Exploring the Cytotoxic Potential of Triterpenoids-enriched Fraction of *Bacopa monnieri* by Implementing *In vitro*, *In vivo*, and *In silico* Approaches

Md. Nasar Mallick[1,2], Washim Khan[1], Rabea Parveen[1], Sayeed Ahmad[1], Sadaf[2], Mohammad Zeeshan Najm[2], Istaq Ahmad[2], Syed Akhtar Husain[2]

[1]Department of Pharmacognosy and Phytochemistry, Bioactive Natural Product Laboratory, School of Pharmaceutical Education and Research, Jamia Hamdard,
[2]Department of Bioscience, Human Genetics Laboratory, Jamia Millia Islamia, New Delhi, India

ABSTRACT

Background: *Bacopa monnieri* (BM) is a herbaceous plant traditionally used from time immemorial in Ayurvedic and folklore medicines. We hypothesized that the extract of the whole plant might contain numerous molecules with having antitumor activities that could be very effective in killing of human cancer cells. **Objectives:** This work investigated anticancer activity of bioactive fraction of BM. **Materials and Methods:** The hydroalcoholic extract of BM was fractionated with different solvent, namely, hexane, dichloromethane (DCM), acetone, methanol, and water. The *in vitro* anticancer activity was performed against various Human Cancer Cell lines, namely, Colon (HT29, Colo320, and Caco2), Lung (A549), Cervix (HeLa, SiHa), and Breast (MCF-7, MDAMB-231). Further, DCM fraction was evaluated *in vivo* for anticancer activity against Ehrlich ascites carcinoma (EAC) tumor-bearing mice since it showed the best cytotoxicity at 72 h (IC$_{50}$ 41.0–60.0 μg/mL). The metabolic fingerprinting of these extract were carried out using high-performance thin-layer chromatography along with quantification of bacoside A, bacoside B, cucurbitacin B, cucurbitacin E, and bittulinic acid. **Results:** Oral administration of DCM fraction at a dose of 40 mg/kg rendered prominent reduction of tumor regression parameters such as tumor weight, packed cell volume, tumor volume and viable tumor cell count as compared to the untreated mice of the EAC control group. The anticancer activity of DCM fraction may be due to the presence of large amount of bacoside A, B and cucurbitacins. The molecular docking studies of major metabolites with targeted proteins predicted the anticancer activity of DCM fraction which was in support of *in vivo* activity. **Conclusion:** The *in vitro*, *in vivo*, analytical and in silico studies on DCM fraction of *Bacopa monieri* has proved its great potential for development of anticancer phytopharmaceuticals.

Key words: Anticancer, *Bacopa monnieri*, high-performance thin-layer chromatography, *in silico* screening

SUMMARY

- A new HPTLC method has been developed and validated for the qualitative and quantitative analysis of bacoside A, B, cucurbitacin B, D, E and bittulinic acid in Bacopa monnieri extract. Enrichment of active anticancer metabolites was done by polarity based fractionations of hydroalcoholic extract of Bacopa. DCM fraction of a hydroalcoholic extract of Bacopa showed anticancer potential against human cancer cell line (IC50 41.0-60.0 μg/mL) and in EAC treated mice (at a dose of 40 mg/kg body weight). The anticancer activity of Bacopa may be due to the presence of bacosides and cucurbitacin and it was confirmed by in silico screening.

Qualitative and quantitative analysis by HPTLC

In silico screening of bacosides and cucurbitacin

In vivo anticancer activity in EAC bearing Balb/c mice
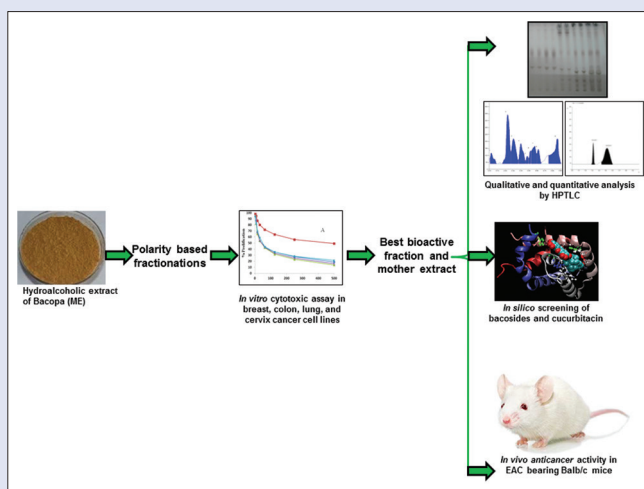
Abbreviations used: DBM: DCM fraction of Bacopa monnieri; DCM: Dichloromethane; EAC: Ehrlich ascites carcinoma; HCT: Hematocrit; HGB: Hemoglobin; HPTLC: High performance thin layer chromatography; ICH: International council for Harmonisation; LOD: Limit of detection; LOQ: Limit of quantification; LYM: Lymphocytes; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular haemoglobin concentration (MCHC); MCV: Mean corpuscular volume; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PLT: Platelet; RBC: Red blood cell; RDW: Red blood cell distribution width; RSD: Relative standard deviation; WBC: White blood cells.

Correspondence:

Dr. Sayeed Ahmad,
Department of Pharmacognosy and Phytochemistry,
Bioactive Natural Product Laboratory, School of
Pharmaceutical Education and Research, Jamia
Hamdard, New Delhi - 110 062, India.
E-mail: sahmad_jh@yahoo.co.in
Dr. Syed Akhtar Husain,
Department of Bioscience, Human Genetics
Laboratory, Jamia Millia Islamia,
New Delhi - 110 025, India.
E-mail: akhtarhusain2000@yahoo.com
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INTRODUCTION

Cancer is considered as one of the terrified diseases and it is a class of disorders characterized by uncontrolled cell proliferation and tissue invasion or metastasis of abnormal cell in the body. Cancer is the primary cause of death throughout the world and represents a foremost public health burden.[1] It has been estimated that the overall figure of fresh instances of cancer will increase from 10 million in the year 2000 by

almost 25% in every decade and may cross 24 million fresh cases in the year 2050; the aggregate figure of mortalities will ascent from 6 million in the year 2000–2010 million in 2020 to over 16 million in the year 2050. In less developed countries 17 million and in more developed countries 7 million of new cases of cancer will occur.[2]

Over the years, different approaches have been employed and are still in use, individually or in combination, in the treatment of cancer. These include chemotherapy, radiotherapy, surgery, and immunotherapy. Apart from surgery and radiation therapy physicians are usually preferred chemotherapy in most of the cases. Chemotherapeutic agents are cytotoxic and apart from affecting tumor cells, these active principles also deleteriously impact on rapidly proliferating normal cells, including those localized in the gastrointestinal tract, hair, and bone marrow, thus eliciting gastrointestinal side effects such as nausea and vomiting, alopecia, and myelosuppression. These antitumor therapies have also been associated with the development of secondary malignancy. All of the drawbacks presently associated with available chemotherapeutic agents are momentum for the quest for novel, more effective, and superior endured drugs. Natural products, especially the plant kingdom, offer an inexhaustible reservoir for investigation.[3] With above view, numerous new strategies have been developed to control and treat cancer. A combination of phytochemicals with a chemotherapeutic agent could be efficacious while reducing toxicity.

The plant *Bacopa monnieri* (BM) (L.), a traditional Ayurvedic plant, used for centuries as a memory enhancing, anti-inflammatory, antipyretic, sedative, antiepileptic agents,[4] nervine tonic, anti-tumor agent, anti-Parkinson's agent,[5] cardiotonic agent, protective effect, anti-Alzheimer's drug, hepatoprotective agent, antimicrobial agent, and adaptogenic agent.[6] It is found in wetlands throughout the Indian subcontinent and locally known as Brahmi or Jalanimba. Antioxidant properties of *Bacopa* may postulate protection from free radical damage in cardiovascular disorder and certain types of cancer.[7] The plant is reported to have cytotoxic potentials due to the presence of triterpenoid saponins such as bacoside A, bacoside B, brahmine, and herpestine.[8]

The traditional system of medicine treasures a host of medicinal formulations that have been shown to possess cytotoxic and cytostatic effects on tumor cell lines. However, there are few experimental studies, which validate the possible antitumor properties of *Bacopa*.[9,10] However, no systematic reports are available on the antitumor activity of bioactive fraction along with their qualitative and quantitative analysis. Therefore, in response to the quest for search of novel anticancer agents, the aim of the present research work was to carry out the *in vitro* cytotoxic potential of dichloromethane (DCM) fraction and aqueous extracts of BM against breast cancer (MCF-7, MDA-MB 231), Colon cancer (HT-29, Caco2, Colo 320), Lung cancer (A549), and cervix cancer (HeLa, SiHa). Bioassay-guided fractionations from hydroalcoholic extract of BM through *in vitro* screening have been carried out to isolate the active fractions enriched with metabolites responsible for the anticancer activity. Apart from *in vitro* cell line activity, this study also correlates the antitumor activity in mice and their preassumed mechanism of action through *in silico* modeling. Later on, the fraction from the mother extract which showed highest cytotoxicity was further analyzed for its *in vivo* anticancer potential after their quality control for multiple marker-based analysis for its bacoside A, B, cucurbitacin B, D, E and bittulinic acid contents using newly developed and validated simultaneous High-performance thin-layer chromatography (HPTLC) methods.[10] Apart from *in vitro* and *in vivo* anti-cancer activity, probable mechanism of DCM fractions has been predicted through *in silico* screening.

MATERIALS AND METHODS

Chemicals

RPMI-1640, phosphate buffered saline (PBS), Fetal bovine serum (Gibco, USA), Trypsin-EDTA, Trypan blue, penicillin-streptomycin and amphotericin and dimethyl sulfoxide (DMSO) were purchased molecular biology grade. Cucurbitacin B and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and betulinic acid were procured from Sigma-Aldrich, USA, whereas cucurbitacin D, cucurbitacin E and bacoside A were obtained from Chromodex, USA. Bacoside B (Provided by Sami Labs, Bengaluru, India) was a gift sample. All the reference standard markers used had more than 98% purity.

Plant material

The plant materials were procured from local market of New Delhi, India, and the specimen (Ref. NISCAIR/RHMD/Consult/-2010-11/1563/161/27/10-10) authenticated by botanist Dr. H. B. Singh, Scientist F and Head Raw Material Herbarium and Museum, NISCAIR, New Delhi.

Preparation of hydroalcoholic extract (mother extract) and its fractionation

The extraction was done by taking 500 g of powdered drug and extracting it using 70% ethanol as a solvent in soxhlet extractor on a water bath for 5 h. It was then filtered and vaporized to dryness under vacuum. Further, it was weighed to calculate percentage yields and extractive values. The hydroalcoholic extract of BM whole plant thus obtained was suspended in double distilled water and sonicated for 15 min at 45°C. Further, it was subjected to partitioning with different solvent, namely, hexane, DCM, and acetone. The extract left after partitioning was evaporated to dryness and excess was sonicated with acetone, methanol and water for 20 min each to prepare different fractions. All the fractions were vaporized to dryness under reduced pressure and yield percentage were calculated. The dried extract and fractions were further used for analysis and bioactivity.

Quality control analysis of extracts and fractions by high-performance thin-layer chromatography fingerprinting

HPTLC fingerprints of mother extracts and its fractions such as hexane, DCM, acetone, methanol, and water fractions were carried out for their quality control and determination of number of compounds present in them. ICH guidelines and several reported laboratory methods were followed for quality control of herbal drugs and botanicals.[10]

Sample preparation and chromatographic conditions

The dried mother extract and fractions (100 mg each) of BM were reconstituted using HPLC grade methanol in a 10 mL volumetric flask to get 10 mg/mL solution. These were sonicated and before being used for HPTLC analysis, 0.22 μM syringe filter was used for filtration. In triplicate (8.0 μL each), samples were applied, and the width of the track was 4.0 mm on precoated silica gel 60 F_{254} plates (E. Merck, 0.20 mm thickness), using Linomat V (HPTLC sample applicator). Linear ascending development was performed in 10 cm × 20 cm twin trough-glass chamber (Camag, Switzerland). The chamber saturation time for the solvent system [Table 1] was optimized and it was found 30 min at 25°C and 60% of relative humidity. The chromatogram was developed up to 85% of total TLC plate height. Developed chromatograms were scanned at 254 nm for DCM extract without derivatization, whereas at 520 nm for other extracts after derivatization with anisaldehyde sulfuric acid [Table 1]. The wavelengths for fingerprinting were selected by multi-wavelength scanning showing the highest number of peaks.

Table 1: High-performance thin-layer chromatography fingerprint data of mother extract and different fractionation of *Bacopa monnieri*

Extracts	Solvent system	Visualization	Number of major spots and R_f values
Hydroalcoholic extract of BM	Toluene: Ethyl acetate: Methanol: Ammonia (5:2:3:0.2 v/v/v/v)	Anisaldehyde sulfuric acid at 520 nm	(08) 0.16, 0.28, 0.37, 0.46, 0.50, 0.53, 0.60, 0.75
Hexane fraction	Toluene: Ethyl acetate (1.8:0.1 v/v)	Anisaldehyde sulfuric acid at 520 nm	(10) 0.10, 0.15, 0.22, 0.26, 0.43, 0.56, 0.62, 0.67, 0.73, 0.85
DCM fraction	Toluene: Ethyl acetate: Formic acid (7:3:1v/v/v)	At 254 nm	(07) 0.16, 0.22, 0.34, 0.38, 0.49, 0.57, 0.68
Methanol fraction	Toluene: Ethyl acetate: Methanol: Ammonia (3:2:3:0.5 v/v/v/v)	Anisaldehyde sulfuric acid at 520 nm	(07) 0.12, 0.25, 0.41, 0.45, 0.55, 0.79, 0.86
Acetone fraction	Toluene: Ethyl acetate: Methanol: Ammonia (3:2:3:0.5 v/v/v/v)	Anisaldehyde sulfuric acid at 520 nm	(07) 0.13, 0.21, 0.40, 0.43, 0.55, 0.80, 0.86

BM: *Bacopa monnieri*; DCM: Dichloromethane

Cytotoxicity testing by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay of *Bacopa monnieri* extract and its fractions

The cytotoxicity assay of mother extract and its hexane, DCM, acetone, methanol, and water fractions were carried out to find out a best active fraction.

Preparation of sample for *in vitro* activity

500 mg of each extract was dissolved in 10 mL of DMSO and then passed through a 0.45 μ membrane filter and stored at 4°C until used. The 50 mg/mL solutions of every extract/fractions as prepared above were diluted fifty times using RPMI-1640 media (1 mL to 50 mL) to get a concentration of 1 mg/mL of every extract/fractions. It was passed through 0.22 μ membrane filter before using for *in vitro* activity on different cell lines. Similarly, DMSO control was also prepared and used in every cell line.

3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cell proliferation assay was carried out using MTT formazon assay.[11] 1×10^4 cells of A549, HT-29, Caco2, Colo320, MCF-7, HeLa, SiHa, MDA-MB231 cell lines were inoculated onto 96-well plates filled out with 100 μL of the relevant culture media and the plates are then incubated at 37°C. After 24 h, it was replaced by 100 μL of freshly prepared media along with different concentrations of the extract/fractions (3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, and 500 μg/mL). The culture plates were subsequently incubated at 37°C for 24 h for three intervals separately at 37°C, by changing fresh media containing extracts/fractions at every interval. After the incubation period, media was replaced with fresh media along with 20 μL of MTT reagent was added to all the wells, followed by incubation for 3 h. The 100 μL of DMSO was then further added to every well and cells were put in the dark for around 1 h at room temperature. Through a microplate reader (Bio-Rad, USA), 570 nm was used to measure the intensity of purple color. Percentage cytotoxicity of these extracts was determined by exploiting the formula:

$$\{(A_c - A_s)/A_c\} \times 100.$$

where A_c and A_s are the absorbances of the control and sample respectively. All assays were executed in triplicate and reiterated, at least, three times.

Development and validation of high-performance thin-layer chromatography method for bacoside A, B analysis

For quantitative estimation of bacosides, a new, simple, precise, and economic HPTLC method has been developed. ICH guidelines and several reported laboratory methods were emanated for validation of developed HPTLC method.[10]

Preparation of standard solutions and their application on high-performance thin-layer chromatography

500 μg/mL of stock solution of bacoside A, B was made in HPLC grade methanol. A concentration of 250.0 μg/mL of each standard was obtained by mixing an equal volume of each stock solution (bacoside A, B). These mixed stock solutions were applied in triplicate in different volumes (0.1–10 μL) on HPTLC plate for the development of simultaneous HPTLC method and calibration plot. Ethyl acetate:methanol:water (70:20:10, v/v/v) was used as a solvent system for simultaneous elution of bacoside A and B. Developed chromatograms were derivatized by spraying with anisaldehyde sulfuric acid and then scanned at 450 nm for both qualitative and quantitative analysis of bacosides. A linear least square regressions equation were obtained by plotting a graph of peak area versus drug concentration and concentration range showing best regressions were considered for linearity and using this plot, concentration of unknown sample were determined.

Validation of developed methods

Validation of developed HPTLC method was followed ICH guidelines for calibration, linearity, robustness, precision, specificity, accuracy, limit of detection (LOD), and limit of quantification (LOQ), similar to the other methods reported from the laboratory.[10] The LOD was expressed as (LOD = 3.3 σ/slope), whereas LOQ was expressed as (LOQ = 10 σ/slope of calibration curve).

Precision

The precision of an analytical procedure was obtained from several sampling of the homogeneous sample under the similar conditions. The exactness of the proposed methods was obtained by repeatability and intermediate precision. Between day and intra-day, precisions were carried out by preparing and applying three separate concentration of standard (in triplicate) around the same time and in three separate days, individually. The inter-analyst precisions were carried out by repeating same procedure using a different system of the same make and by a different analyst. Precision studies were done at three concentration levels. The method of precisions was determined and reported in terms of % reflex sympathetic dystrophy (RSD).

Robustness of method

Robustness of the methods was carried out by introducing small changes in the different method parameters such as compositions of mobile phase and detection wavelength. The effect on the results was studied as % RSD.

Specificity

The specificity of the methods was determined by analyzing standard drug and sample. The detection of a spot for bacoside A and B in mother

extract and DCM fraction was confirmed by matching R_f and spectra of the spot with that of standards. The purity of peak was evaluated by looking at the spectra at three different levels, i.e., peak start, peak apex and peak end of the spot.

Accuracy as recovery

The accuracy of an analytical technique is the closeness of test outcomes got by that method to the hypothetical worth. Accuracy may often be expressed as percent recovery by the assay of added known amounts of analyte. The preanalyzed specimens were spiked with standard with four different concentration levels separately, i.e., 0, 50, 100, and 150% and the mixtures were reanalyzed by the developed methods.

Quantitative estimation of cucurbitacin B, D, E, betulinic acid and bacoside A and B in dichloromethane fraction

The previously developed methods for simultaneous estimation of cucurbitacin B, D, E, and betulinic acid[10] and newly developed method for bacoside A and B were used for quantitative determination of metabolite content. The samples were applied in triplicate on HPTLC plates with each standard separately and the contents of metabolites were analyzed, using regression equations obtained from calibration plots, and yield expressed as %w/w.

In vivo antitumor activity in Balb/c mice

The *in vivo* study was performed to carry out the anticancer activity of DCM fraction (in 1% carboxy methyl cellulose [CMC]) of hydroalcoholic extract of BM after oral administration to female Balb/c mice (25–30 g) as per the standard protocol. The dose of DCM fraction was decided as per its extractive value equivalent to the dose of the drug (4 g/daily). The activity was carried out under strict guiding principles of institutional animal ethics committee (CPCSEA) as per the approved protocol (project no 915/22.10.2012).

Animals and treatment schedule

Twenty-four female Balb/c mice were obtained from central animal house facility of University and apportioned into four groups, and each group had six animals each. Group I, served as control (nontumor mice, untreated), receiving 1% CMC (0.2 mL oral, once daily for 40 days). Other groups received Ehrlich ascites carcinoma (EAC) cells (2×10^6 cells/mouse, intraperitoneally (i.p.), which was obtained generously from Cell Culture Laboratory of Dr. Dwarka Nath, INMAS, New Delhi. Group II served as toxic control (tumor induced, untreated mice), whereas Group III received a suspension of DCM fraction (40 mg/kg body weight, orally), once daily. However, Group IV received standard 5-fluorouracil (5-FU) (20 mg/kg body weight, i.p.) once daily for 10 d, after 24 h of EAC transplantation.[12]

Analysis of tumor regression and hematological parameters after oral administration of dichloromethane fraction

The tumor regression parameters (packed cell volume [PCV], tumor volume, tumor weight, viable, and nonviable cell count) were analyzed after oral administration of the last dose. The mice from the individual group were kept fasting for 18 h and blood samples were collected in ethylenediamine tetra acetic-coated vials the following anesthesia with ketamine-xylazine by cardiac puncture for the estimation of hematological toxicity. The animals were then sacrificed by cervical dislocation for the study of antitumor activity. An automated hematologic

analyzer (MS9 Differential Cell Counter 3 Part, HD Consortium, India) were used hematologic examination.

Ascetic fluid was collected from the peritoneal cavity dissected mice. The tumor volume was determined by taking it in a graduated centrifuge tube and stated in a milliliter. The PCV was determined by centrifuging the ascetic fluid at 10,000 rpm for 5 min in a centrifuge tube. The volume of packed cells divided by the total volume of the ascetic fluid gives the % PCV. The tumor weight was determined by taking the weight of the mice before and after the collection of ascetic fluid from the peritoneal cavity and expressed in grams. The ascetic fluid was taken through a syringe and diluted 20 times with PBS. Then, a drop of diluted cell suspension was placed in the Neubauer's chamber, and the cells' number in 64 small squares was counted. Trypan blue assay was used for viability and nonviability of the cells. Trypan blue (0.4% in normal saline) dye was used for staining of the cell. Viable cell did not take up dye and nonviable cell took dye. Viable and nonviable cells were counted as:

Cell count = [number of cells × dilution factor]/area × thickness of the liquid film)

The hematological parameters such as white blood cells (WBC), total red blood cell (RBC), platelet (PLT), lymphocytes (LYM), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume, mean corpuscular hemoglobin (MCH), red blood cell distribution width (RDW) MCH concentration (MCHC), and MID cells (less frequently occurring and rate cells correlating to monocytes, eosinophils, basophils, etc.) were analyzed using a blood automatic analyzer.[10]

In silico screening

To have a better understanding of the mode of interactions of the phytochemical compounds present in DCM fraction, docking analysis was accomplished using the MOPAC 6 software package (Stewart Computational Chemistry, Colorado Springs, CO, USA). Different proteins were assumed that they might have interacted with targeted molecules (bacoside, betulinic acid, and cucurbitacin E). From the literature review, eight different proteins such as MDM2-p53 auto-regulatory loop,[13] Caspase-3, p53,[14] Bcl2 isoforms,[15] checkpoint kinase-1,[16] and palmitoyl protein thioesterase1[17] were selected for docking analysis.

Calculations of docking were performed on different protein model. Solvation parameters, kollman united atom type charges, and Essential hydrogen atoms were added with the aid of AutoDock tools.[18] Autogrid program was used for generation of affinity (grid) maps of × Å grid points and 0.375 Å spacing.[18] The van der Waals and the electrostatic terms were calculated by auto Dock parameter set and distance-dependent dielectric functions, respectively. Simulations of docking were executed using the Lamarckian genetic algorithm and the Solis and Wets local search method.[19] A random selection has been done for initial position, orientation, and torsions of the ligand molecules. During docking, all rotatable torsions were released. Individual docking experiment was originated from 2 different runs that were set to terminate after a maximum of 250,000 energy assessments. The population extent was set to 150. A translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied during the search. The structure of molecules in mol format was drawn in ChemDraw Ultra 7.0.1 (CambridgeSoft Corporation, Cambridge, USA) and converted to input ligand format (pdb) for docking by OpenBabel version 2.3.2 Open Babel: An open chemical toolbox (Journal of Cheminformatics 2011, 3:33).

Statistical analysis

Values were expressed as mean ± standard deviation. One-way analysis of variance followed by Dunnet's test for comparison analysis (GraphPad, San Diego, CA, USA) was used for the assessment of statistical

significance level. All the treatment groups were paralleled with the toxic control group. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The plant material was extracted using ethanol (70% v/v) by maceration and soxhlet extraction after optimization. The hot extraction was selected for study due to its high yields and called as mother extract (18.5%). Mother extract was further fractionated using hexane (9.9% w/w), DCM (28.6% w/w), acetone (14.7% w/w), methanol (24.6% w/w), and water (17.5% w/w). However, 4.2 g of hydroalcoholic extract (4.54% w/w) of the drug was lossed during the processing [Figure 1].

Hydroalcohalic extract (Mother extract): 500 gm of powdered herb extracted by reflux using 70 % alcohol on water bath (yielded 92.58gm, Extractive value 18.5 % w/w)

Hexane fraction: Yielded 9.2 gm, extractive value 9.9 % w/w of mother extract, 1.8% w/w of drug

Dichloroform (DCM) fraction: Yielded 26.5 gm, extractive value 28.64 % w/w of mother extract, 5.3% w/w of drug

Acetone fraction: Yielded 13.6 gm, extractive value 14.7 % w/w of mother extract, 2.72 % w/w of drug

Methanol fraction: Yielded 22.8 gm, extractive value 24.64 % w/w of mother extract, 4.56 % w/w of drug

Water fraction: Yielded 16.2 gm, extractive value 17.5 % w/w of mother extract, 3.24 % w/w of drug

Figure 1: Schematic representation of extraction and fractionation of hydroalcoholic extract (mother extract) of *Bacopa monnieri* showing extractive values

High-performance thin-layer chromatography fingerprinting of mother extract and different fractions

The HPTLC fingerprinting of mother extract, and different fractions were developed on silica gel. The DCM fraction showed maximum number of ultravilot active compounds and thus detected at 254 nm, whereas other fractions and mother extract were detected at 520 nm after visualization using anisaldehyde sulfuric acid reagents [Figure 2 and Table 1] showed different solvent system used for fingerprinting of extract/fractions with a number of spots present in them with their respective R_f values.

Cytotoxicity assay

The cytotoxicity of hydroalcoholic extracts and its fractions of BM on certain cancer cell lines were determined by MTT assay. The results of cytotoxicity assay of mother extract with hexane, DCM, acetone, methanol and water fractions are summarized in Table 2. DCM fraction (IC_{50} ranged from 41 to 60 µg/mL at 72 h) showed best cytotoxic activities for all cancer cell lines as compared to others. The best cytotoxic activity of DCM fraction from *Bacopa monnieri* (DBM) may be attributed due to the existence or synergistic activities of phytochemical components including sterol, triterpenes and polyphenols.[14,15,20] However, betulinic acid may have been attributed this activity since DBM is rich in same as proved by our analytical studies. MTT assay results of all four cell lines proved that cytotoxicity was highest in DCM followed by Methanol fraction and mother extract at 72 h as shown in Figure 3, similar to results of 24 h and 48 h as shown in Table 2. The water fraction did not produce any cytotoxicity up to 500 µg, whereas hexane fraction showing only poor cytotoxicity.
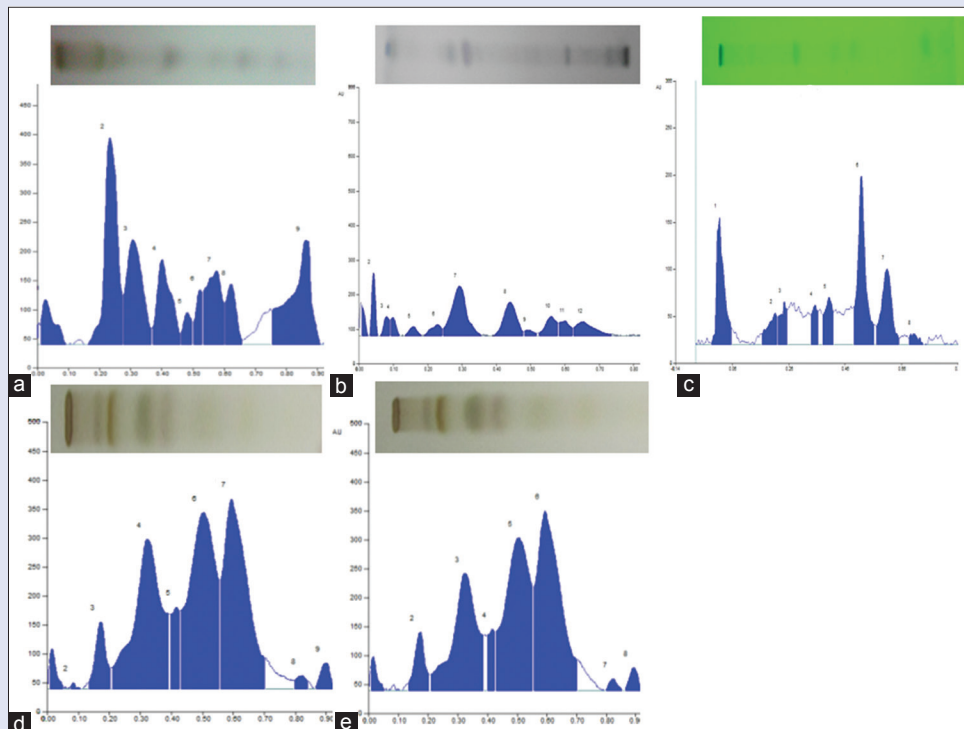

Figure 2: Developed thin-layer chromatography plates and high-performance thin-layer chromatography chromatogram for hydroalcoholic extract (a), hexane fraction (b), dichloromethane fraction at 254 nm underivatised (c), methanol fraction (d) acetone fraction (e) of *Bacopa monnieri* at 520 nm after derivatisation with anisaldehyde sulfuric acid showing peaks of separated compounds
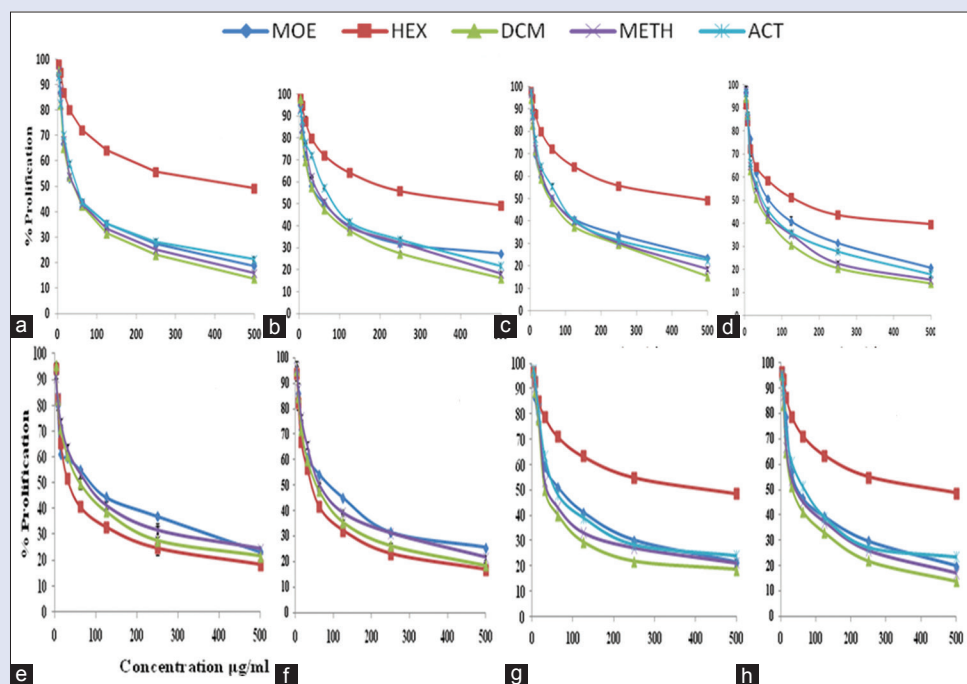
Figure 3: Scatter graph showing concentration of extract/fractions of *Bacopa monnieri* vs percent proliferation of HeLa (a), MCF-7 (b), SiHa (c), MDA-MB231 (d), Colo 320 (e), Caco2 (F), HT-29 (g) and A-549 (h) cell lines at 72 h
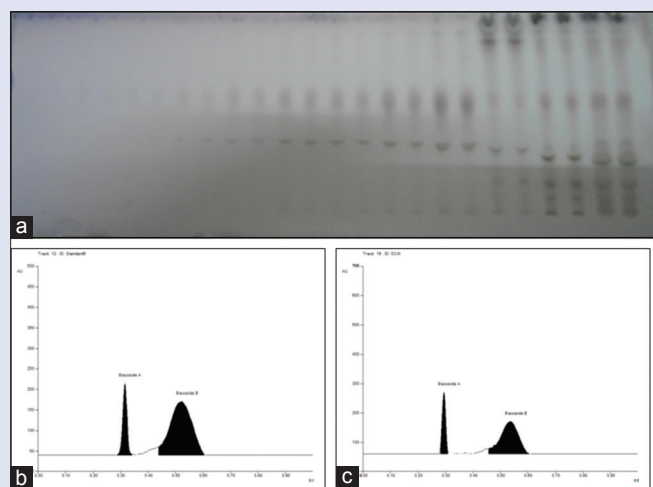

Figure 4: Developed high-performance thin-layer chromatography plate at 450 nm showing spots of bacoside A, B at different concentrations in standard and in sample (a). High-performance thin-layer chromatography chromatograms of standard bacoside A, B (b), sample (c)

Validation of validated high-performance thin-layer chromatography method for quantitative analysis of bacoside A and B

Ethyl acetate:methanol:water (70:20:10, v/v/v) as the solvent system was developed for separation and quantification of bacoside A, B which gave good separation of components. The plate was scanned at 450 nm wavelengths after derivatization with anisaldehyde sulfuric acid, which produces exceptionally well-characterized peaks of bacoside A, B at R_f values 0.32 ± 0.01, 0.52 ± 0.02, respectively [Figure 4]. Validated HPTLC

method was used for the analysis of bacoside A and B in DCM fraction. The peak areas of samples were used for quantitative analysis of bacoside A and B by exploiting regression equation obtained from the linearity plot. Content obtained for different markers are reported in Table 3.

Calibration curves and linearity

The newly developed methods for simultaneous estimation of bacoside A, B were found linear in the wide range of concentration with good regression coefficient (>0.99). The linearity data of all the biomarkers developed such as range linearity, regression equation, regression coefficient, slope, intercept, LOD, and LOQ are shown in Table 3.

Precision

Precisions of developed method such as intra-day and inter-day precision were determined and reported in terms of % RSD at three different concentration levels. The percentage RSD of inter-day precision and intra-day precision were found in the range of 1.74–2.01 for all compounds as reported in Table 4.

Robustness of the method

The low values of % RSD obtained after incorporating slight but thoughtful changes in the composition of the solvent system and wavelength indicated the robustness of the methods [Tables 5a and 5b] at 3 different concentration levels.

Specificity

The specificity of the methods was determined by analyzing standard drugs and samples. The detection of a spot for bacoside A, B in DCM fraction was confirmed by matching R_f and spectra of the spot with that standard. The peak purity was evaluated by evaluating the spectra at three different levels i.e., peak start, peak apex, peak end positions of the spot.

Limit of detection and limit of quantification

The LOD and LOQ of different markers were computed as per reported standard protocol[10] and are reported in Table 3. The LOD of markers are in the range of 33.0–66.5 ng, indicating the good sensitivity of methods for simultaneous quantification of compounds.

Accuracy as recovery

Accuracy in terms of recovery of developed method was calculated by standard addition method by spiking 0%, 50%, and 100%, 150% of analyte in analyzed samples, which showed better recovery of all biomarkers used and are in the range of 99%–101.4% [Table 6].

Quantitative estimation of cucurbitacin B, D, E, betulinic acid and bacoside A and B in dichloromethane fraction

The peak areas of triplicate samples were evaluated by regression equation obtained from the linearity plot. Content obtained for different phytoconstituents is shown in Table 7. Cucurbitacin D was found absent whereas bacoside A content was maximum (3.89 ± 0.016) in DBM.

In vivo anticancer activity of dichloromethane fraction from *Bacopa monnieri*

DBM showed a significant effect on tumor regression parameters of EAC cell-bearing mice. The DCM fraction significantly ($P < 0.01$) reduces the tumor volume, % PCV, tumor weight at a dose 40 mg/kg body weight in compare to EAC control groups as shown in Figure 5. The results were almost comparable to that of 5-FU, a standard drug. It was observed that there was a significant decrease in no of tumor cells on treatment with DCM fraction and 5-FU in tumor-bearing mice as compared to EAC control. Similarly, percentage viable cells were decreased significantly in treatment groups as compared to untreated EAC control [Table 8].

Hematological parameters of EAC tumor-bearing and treatment group mice were studied on the 14th day, which showed significant changes in the number of WBCs only and that was reversed in treated groups as compared to untreated EAC control. Other parameters such as Hb, RBC, LYMs, HCT, RDW, PLT, etc., were found to near normal and did not produce any significant alteration [Table 9].

The reliable principles for arbitrating the healthier value of any anticancer drug are the loss of leukemic cells from the blood, reduction of solid tumor volume, and prolongation of life span. Tumor grafting includes a local inflammatory reaction, with increasing vascular penetrability resulting in an intense ascetic fluid accumulation.[2] Our results showed a significant reversal of tumor regression parameters complemented by a reduction in WBC count in DBM treated mice. The DCM/enriched fraction also constrained the accumulation of ascetic fluid in the peritoneal cavity of the tumor-bearing animals. Above results clearly revealed the antitumor effect of *BM* on EAC tumor cells.

In silico screening

Considering results obtained from *in vitro* and *in vivo* study, it was thought worthy to perform molecular docking studies which correlate *in silico* results with *in vivo* and *in vitro* results. Docking studies are used to predict a ligand-receptor interaction and also to rank the compounds based on the binding energies or fitness score.[20] In the present study,

Table 2: Cytotoxicity assay of hydroalcoholic extract/fractions of *Bacopa monnieri* in different cell line showing IC_{50} at 24, 48, 72 h

Extract/fractions	Cell line	IC_{50} (µg/mL)		
		24 h	48 h	72 h
Hydroalcoholic extract (mother extract)	A 549	122.0	86.0	68.0
	HT-29	116.0	91.0	74.0
	Caco 2	125.0	105.0	77.0
	Colo 320	121.0	95.0	72.0
	HeLa	72	63	52.0
	MCF-7	128.0	106.0	73.0
	SiHa	110.0	86.0	75.0
	MDA-MB 231	175.0	91.0	77.0
Hexane fraction	A 549	NC	NC	370.0
	HT-29	NC	NC	373.0
	Caco 2	NC	NC	NC
	Colo 320	NC	NC	NC
	HeLa	NC	422.0	383.0
	MCF-7	NC	418.0	380.0
	SiHa	NC	421.0	384.0
	MDA-MB 231	484.0	382.0	149.0
DCM fraction	A 549	74.0	59.0	45.0
	HT-29	78.0	66.0	53.0
	Caco 2	80.0	64.0	50.0
	Colo 320	82.0	65.0	53.0
	HeLa	63.0	51.0	44.0
	MCF-7	67.0	65.0	56.0
	SiHa	74.0	65.0	60.0
	MDA-MB 231	61.0	44.0	41.0
Acetone fraction	A 549	104.0	75.0	64.0
	HT-29	132.0	100.0	74.0
	Caco 2	99.0	85.0	71.0
	Colo 320	109.0	94.0	75.0
	HeLa	83.0	70.0	57.0
	MCF-7	136.0	105.0	92.0
	SiHa	90.0	85.0	81.0
	MDA-MB 231	85.0	78.0	56.0
Methanol fraction	A 549	89.0	65.0	55.0
	HT-29	100.0	82.0	63.0
	Caco 2	96.0	74.0	64.0
	Colo 320	103.0	75.0	68.0
	HeLa	72.0	60.0	50.0
	MCF-7	110.0	84.0	72.0
	SiHa	84.0	75.0	69.0
	MDA-MB 231	71.0	56.0	49.0
Water fraction	A 549	NC	NC	NC
	HT-29	NC	NC	NC
	Caco 2	NC	NC	NC
	Colo 320	NC	NC	NC
	HeLa	NC	NC	NC
	MCF-7	NC	NC	NC
	SiHa	NC	NC	NC
	MDA-MB 231	NC	NC	NC

NC: Noncytotoxic upto 500 µg/mL, DCM: Dichloromethane

Table 3: Linearity data of chromatographic high-performance thin-layer chromatography method for bacoside A and B ($n=3$)

Biomarkers	Solvent system	Linearity (ng/spot)	Equation	Regression±SD	Slope±SD	Intercept±SD	LOD (ng/spot)	LOQ (ng/spot)
Bacoside A	Ethyl acetate: methanol:water (70:20:10, v/v/v)	100-2500	$Y=155.80+1.006x$	0.994±0.002	1.006±0.004	155.80±0.04	33.0	100.0
Bacoside B		200-2500	$Y=390.0+3.983x$	0.997±0.001	3.96±0.03	390.00±0.604	66.47	201.44

SD: Standard deviation; LOD: Limit of detection; LOQ: Limit of quantification

Table 4: Precision of the method for the estimation of bacoside A and B

Concentration (ng/spot)	Inter-day precision		Intra-day precision	
	Mean peak area±SD	% RSD	Mean peak area±SD	% RSD
Bacoside A				
250	412.66±8.02	1.94	409.88±7.50	1.82
500	681.0±12.52	1.83	684.33±12.22	1.78
1000	1375.33±24.11	1.75	1380±24.06	1.74
Bacoside B				
250	1494.67±29.50	1.97	1490.33±30.0	2.01
500	2529.0±47.14	1.86	2519.33±46.45	1.84
1000	4650.67±90.74	1.95	4654.67±83.51	1.79

SD: Standard deviation; RSD: Relative standard deviation

docking of tested compounds with targeted proteins was performed, and the corresponding fitness score was also determined as shown in Table 10. The Estimated free energies for interaction followed the order of the best fitness score. Highest fitness scored compound was further subjected to compare its interaction surface and total intermolecular energy with targeted molecules and proteins separately.[21] Protein-ligand interaction in docking studies resulted with lowest free energy and highest frequency. Estimated free energy of ligand-protein interaction was found < -5.2 for MDM2-p53 auto-regulatory loop, Caspase-3, p53, Bcl2 alpha isoform, checkpoint kinase-1, palmitoyl protein thioesterase 1 and this interaction indicating that expression of these proteins might be changed after oral administration of DCM fraction. Interaction of protein and ligand were shown in Figures 6-8. Some previous study suggested that anti-tumor

Table 5a: Robustness of the high-performance thin-layer chromatography method for estimation of bacoside A and B by changing detecting of wavelengths

Parameters				Mean area±SD	Percentage of RSD of area
	Components	Concentration (ng/spot)	Wavelength used		
Detecting wavelength (nm) 450	Bacoside A	250	448	409.66±8.62	2.10
			452	412.66±8.02	1.94
		500	448	687.33±13.50	1.96
			452	691.33±13.02	1.88
		1000	448	1387.66±25.10	1.80
			452	1375.33±24.11	1.75
	Bacoside B	250	448	1493.66±28.02	1.87
			452	1496.0±28.51	1.90
		500	448	2528.66±48.41	1.91
			452	2519.33±46.45	1.84
		1000	448	4646.66±83.51	1.79
			452	4653.66±88.68	1.90

SD: Standard deviation; RSD: Relative standard deviation
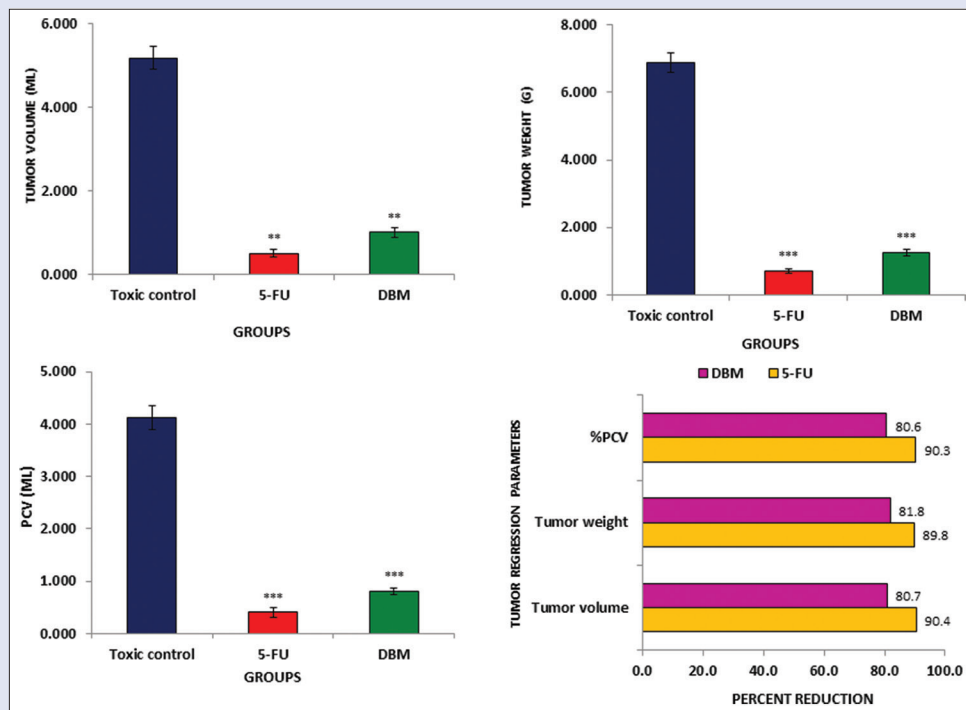

Figure 5: Tumor regression parameters as obtained on Balb/c mice after fourteen days oral administration of DBM

Table 5b: Robustness of the high-performance thin-layer chromatography method for estimation of bacoside A and B by changing detecting of mobile phase composition

Parameters			Mean area±SD	% RSD of area
Components	Concentration (ng/spot)	Mobile phase composition (ethyl acetate: Methanol:water 70:20:10, v/v/v)		
Bacoside A	250	72:18:10	409.66±8.62	2.10
		68:22:10	418.0±7.93	1.89
		70:22:08	411.0±7.54	1.86
	500	72:18:10	691.33±13.57	1.96
		68:22:10	682.66±12.50	1.83
		70:22:08	689.66±13.5	1.95
	1000	72:18:10	1371.66±25.16	1.83
		68:22:10	1382.33±23.54	1.70
		70:22:08	1385.0±27.83	2.01
Bacoside B	250	72:18:10	1493.33±29.09	1.94
		68:22:10	1490.33±29.50	1.97
		70:22:08	1495.0±29.51	1.97
	500	72:18:10	2528.66±48.41	1.91
		68:22:10	2520.33±46.45	1.84
		70:22:08	2513.33±49.40	1.96
	1000	72:18:10	4651.0±87.0	1.87
		68:22:10	4653.33±92.73	1.99
		70:22:08	4648.66±82.0	1.76

SD: Standard deviation; RSD: Relative standard deviation

Table 6: Accuracy of the high-performance thin-layer chromatography methods for the estimation of bacoside A and B

Percentage of standard spiked to the sample	Theoretical content (µg/mL)	Amount of drug recovered (µg/mL)	Percentage of drug recovered	% RSD
Bacoside A				
0	390	388.76	99.68	0.47
50	585	585.83	100.14	0.25
100	780	782.0	100.25	0.18
150	975	977.73	100.28	0.21
Bacoside B				
0	230	229.96	99.98	0.72
50	345	345.4	100.11	0.37
100	460	460.83	100.18	0.44
150	575	575.70	100.12	0.35

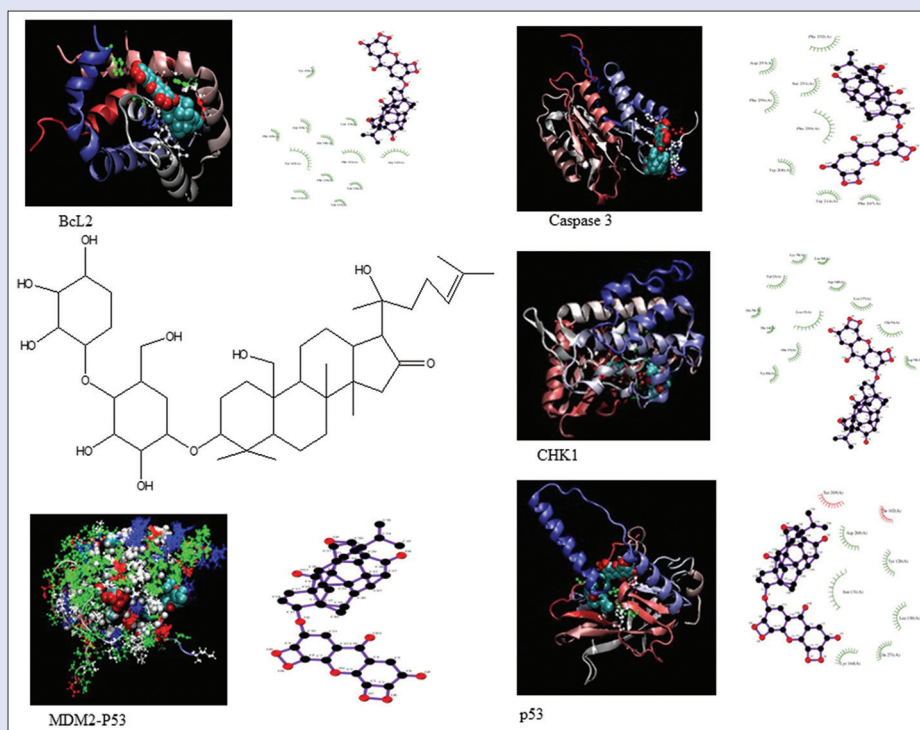RSD: Relative standard deviation

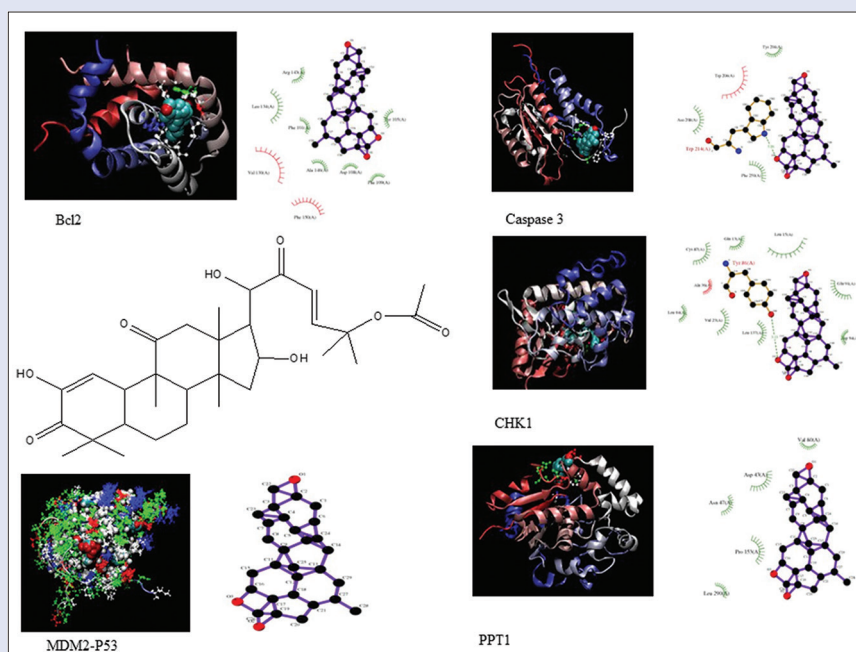**Figure 6:** Superimposed and two-dimensional interaction overview of bacoside with Bcl2, Caspase 3, CHK1, MDM2-P53 protein

Figure 7: Superimposed and two-dimensional interaction overview of bittulinic acid with Bcl2, Caspase 3, CHK1, MDM2-P53, PPT1
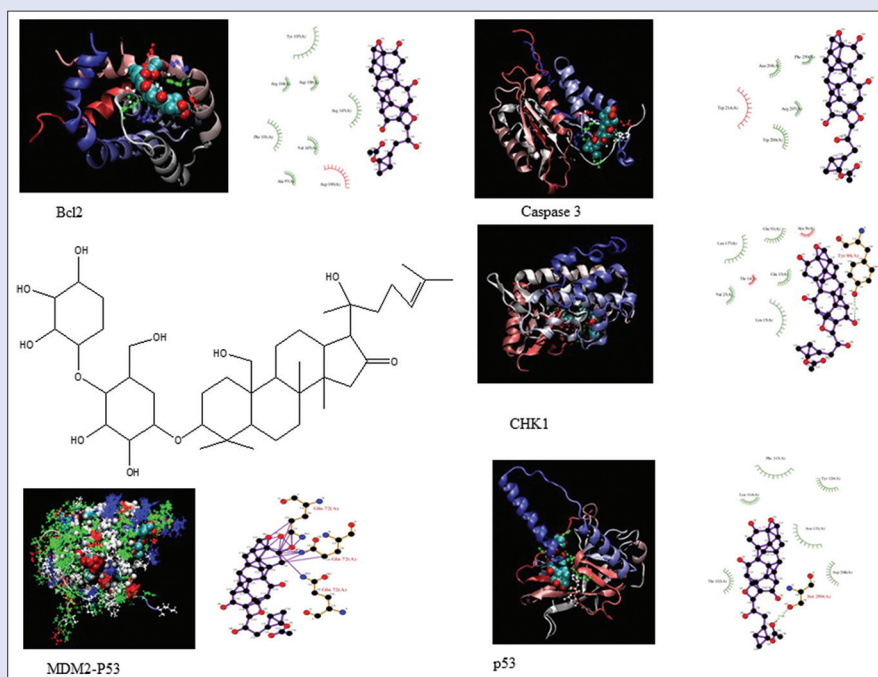

Figure 8: Superimposed and two-dimensional interaction overview of cucurbitacin E with Bcl2, Caspase 3, CHK1, MDM2-P53, p53

Table 7: Content of cucurbitacin B, D, E, bittulinic, bacoside A and bacoside B acid in dichloromethane fraction of *Bacopa monnieri*

Components	Percentage yield from DBM (%w/w)
Cucurbitacin B	0.022±0.00152
Cucurbitacin E	1.311±0.0075
Cucurbitacin D	Nil
Betulinic acid	0.841±0.0025
Bacoside A	3.89±0.016
Bacoside B	2.31±0.0251

DBM: Dichloromethane fraction of *Bacopa monnieri*

activity of *Bacopa* due to bacosides,[9,22] cucurbitacins,[23,24] and also betulinic acid.[25] This docking study presented probable mechanism of action of anti-tumor activity of DBM. Further, a molecular-based study is needed for confirmation of this above-proposed mechanism.

CONCLUSION

Through the above results, it can be concluded that mother extract and all other fractions of BM show cytotoxic potential, yet it was DCM fraction which expressed most significant cytotoxic strength. DCM fractions

Table 8: Tumor cell count of all the groups (mean±standard deviation, *n*=3)

Groups	Total cells/mL × 10^7±SD	Viable cells/mL × 10^7±SD	Nonviable cells/mL × 10^7±SD	Percentage of viable cells±SD	Percentage of nonviable cells±SD
EAC	9.93±0.81	9.43±0.57	0.50±0.59	95.22	4.77
5-FU	2.91±0.33	0.53±0.17	2.38±0.31	18.28	81.71
DBM	3.15±0.61	1.16±035	1.98±0.72	38.6	61.34

EAC: Ehrlich ascites carcinoma; 5-FU: 5-fluorouracil; DBM-Dichloromethane fraction of *Bacopa monnieri*; SD: Standard deviation

Table 9: Comparative hematological profile of ehrlich ascites carcinoma, control, standard and dichloromethane fraction of *Bacopa monnieri* after drug administration

Parameters	Control±SD	EAC control±SD	5-FU±SD	DBM±SD
WBC (×10^3/μL)	5.20±0.26	9.50±0.47	3.10±0.155	0.43±0.01
RBC (×10^6/μL)	9.36±0.46	7.29±0.36	8.79±0.440	8.85±0.35
HGB (g/dL)	13.40±0.67	10.70±0.53	13.00±0.650	12.57±0.50
HCT (%)	45.80±2.29	36.30±1.81	45.00±2.250	44.10±1.76
MCV (fL)	48.90±2.44	49.80±2.49	51.20±2.560	49.80±1.99
MCH (pg)	14.30±0.71	14.70±0.73	14.80±0.740	14.60±0.58
MCHC (g/dL)	29.30±1.46	29.30±1.46	28.90±1.445	29.10±1.16
PLT (×10^5/μL)	7.08±0.35	11.84±0.59	6.83±0.342	7.130±2.85
RDW (fL)	29.20±1.40	29.90±1.49	30.40±1.520	29.40±1.17
PDW (fL)	9.10±0.45	10.40±0.52	9.00±0.450	9.40±0.37
MPV (fL)	7.40±0.37	7.90±0.39	6.90±0.345	7.10±0.28
P-LCR (%)	9.80±0.49	11.90±0.59	6.10±0.305	9.20±0.36

WBC: White blood cell; RBC: Red blood cell; HGB: Hemoglobin; HCT: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; PLT: Platelet; RDW: Red blood cell distribution width; MPV: Mean platelet volume; PDW: Platelet distribution width; P-LCR: Platelet-to-large-cell ratio; SD: Standard deviation; EAC: Ehrlich ascites carcinoma; 5-FU: 5-fluorouracil; DBM-Dichloromethane fraction of *Bacopa monnieri*

Table 10: Docking summary

Metabolites	Parameters	Bcl2	Bcl2 X1	Caspase 3	CHK1	MDM-p53	p53	PPT1	TRPV-1
Bacoside A	Estimated free energy of binding ($E_{FreeBind}$)	−10.46	−1.73	−7.85	−8.78	−8.05	−5.58	+65.21	+0.16
	Interaction surface	1066.9	271.63	790.8	1004.76	873.34	841.98	989.85	818.65
	Final intermolecular energy ($E_{InterMol}$)	−11.34	−2.39	−8.62	−9.58	−9.08	−6.72	+64.45	−0.64
	E_{VHD} (vdW + Hbond + desolv Energy)	−11.20	−252	−8.78	−9.84	−9.01	−6.31	+64.4	−0.21
Betulinic acid	Estimated free energy (kcal/mol)	−9.49	−2.48	−7.72	−10.05	−8.6	−6.05	−6.05	−6.37
	Interaction surface	736.38	269.07	679.52	755.84	726.7	595.19	−689.9	572.19
	Total intermolecular energy (kcal/mol)	−9.49	−2.48	−7.72	−10.05	−8.60	−6.05	−6.05	−6.37
	vdW + Hbond + desolv energy (kcal/mol)	−9.36	−2.46	−7.71	−10.02	−8.57	−6.08	−6.08	−6.35
Cucurbitacin E	Estimated free energy (kcal/mol)	−6.12	−1.03	−6.81	−8.29	−5.87	−5.49	+3.06	−4.76
	Interaction surface	776.36	249.62	748.27	859.01	802.7	711.07	754.6	662.38
	Total intermolecular energy (kcal/mol)	−7.34	−1.96	−8.09	−9.86	−6.93	−6.74	+1.29	−5.82
	vdW + Hbond + desolv Energy (kcal/mol)	−7.42	−1.95	−8.15	−9.8	−7.01	−6.48	+1.5	−5.86

showed therapeutic efficacy against EAC tumor-bearing mice while giving protection against malignancy incited modified physiological conditions.

The developed and validated quantification method of bacoside A and B, cuccurbitacin B, E and betulinic acid can be utilized for quality control and for other drugs containing them as an ingredient. From the above findings, it could be inferred that DCM fraction of BM displayed powerful anticancer action as exhibited from *in vitro* cytotoxicity such as Colon, Lung, Cervix, and Breast cell lines. In addition, EAC tumor model in mice also showed significant reversal of tumor regression parameters which was again supported by *in silico* study.

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Conflicts of interest

There are no conflicts of interest.

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