (Provimi Animal Nutrition India Pvt. Ltd., Doddaballapur, Bangalore) and purified water ad lib. The study protocol was approved by the institutional Animal Ethics Committee.

**Ehrlich ascites carcinoma (EAC) tumor cells induced antitumor studies:** Fifty four mice were devided in to 5 groups, group 1 containing 6 animals, served as normal control, for which inoculation of tumor cells was not done. The remaining animals were inoculated with EAC tumor cells ($1 \times 10^6$ cells /mouse) intraperitoneally and divided into 4 groups containing 12 mice in each group. Group II, served as the tumor control. Groups I (Normal control) and II (EAC control) received an oral dose of 10 mL $kg^{-1}$

b.wt. Sodium CMC suspension (0.3%). Group III, served as a reference drug, was treated orally with 5-Flurouracil (5-FU) at the dose of 20 mg $kg^{-1}$ body weight. Groups IV and V were treated with BM-2B fraction at 250 and 500 mg $kg^{-1}$ body weight respectively. All the treatments were given orally at 24 h after tumor inoculation and continued once daily for 10 days. On the $11^{th}$ day, six animals from each group was anesthetized with anesthetic ether and blood was collected by retro-orbital puncture for evaluation of hematological parameters, which included Hemoglobin (HB) content, Red Blood cell count (RBC), White Blood Cell count (WBC) and differential leucocyte count. The remaining animals in each of the groups were kept to check the Mean Survival Time (MST) and percent increase in life span of the tumor bearing hosts (Natesan *et al*., 2007).

**Tumor growth response:** Antitumor effect of BM-2B was assessed by observation of change with respect of body weight, ascitic tumor volume, packed cell volume and viable tumor cell count, Mean Survival Time (MST) and percentage Increase In Life Span (%ILS).

**Tumor cell count and packed cell volume:** The mice were dissected for collecting ascitic fluid from peritoneal cavity. The transplantable murine tumor was carefully collected with the help of 5 mL sterile syringe to measure the tumor volume and the ascitic fluid was transferred to a graduated glass centrifuge tube and packed cell volume was determined by centrifuging at 1000 rpm for 5 min.

**Viable and non viable cell count:** Viable and non viable cell counting of ascitic cells were performed by dye exclusion test using trypan blue stain (0.4% in normal saline). The cell counts were determined in a Neubauer counting chamber.

**Mean Survival Time (MST) and percent increase in life span:** The effect of BM-2B on tumor growth was observed by MST and %ILS. MST of each group containing 6 mice were monitored by recording the mortality daily for 6 weeks and % ILS was calculated by using following equation (Natesan *et al*., 2007; Gothoskar and Ranadive, 1971; Joharapurkar *et al*., 2003; Vijayabaskaran *et al*., 2010):

$$\text{Death MST} = \frac{\text{Day of first death} + \text{Day of last}}{2}$$

**Statistical analysis:** The experimental results were expressed as mean ± SEM. Data was assessed by ANOVA followed by the Dunnet't multiple comparison post hoc test. Value of $p<0.05$ was considered as statistically significant. The $IC_{50}$ concentrations were calculated using linear regression analysis. The statistical analysis was performed using GraphPrism software (version 4.03).

## RESULTS

**Identification and estimation of major terpenoids (bacosides):**

**Thin Layer Chromatography (TLC):** The terpenoidal compounds of BM were distributed between BM-1, BM-2 and BM-3 extracts. Some terpenoidal compounds were present in BM-1 extract, were equally distributed between BM-1A but BM-1B contained negligible terpenoidal compounds. BM-2 extract contained maximum compounds of terpenoids or bacosides, which when fractionated with ethyl acetate remained in the BM-2B. BM-3 extract that contained negligible or nil content of bacosides compounds were portioned between BM-3A and BM-3B.

**High Performance Liquid Chromatography (HPLC):** BM-1, BM-1A and BM-1B did not contain any major Bacosides compounds, BM-2 and its fraction BM-2B (Fig. 2) contained major bacosides. The BM-2 contains 25.97% of Bacoside A (containing 5.55 % Bacoside A3), BM-2B contains 31.38 % of Bacoside A (containing 8.09 % Bacoside A3), but BM-2A, BM-3, BM-3A and BM-3B was containing negligible or nil Bacoside A (Table 1).

**Isolation and characterization of Bacoside A:** As per the TLC, out of BM2B-$F_1$ to $F_{11}$ fractions only BM2B-$F_8$ (Fig. 1) fraction was rich in Bacoside A. BM2B-$F_8$ fraction was subjected to repeated column chromatography followed by repeated crystallization until to get principal spot by TLC (Fig. 2) and peaks by HPLC (Fig. 3). The purified compound after crystallisation yielded 7 g of Bacoside A (BM2B$F_8$-BSD) with purity 76.44 % containing 13.79 % Bacoside A3. The co-chromatography of isolated BM2B$F_8$-BSD was exactly matched with chromadex Bacoside A standard (Fig. 2-3). The same was confirmed by mass spectra.

Table 1: Comparative analysis of Bacoside A (containing Bacoside A3) in different extracts, fractions and isolated compound

| Extract/fraction | Bacoside A3 % w/w | Bacoside A % w/w |
|---|---|---|
| BM-1 extract | Nil | Nil |
| BM-1A fraction | Nil | Nil |
| BM-1B fraction | Nil | Nil |
| BM-2 extract | 5.55 | 25.97 |
| BM-2A fraction | 0.90 | 1.26 |
| BM-2B fraction | 8.09 | 31.38 |
| BM2B$F_8$-BSD (Bacoside A) | 13.79 | 76.44 |
| BM-3 extract | Nil | Nil |
| BM-3A fraction | Nil | Nil |
| BM-3B fraction | Nil | Nil |

Table 2: *In vitro* cytotoxicity of BM-2 extract, fraction BM-2B and $BM2BF_8$-BSD (Bacoside A)

| | | Cytotoxicity (%) | | | $CTC_{50}$ (µg $mL^{-1}$) | | |
|---|---|---|---|---|---|---|---|
| Extract/ fraction | Concentration (µg $mL^{-1}$) | MCF-7 | HT-29 | A-498 | MCF-7 | HT-29 | A-498 |
| BM-2 extract | 200.0 | 76.53 | 63.25 | 94.11 | | | |
| | 100.0 | 37.71 | 32.10 | 47.66 | | | |
| | 50.0 | 18.56 | 16.04 | 24.51 | 131.21 | 157.60 | 105.56 |
| | 25.0 | 9.42 | 8.11 | 11.95 | | | |
| | 12.5 | 5.21 | 4.05 | 5.87 | | | |
| BM-2B fraction | 200.0 | 98.64 | 99.46 | 99.15 | | | |
| | 100.0 | 70.58 | 51.78 | 68.48 | | | |
| | 50.0 | 38.59 | 26.84 | 44.21 | 85.24 | 99.16 | 77.51 |
| | 25.0 | 15.96 | 13.05 | 28.28 | | | |
| | 12.5 | 7.43 | 6.58 | 12.45 | | | |
| BM2BF8-BSD | 200.0 | 98.75 | 99.47 | 99.15 | | | |
| (Bacoside A) | 100.0 | 82.47 | 72.36 | 78.95 | | | |
| | 50.0 | 72.31 | 54.21 | 68.67 | 46.84 | 76.31 | 38.04 |
| | 25.0 | 36.42 | 17.45 | 48.76 | | | |
| | 12.5 | 18.42 | 9.26 | 22.47 | | | |

Table 3: Effect of BM-2B extract of *Bacopa monnieiri* on survival time of EAC bearing mice

| Experimental groups | Mean Survival Time (MST) days | (%) Increase in life span |
|---|---|---|
| Normal control | - | - |
| EAC control | 21.45±1.58 | - |
| EAC+BM-2B (250 mg $kg^{-1}$) | 22.48±1.64 | 4.80 % |
| EAC+BM-2B (500 mg $kg^{-1}$) | 24.95±1.48* | 16.31% |
| EAC+5-flurouracil | 27.48±1.40** | 28.11% |

Values are mean ± SEM; Number of mice in each group (n=12); *p<0.05, **p<0.01 as compared with EAC control

Table 4: Effect of BM-2B fraction of *Bacopa monnieiri* on tumor volume, packed cell volume, viable and non viable tumor cell count on EAC bearing mice

| Parameters | EAC control | BM-2B (250 mg $kg^{-1}$) | BM-2B (500 mg $kg^{-1}$) | 5- FU (20 mg $kg^{-1}$) |
|---|---|---|---|---|
| Body weight (g) | 27.51±0.52 | 25.58±0.18* | 23.81±0.27** | 22.66±0.83** |
| Tumor volume (mL) | 5.60±0.1 | 4.80±0.5* | 4.10±0.6** | 3.50±0.1** |
| Packed cell volume (mL) | 2.90±0.1 | 2.60±0.5* | 2.30±0.30** | 1.70±0.10** |
| Viable tumor cell count (×$10^7$ cells $mL^{-1}$) | 11.34±0.03 | 8.60±0.03* | 5.45±0.05** | 4.82±0.01** |
| Non viable tumor cell count (×$10^7$ $m^{-1}$l) | 0.54±0.01 | 0.9±0.01* | 1.24±0.02** | 1.52±0.01** |

Values are mean ± SEM; Number of mice in each group (n = 12); *p<0.05, **p<0.01 as compared with EAC control

**In vitro cytotoxic activity of Bacoside A (Bacosides):**

**Cytotoxicity of Bacoside A:** Since only BM-2 and its fraction BM2B, which were rich in Bacoside A was subjected to cytotoxicity along with isolated compound $BM2BF_8$-BSD (Bacoside A). BM-2 extract showed an $IC_{50}$ concentrations 131.21, 157.60 and 105.56 µg $mL^{-1}$ against MCF-7, HT-29 and A-498 cell lines respectively. BM-2B fraction showed an $IC_{50}$ concentrations 85.24, 99.16 and 77.51 µg $mL^{-1}$ against MCF-7, HT-29 and A-498 cell lines respectively. Bacoside A ($BM2BF_8$-BSD) compound exhibited an $IC_{50}$ concentrations 46.84, 76.31 and 38.04 µg $mL^{-1}$ against MCF-7, HT-29 and A-498 cell lines respectively (Table 2).

***In vivo* anticancer activity of bacosides:**

**Ehrlich ascites carcinoma (EAC) induced antitumor studies:** Effect on tumor growth: In the Ehrlich ascites carcinoma (EAC) tumor control group, the average life span of animals was found to 21.45±1.58 days. BM-2B at the doses of 250 mg $kg^{-1}$ body weight no significant change in the average life span as compared to EAC control. BM-2B at a dose of 500 mg $kg^{-1}$ body weight showed a significant change in the average life span as compared to EAC control. The average life span of 5-FU treatment was found to be 27.48 ± 1.40 days, indicating its potent antitumor nature (Table 3). The antitumor nature of BM-2B at both doses was evidenced by the significant reduction in increase in body weight as compared to EAC tumor bearing mice. Treatment with BM-2B also showed a significant reduction in tumor volume, packed cell volume and viable tumor cell count and non-viable tumor cell count in both the doses as compared to the EAC tumor control (Table 4).

**Effect of hematological parameters:** EAC tumor bearing mice showed a significant decrease in the RBC count and a significant increase in the WBC count as compared to the normal control.

Table 5: Effect of BM-2B fraction *Bacopa monnieiri* on hematological parameters of EAC bearing mice

| Parameters | Normal control | EAC control | EAC+ BM-2B (250 mg $kg^{-1}$) | EAC+ BM-2B (500 mg $kg^{-1}$) | EAC + 5 FU (20 mg $kg^{-1}$) |
|---|---|---|---|---|---|
| Hemoglobin (gm) | 12.36 ± 0.16** | 9.61 ± 0.13 | 10.85 ± 0.06* | 11.54 ± 0.0** | 11.68 ± 0.10** |
| Total RBC count (million/cu.mm) | 7.51 ± 0.12** | 3.46 ± 0.06 | 4.48 ± 0.12* | 5.52 ± 0.06** | 6.01±0.02** |
| Total WBC count ($10^3$/cu.mm) | 7.96 ± 0.05** | 18.08 ± 0.09 | 11.52 ± 0.15* | 9.48 ± 0.05** | 8.96 ± 0.15** |
| Differential leucocyte count (%) | | | | | |
| Lymphocyte | 68.34 ± 0.52** | 35.62 ± 0.16 | 45.45 ± 0.36 | 54.27 ± 0.32** | 63.63±0.60** Monocyte |
| | 2.96 ± 0.03** | 0.96 ± 0.01 | 1.42 ± 0.02* | 1.82 ± 0.01** | 1.76 ± 0.02** |
| Granulocyte | 27.46 ± 0.26** | 65.20 ± 0.61 | 52.18 ± 0.62* | 35.42 ± 0.81** | 33.48 ± 0.12** |

Values are mean ± SEM; Number of mice in each group (n=6); *p<0.05, **p<0.01 as compared with EAC control

There was also a significant decrease in the lymphocyte count with an increase in the neutrophil counts in EAC tumor bearing mice as compared to normal control. Treatment with BM-2B significantly reversed EAC tumor-induced changes in the hematological profiles (Table 5). Similar observations were observed with 5-FU (Reference drug).

## DISCUSSION

Natural products have been regarded as important sources that could produce potential chemotherapeutic agents. Plant derived compounds; in particular have gained importance in anticancer therapy and some of the new chemotherapeutic agents currently available for use includes paclitaxel, vincristine, podophyllotoxin and camptothecin, a natural product precursor from water soluble derivatives. Obviously natural products are extremely an important source of medicinal agents. Although there are some new approaches to drug discovery, such as combinatorial chemistry and computer based molecular modeling design, none of them can replace the importance of natural products in drug discovery and development (Jagetia and Rao, 2006).

The present investigation was carried out to evaluate the anticancer activity of Bacoside A (containing Bacoside A3) isolated from BM-2B fraction of *Bacopa monnieiri*. *In vitro* cytotoxicity study using MCF-7, HT-29 and A-498 cell lines showed potent activity with BM-2B as well as its isolated compound $BM2BF_8$-BSD (Bacoside A). These observations provided a basis for the conduct of *in vivo* study of BM-2B using EAC tumor in mice.

The reliable criteria for judging the potency of an anticancer drug is the prolongation of the life span of tumor bearing animals. There is also a significant increase in the body weight in the EAC tumor bearing mice due to the regular rapid increase in the ascitic tumor volume (Price and Greenfield, 1958). The mice bearing EAC tumor when orally administered with BM-2B showed a significant increase in the life span and also a significantly prevented the increase in body weight that was observing in the EAC control mice. Treatment with BM-2B also showed a significant decrease in the tumor volume, packed cell volume and viable tumor cell count, thereby indicating the anticancer nature of BM-2B. These results indicate either a direct cytotoxic effect on the tumor cells or local cytotoxic effect of BM-2B.

In cancer chemotherapy the major problem are of myelosuppression and anemia (Maseki *et al*., 1981; Badami *et al*., 2003). The anemia encountered in the tumor bearing mice is due to the reduction in the RBC count or hemoglobin percentage and this may occur due to iron deficiency or due to hemolytic or myelopathic conditions. Treatment with BM-2B restored the hematological profiles as compared to EAC mice. This indicates BM-2B possess protective action on the hemopoietic system.

Excessive production of free radical resulted in oxidative stress, which leads to damage of macromolecules such as lipids can induce lipid peroxidation *in vivo*. Increased peroxidation would cause degeneration of tissues. Lipid peroxide formed in the primary site would be transferred through the circulation and provoked damaged by propagating the process of lipid peroxidation. Malondialdehyde (MDA), the end product of lipid peroxidation was reported to be higher in cancer tissues than in non diseased organs. The methanolic extract of this plant was reported significant protective effect on $H_2O_2$-induced toxicity and DNA damage in human non-immortalized fibroblasts. It has shown dose dependent free radical scavenging activity. Since bacosides induced elevation of antioxidant activity could be responsible for anticancer activity (Maseki *et al*., 1981; Rosso *et al*., 2003).

## CONCLUSION

From the above findings it could be concluded that Bacosides rich fraction BM-2B (31.38 % Bacoside A containing 8.09% Bacoside A3) from *Bacopa monnieiri* whole plant exhibited potent anticancer activity as demonstrated by *in vitro* cytotoxicity using MCF-7, HT-29 and A-498 cell lines as well as EAC induced tumor in mice.

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