In vitro Safety Evaluation and Anticlastogenic Effect of BacoMind™ on Human Lymphocytes

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Objective BacoMind™ (BM) is a standardized extract of *Bacopa monnieri*, which belongs to the family Scrophulariaceae and is a creeping annual plant found throughout the Indian subcontinent. It has been used by Ayurvedic medicinal practitioners in India for almost 3000 years and is classified as a *medharasayana*, a substance which improves memory and intellect. With the widespread traditional use as well as scientific validation of *Bacopa monnieri* for nootropic activity, a bioactive-rich unique phytochemical composition-BacoMind™ was developed from *B. monnieri* for use as a cognition and memory enhancing agent. The present study aimed to investigate the *in vitro* toxicity of this formulation of BacoMind™ on human lymphocytes and to rule out its possible contribution to mutagenicity. **Methods** In the present investigation the active ingredients present in BM were identified and quantified by high performance liquid chromatography (HPLC) and high performance thin-layer chromatography (HPTLC). Antioxidant and anticlastogenic properties of BM were studied *in vitro* with and without metabolic activation. Doses of BM were chosen on the basis of mitotic index (MI) and cytokinesis-block proliferation index (CBPI). Clastogenicity assays were performed at 31.2 μg/mL, 62.5 μg/mL, and 125 μg/mL, while the *Salmonella* reverse mutation assay (Ames test) was performed at doses of 61.72, 185.18, 555.55, 1666.67, and 5000.00 μg/plate. **Results** HPLC and HPTLC analysis of BM revealed the presence of bacoside A_3, bacopaside I, bacopaside II, jujubogenin isomer of bacopasaponin C, bacosine, luteolin, apigenin, bacosine, and β-sitosterol D glucoside. BM demonstrated significant antioxidant activity. The number of chromosomal aberrations and the frequency of micronuclei induced by BM were not statistically significant up to a dose of 62.5 μg/mL. A subsequent dose of 125 μg/mL prior to metabolic activation induced mild clastogenicity, but it was found to be biologically insignificant as this effect was not seen post metabolic activation. BM also demonstrated a dose-dependent protection against the clastogens used in this study using the above tests for clastogenicity. Maximum protection was observed in presence of metabolic activation. Moreover, BM demonstrated no mutagenic effect on the tested strains, as observed in the Ames test. **Conclusion** BM protected human lymphocytes against various clastogens. BM also exhibited high antioxidant activity which might be responsible for the observed protective effects against the clastogens since the used clastogens are known to induce their clastogenic effects *via* production of oxidative radicals.

Key words: BacoMind™; Cytotoxicity; Chromosomal aberration; Ames test; Micronucleus; Clastogens; Antioxidant; High performance liquid chromatography; High performance thin-layer chromatography (HPTLC)

INTRODUCTION

Bacopa monnieri has been used by Ayurvedic medical practitioners in India for almost 3000 years and is classified as a *medhyarasayana*, a drug used to improve memory and intellect (medhya). *Bacopa monnieri* has been mentioned in several ancient Ayurvedic treatises including the *Caraka Samhita* (6th century AD) and the *Bravprakash Var-Prakarana* (16th century AD), in which it is recommended for the management of a range of mental conditions including anxiety, poor cognition and lack of concentration. *Bacopa monnieri* is currently recognized as being effective in the treatment of mental illness and epilepsy[1].

Bacopa monnieri, which belongs to the family Scrophulariaceae, is a creeping annual plant found throughout the Indian subcontinent[2]. This medicinal plant is locally known as 'Brahmi'. It is a small herb with purple flowers, which grows in wet and sandy areas and near streams in tropical regions. Extracts of *B. monnieri* were reported to have several pharmacological effects, such as anxiolytic[3], anti-oxidant[4-5], anti-ulcerogenic[6], anti-*Helicobacter*

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pylori[7], and mast cell stabilizing activities[8].

Literature survey also reveals the memory and cognition enhancement property of *B. monnieri*. Preliminary studies have established that the treatment with *B. monnieri* plant, its aqueous decoction and the alcoholic extract enhance learning ability in rats. Preclinical studies have reported cognition enhancing effects with various extracts of *B. monnieri*[9-11].

In view of the importance of this plant in the indigenous system of medicine, systematic chemical examinations of the plant have been carried out by several groups of researchers. Earlier studies reported the isolation of alkaloid "brahmine", nicotine, herpestine, hersaponin, jujubogenin, pseudojujubogenin, bacopasaponin G, and bacoside A (major chemical entity) from *B. monnieri*, which are responsible for the memory – facilitating action of *B. monnieri*[12].

With the widespread traditional use as well as scientific validation of *Bacopa monnieri* for nootropic activity, a bioactive-rich unique phytochemical composition-BacoMind™ was developed from *B. monnieri* for use as a cognition and memory enhancing agent. BacoMind™ differs from previously reported standardized extracts in that it has been standardized with reference to nine different bioactive constituents.

Since the use of an herbal extract for animal or human consumption necessitates the generation of safety data, the present study was performed to evaluate the safety of BacoMind™ using the common methods of genotoxicity and mutagenicity evaluation in human lymphocytes and bacteria, respectively.

MATERIALS AND METHODS

Chemicals and Media

DMEM culture media, penicillin, streptomycin, and phytohemagglutinin M were purchased from Gibco-BRL, Invitrogen Corporation (UK). Heat-inactivated fetal bovine serum was obtained from Life Technologies, (UK). Mitomycin C (MMC), benzo[a]pyrene (B[a]P), apigenin, luteolin, cytochalasin-B (Cyt-B), colchicine, dimethyl sulfoxide (DMSO), Aroclor-1250, 2-amino anthracene (2-AA), 2 amino fluorene (2-AF), danthron, methyl methane sulphonate (MMS), N-naphthyl ethylene diamine, superoxide dismutase (SOD), 2.2-azinobis-ethyl-benzothiozoline-sulphonic acid diammonium salt (ABTS), 2.2'-azo-bis (2-amidino propane) dihydrochloride (AAPH), thiobarbituric acid (TBA), catechins, 1.1-diphenyl-2-picryl hydrazyl (DPPH), lipopolysaccharide (LPS), and sulfanilamide were procured from Sigma, (St. Louis, MO, USA). 4-Nitro quinolene-N-oxide (NQNO) and sodium azide were obtained from Fluka, Germany. All other chemicals and solvents used in this study were of the highest analytical grade available.

Bacterial Strains

Histidine auxotrophic TA 97a, TA 98, TA 100, TA 1535, and TA 102 strains of *Salmonella typhimurium* were obtained from Bruce Ames Laboratory, University of California, Berkeley, USA.

Preparation of BacoMind™

BacoMind™, an enriched phytochemical composition of *B. monnieri,* developed by Natural Remedies Pvt. Ltd., (patent pending) was standardized to the content of the following bioactive constituents, *viz*., bacoside A_3 (>5.0% w/w), bacopaside I (>7.0% w/w), bacopaside II (>5.5% w/w), jujubogenin isomer of bacopasaponin C (>7.0% w/w), bacopasaponin C (>4.5% w/w), bacosine (>1.5% w/w), luteolin (>0.2% w/w), apigenin (>0.1% w/w), bacosine (>1.5% w/w) and β-sitosterol-d-glucoside (>0.3% w/w). It was further standardized using the following *in vitro* bioassays *viz*., lipoxygenase inhibition assay (IC_{50}<600 µg/mL), ABTS radical scavenging assay (IC_{50}<100 µg/mL), DPPH assay (IC_{50}<200 µg/mL) and butyrylcholinesterase inhibition assay (IC_{50}<3000 µg/mL).

HPLC Analysis

Estimation of bacoside A_3, bacopaside I, bacopaside II, jujubogenin isomer of bacopasaponin C, bacopasaponin C, bacosine, luteolin and apigenin

The HPLC system comprised a Shimadzu LC10A pump, a SPD-M 10A*vp* photo diode array detector with Class-VP software and a column [LiChrospher 100, RP-18e (5µm) 250 × 4 mm (id)] Merck, Germany. Quantitative determination was done at 205 nm.

The mobile phase comprised a gradient with two solvents – Solvent A and Solvent B.

a) Solvent A was prepared by dissolving 0.136 g of anhydrous potassium dihydrogen orthophosphate (KH_2PO_4) in 900 mL of HPLC grade water (obtained from Millipore, Milli-Q water purification system) and adding 0.5 mL of orthophosphoric acid to it. The solution was made up to 1000 mL with water, filtered through a 0.45 µm membrane filter and degassed in a sonicator for 3 min.

b) Solvent B was acetonitrile.

The gradient system adopted was as follows:

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Time (min)	Buffer Concentration (Solvent A)	Acetonitrile Concentration (Solvent B)
0.01	70	30
25	60	40
35	40	60
40	60	40
43	70	30
45	70	30

Estimation of Bacosine

Identification and quantification of the bacosine was carried out with a mobile phase consisting of a mixture of 5% acetonitrile in water adjusted to pH 2.8 with orthophosphoric acid and methanol (1: 9).

The injected sample volume was 20 μL. The column temperature was maintained at 35℃ and the flow rate of the mobile phase was maintained at 1.5 mL/min throughout the experiment. Retention time and peak areas of compounds were compared with those of standards for the purpose of their identification and quantification.

HPTLC Analysis

The system comprised a spotting device (Linomat N, CAMAG) and a dual wavelength flying spot scanning densitometer (CS 9301 PC, Shimadzu). TLC plates comprised precoated silica gel on aluminium sheets (SilicaGel 60F254, 20 × 20 cm, Merck). Samples were spotted on the TLC plates using the Linomat N, CAMAG device. Plates were run on a glass chamber containing a solvent of chloroform: methanol (9:1). The plates were dried, sprayed with anisaldehyde sulfuric acid reagent and heated at 100℃ for 10 min. They were then analyzed using a densitometer at a wavelength of 530 nm.

Antioxidant Status

Hydroxyl radical scavenging assay The hydroxyl radical scavenging assay was performed according to the method of Halliwell *et al.*[13]. The reaction volume of 1.0 mL contained 5.6 mmol/L deoxyribose, 2.8 mmol/L H_2O_2, 40 μmol/L ferric chloride, 100 μmol/L EDTA and varying concentrations of BacoMind™ in 2.5 mmol/L phosphate buffer (pH 7.4). Initiation of the reaction was performed by the addition of 0.1 mmol/L ascorbic acid. The mixture was then incubated for 90 min at 37℃. This was followed by the addition of 1 mL of thiobarbituric acid (0.7% in 0.05 N KOH) and 1 mL of 2.5% trichloroacetic acid to the mix. It was then heated at 100℃ for 8 min, cooled, and the pink color formed was measured spectrophotometrically at 532 nm. Controls were run which were devoid of test samples. Catechin was used as the reference standard and IC_{50} values were calculated for BM using Log-Probit regression analysis.

Superoxide scavenging activity using the PMS-NADH assay system The PMS-NADH assay was carried out according to the method of Yen *et al.*[14]. 250 μL of test sample BM/reference standard (various concentrations) in 0.1 mol/L phosphate buffer (pH 7.4), 250 μL of NADH solution (468 μmol/L), 250 μL of NBT solution (150 μmol/L) and 250 μL of PMS (60 μmol/L) were incubated at room temperature for 5 min. The resulting absorbance was measured at 560 nm. Controls were run which were devoid of test samples. IC_{50} values were calculated using Log-Probit regression analysis. Gallic acid and catechin were used as the reference standards.

DPPH scavenging assay The DPPH scavenging assay was performed as described earlier by Vani *et al.*[15]. 25 μL of different concentrations of BM and gallic acid were added to different test tubes containing methanol. Later, 75 μL (1.3 mg/mL) of DPPH stock solution was added to all the test tubes and made to a final volume of 3 mL. Control samples were run which were devoid of test samples. Anti-radical activity was measured by a decrease in the absorbance (516 nm) in the presence of BacoMind™ at different concentrations after 15 min. IC_{50} values were calculated using regression analysis.

ABTS radical scavenging assay The ABTS radical scavenging activity assay was performed as described by Auddy *et al.*[16]. ABTS radical cations were produced by reacting 7 mmol/L ABTS with 2.45 mmol/L ammonium persulphate and by incubating the mixture at room temperature in the dark for 16 h. The solution obtained was further diluted with phosphate buffered saline (PBS) pH 7.4 to give an absorbance of approximately 1.0. Different concentrations of BM and the reference standard (highest volume taken was 50 μL) were added to 950

μL of ABTS working solution to give a final volume of 1 mL, made up by adding PBS. The absorbance was recorded immediately at 734 nm. IC_{50} values were calculated using regression analysis.

Nitric oxide scavenging assay The nitric oxide scavenging assay was carried out according to the method of Sreejayan *et al.*[17]. 200 μL of 10 mmol/L sodium nitroprusside and 200 μL of BM/reference standard of various concentrations were incubated at room temperature for 150 min. Following incubation, 500 μL of Griess reagent (1% sulfanilamide in 5% orthophosphoric acid and 0.1% N-(1-napthyl) ethylenediamine dihydrochloride in a 1:1 ratio) was added to the tubes and they were incubated for 10 min at room temperature. The absorbance was measured at 546 nm. Controls were run devoid of test samples. IC_{50} values (μg/mL) were calculated using Log-Probit regression analysis. Curcuminoids was used as the reference standard.

TABLE 1a

HPLC Data for BacoMind™

Peak	Retention Time	Compound Name
1	8.043	Luteolin
2	10.513	Bacopaside-I
3	12.631	Apigenin
4	17.231	Bacoside A3
5	17.949	Bacopaside II
6	19.510	Jujubogenin of Bacopasaponin C
7	20.608	Bacopasaponin

TABLE 1b

HPLC Data for BacoMind™

Peak	Retention Time	Compound Name
8	7.141	Bacosine

TABLE 1c

HPTLC Data for BacoMind™

Peak	Compound Name
9	β-sitosterol D Glucoside

Statistical Analysis for Antioxidant Assays

The mean value ± SD was calculated for each parameter. IC_{50} values were calculated using Log-Probit regression analysis.

In vitro Human Lymphocyte Metaphase Analysis

The tests were performed according to OECD Guideline 473[18] for chromosomal aberration assay and Guideline 487 for micronucleus assay[19]. Blood samples were collected from three healthy non-smoking individuals (2 males and 1 female) having an age group of ± 28.

Metabolic Activation System

The procedure of Ames *et al.* and Garner *et al.* was used for the preparation of rat liver homogenate (S-9)[20]. Sprague-Dawley rat liver was stimulated following an intraperitoneal (IP) injection of sodium phenobarbital (dissolved in corn oil) at a dose of 80 mg/kg/day for five consecutive days. On the sixth day of induction, following an overnight fasting, the rats were killed for liver homogenate (S-9). The Bradford assay (595 nm) was used to measure the protein concentration in the S-9 fraction. All steps of this preparation were performed at 0-4℃ with cold sterile solutions and glassware. The S-9 fraction was distributed in 2 mL aliquots in small sterile plastic tubes and stored at -80℃. The S-9 mix was prepared following the method of Maron and Ames[21].

Determination of the Cytotoxicity of the Test Substance

The cytotoxic effect on human lymphocytes was determined by measurement of mitotic index among 1000 cells and cytokinesis-block proliferation index among 500 cells examined per dose per subject. BM was tested at 31.2 μg/mL, 62.5 μg/mL, 125 μg/mL, 150 μg/mL, and 250 μg/mL.

Sample Preparation for Clastogenic Assay

Around 0.5 mL of human venous blood was added to 3.5 mL of DMEM (human lymphocytes culture media) supplemented with 20% of fetal bovine serum, to which phytohemagglutanin (PHA) (50 μg/mL), antibiotics (penicillin 100 IU/mL and streptomycin 50 μg/mL) and heparin sodium salt (5000 IU) (0.4 mL/100 mL) were added and incubated at 37℃ for a period of 72 h depending on the experimental conditions. After 24 h (for micronucleus assay) and 48 h (for chromosomal aberration assay) following culture initiation, lymphocytes were treated either with BM at different doses (31.2 μg/mL, 62.5 μg/mL, 125 μg/mL, 150 μg/mL, and 250 μg/mL) or with a combination of BM and clastogens simultaneously. MMC was used at a dose of 0.29 μmol/L for the CA study and at 0.51 μmol/L for the MN study. H_2O_2 was used at a dose of 20 mmol/L for CA and at 1024 μmol/L for MN. Both these clastogens were used in the absence of metabolic activation and cells were treated for a total period of 20 h for CA and 46 h for MN. B[a]P, on the other hand, was used only with metabolic activation (5% S-9 mix). It was used at doses of 30 μmol/L for CA and for MN with 4 h of cell treatment. After 4 h

of treatment (in the presence of the S-9 fraction), the cultures were washed, re-fed with complete medium and further incubated at 37℃ for a total incubation period of 72 h (5% CO_2).

Chromosomal Aberration Assay

Cells were treated with colchicine (0.1 μg/mL) for 2 h before harvesting (at 70th h of incubation). The cultures were processed and the slides were prepared for CA according to the method of Api and San[22]. A hundred well spread metaphases were scored for aberration study *viz.*, chromatid and chromosome breaks, fragments, exchanges, rings, gaps. Mitotic index (MI) was scored from 1000 cells per culture.

Micronucleus Assay

At the 44th h of incubation, cultures were treated with 0.6 μg/mL of Cytochalasin B to arrest the cells in a binucleated state and incubated till the completion of assay (72th h). At the end of the incubation period, the cultures were processed and the slides were stained with 4% Giemsa stain for 10 mins. About 2000 binucleated cells with well-preserved cytoplasm were scored for the presence of micronucleus[23]. Cytokinesis-block proliferation index (CBPI) was calculated among 500 cells per culture, according to the following formula: CBPI= [MI + 2MII + 3(MIII + MIV)]/N as proposed by Surralles *et al.*[24], where MI-MIV represents the number of human lymphocytes with 1-4 nuclei and N is the total number of cells scored (500).

Cytotoxicity Assay for Ames Test

Dose range finding study was performed using tester strain TA 100 in the presence and absence of microsomal enzymes[25]. Six concentrations of the test substances ranging from 61.72 to 5000 μg/plate were assessed for toxicity study. Dimethyl sulphoxide (DMSO) was used as solvent control. BM was considered to be toxic if there was a decrease in the number of revertants and/or thinning or absence of background colonies. There was no cytotoxicity observed up to concentration of 5000 μg/plate. Therefore concentrations of 61.72 μg/plate, 185.18 μg/plate, 555.55 μg/plate, 1666.67 μg/plate, and 5000 μg/plate were used for the study.

Ames Assay (Salmonella Reverse Mutation Assay)

BM was added onto *Salmonella typhimurium* strains TA 1535, TA 97a, TA 98, TA 100, and TA 102 (100 μL/plate of overnight culture) with and without the addition of 0.5 mL of S-9 fraction using plate incorporation assay as described by Maron and Ames[23]. The concentrations of BM used were 61.72, 185.18, 555.55, 1666.67, and 5000 μg/plate. The plate for negative control contained 100 μL of DMSO, with or without S-9 mix. The positive controls for TA 1535 had sodium azide (0.5 μg/plate) without S-9 mix and 2-AA (0.5 μg/plate) with S-9 mix. TA 98 and TA 97a contained 2-AF (10 μg/plate) with S-9 mix and NQNO (0.5 μg/plate) without S-9 mix. TA 100 contained 2-AF (10 mg/plate) with S-9 mix and MMS (1 μL/plate) without S-9 mix. TA 102 contained Danthrox (30 μg/plate) with S-9 mix and MMS (1 μL/plate) without S-9 mix. The mean number of histidine revertants for all the treatment groups were compared with the number of revertants in the respective solvent control group. The colonies were counted manually after 48 h of incubation at 37℃. In this assay a mutagenic substance is one, which at one or more concentrations, shows a reproducible increase in the number of revertant colonies per plate in at least one strain with or without metabolic activation (i.e. with S-9 mix).

Statistical Analysis

The mean value ± SD was calculated for each parameter. The data were subjected to one-way ANOVA test using Graph Pad Prism software. In order to examine the toxicity profile of BM, it was compared with the cell controls. The protective effect of BM was evaluated by comparing simultaneously treated cultures (BM + clastogens) with clastogen treated controls.

RESULTS

Figs. 1a and 1b, and Tables 1a, 1b describe the chromatogram of the HPLC of BacoMind™; while Fig. 1c and Table 1c describe the HPTLC profile of BM. The individual compounds present in this formulation were identified and quantified using respective standards. Quantification of the compounds was carried out on dry weight basis. BM contained bacoside A_3 (80 mg/g), bacopaside I (89 mg/g), bacopaside II (68 mg/g), jujubogenin isomer of bacopasaponin C (90 mg/g), bacopasaponin C (52 mg/g), bacosine (23 mg/g), luteolin (4 mg/g), apigenin (2 mg/g), bacosine (22 mg/g) and β-sitosterol D glucoside (9 mg/g).

Figs. 2, 3, 4, and 5 exhibit the concentration dependent free radical scavenging activity of BM in a variety of *in vitro* assay systems. The hydroxyl radical scavenging activity of BM was determined against biochemically generated hydroxyl radicals. Catechin was used as a reference standard. BM and catechin exhibited IC_{50} values of 4063.13 μg/mL and

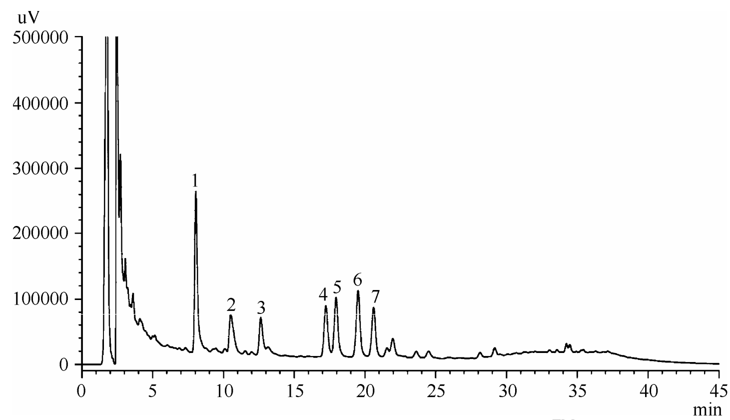

FIG. 1a. HPLC chromatogram of BacoMind^{TM}.

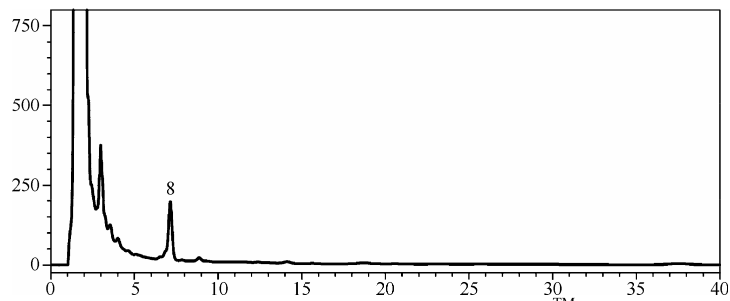

FIG. 1b. HPLC chromatogram of BacoMind^{TM}.

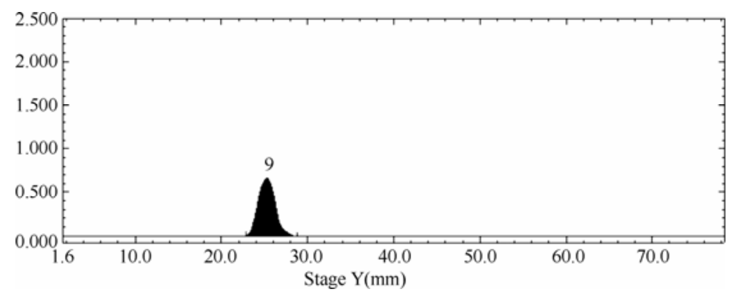

FIG. 1c. HPTLC chromatogram of BacoMind^{TM}.

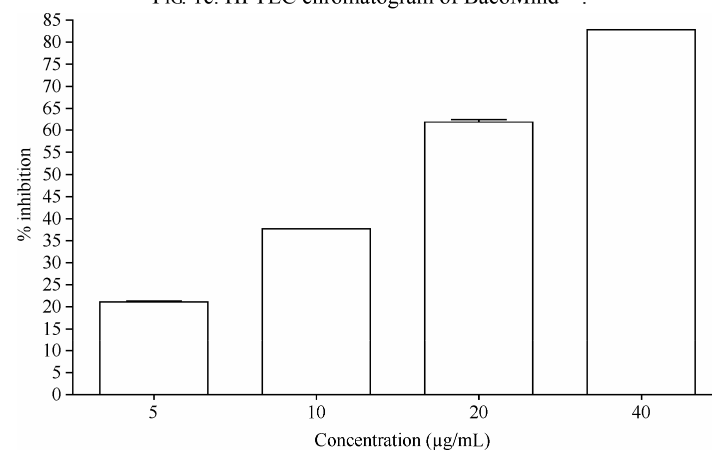

FIG. 2a. Effect of gallic acid on super-oxide scavenging activity.

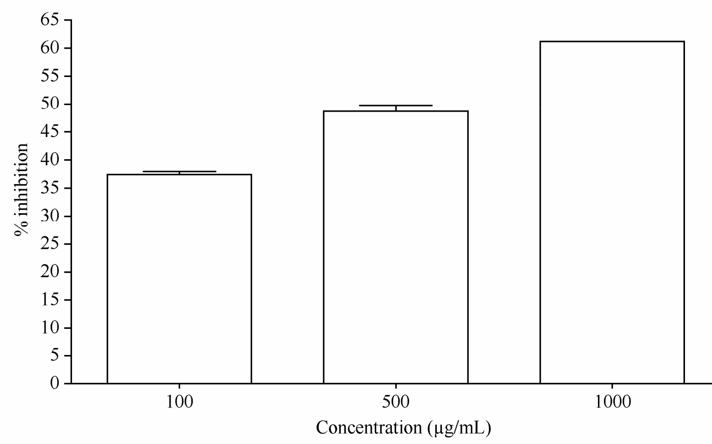

FIG. 2b. Effect of BacoMind™ on super-oxide scavenging activity.

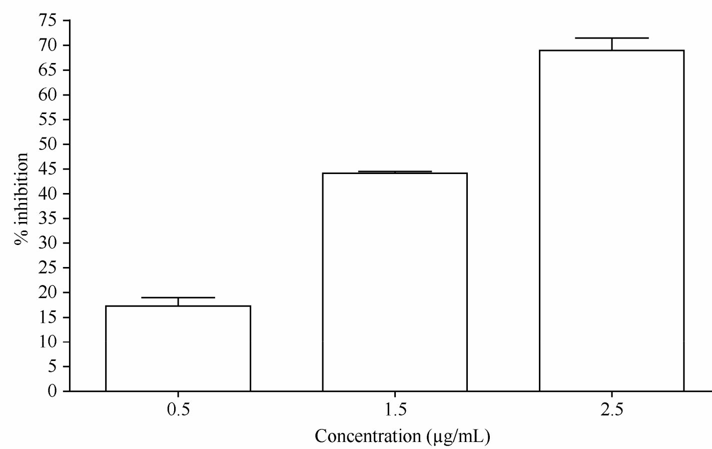

FIG. 3a. Effect of gallic acid on DPPH radical scavenging activity.

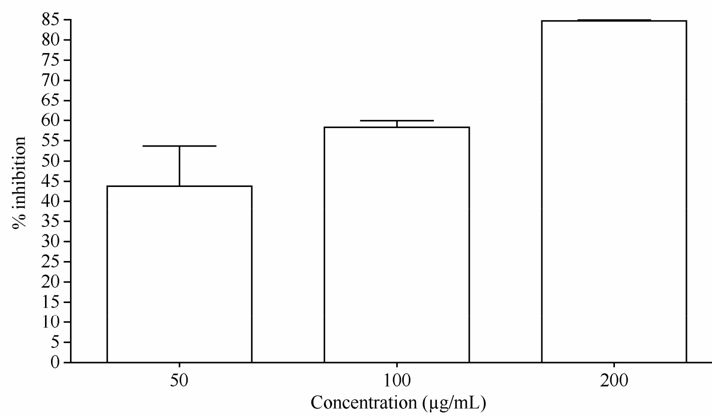

FIG. 3b. Effect of BacoMind™ on DPPH radical scavenging activity.

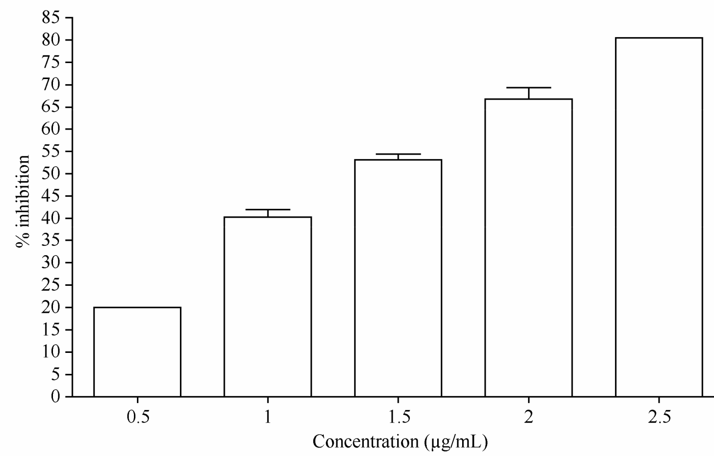

FIG. 4a. Effect of gallic acid on ABTS radical scavenging activity.

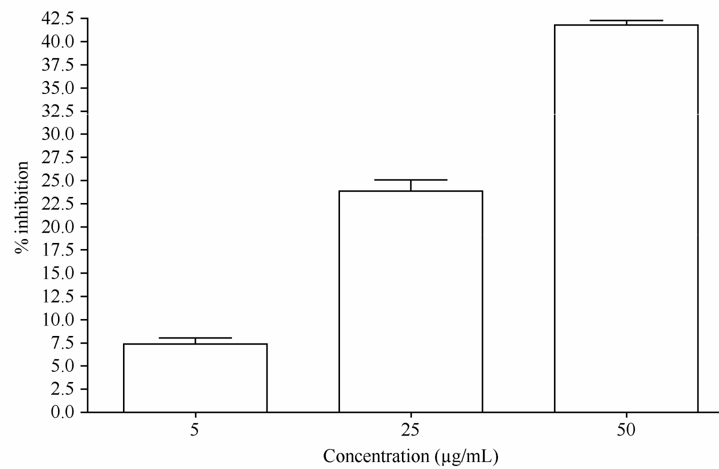

FIG. 4b. Effect of BacoMind^{TM} on ABTS radical scavenging activity.

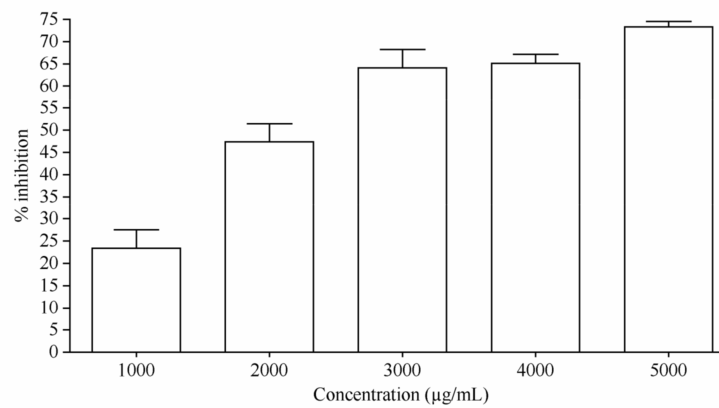

FIG. 5a. Effect of Catechin on hydroxyl radical scavenging activity.

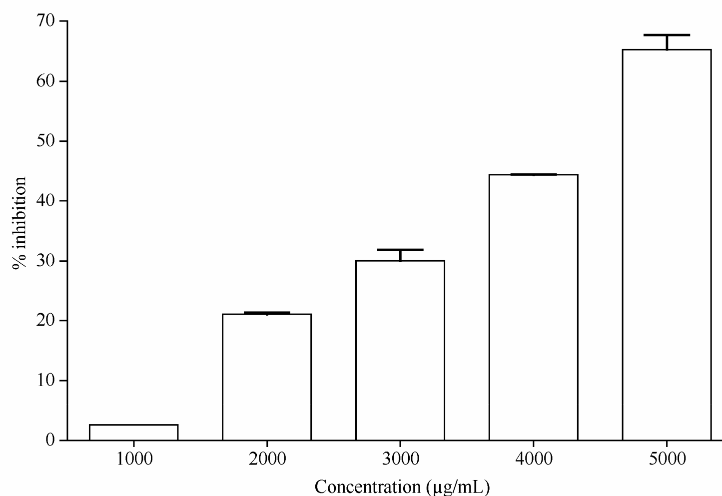

FIG. 5b. Effect of BacoMind™ on hydroxyl radical scavenging activity.

2253.0 µg/mL respectively. BM exhibited superoxide anion scavenging activity in the PMS-NADH system. The IC_{50} value of BM in this assay was 478.7 µg/mL as compared with an IC_{50} of 11.44 µg/mL obtained with reference standard gallic acid. In the DPPH radical scavenging activity assay, an IC_{50} value of 65.57 µg/mL was obtained with BM while standard gallic acid gave an IC_{50} of 1.33 µg/mL. BM also exhibited ABTS radical scavenging activity and the IC_{50} value was observed to be 80.49 µg/mL, whereas the standard (gallic acid) showed an IC_{50} value of 1.49 µg/mL. However, BM was not effective in nitric oxide scavenging assays.

The cytotoxic potential of BM was determined before studying the clastogenic profile of BM on the basis of mitotic index (MI) and cytokinesis block proliferative index (CBPI). BM at concentrations of 150 µg/mL and 250 µg/mL demonstrated a decrease in the level of MI and CBPI, when compared with cell control. However, at doses of 31.2 µg/mL, 62.5 µg/mL, and 125 µg/mL, the reduction in the MI and the CBPI ratios were not significant. Based on the cytotoxicity data, the three concentrations (31.2, 62.5, and 125 µg/mL) were selected for chromosomal aberration (CA) and micronucleus (MN) studies.

Tables 2 and 3 demonstrate the clastogenic potential of BM on human lymphocyte cultures (HLC) as compared with commonly used clastogens MMC, H_2O_2, and B[a]P. MMC and H_2O_2 effects were studied without metabolic activation while B[a]P effect was studied with metabolic activation. Treatment of human lymphocytes with BM (without metabolic activation) at concentrations of 31.2 µg/mL and 62.5 µg/mL had no significant effect on the number of chromosomal aberrations (CA) observed when compared with negative controls. However, BM at 125 µg/mL showed a slight increase in the number of aberrant cells. Treatment of human lymphocytes with BM along with S-9 mix (with metabolic activation) demonstrated no clastogenic effect on BM at doses 31.2 µg/mL to 125 µg/mL.

Studies were also performed to determine the protective effects of BM against clastogenic damage induced by MMC, H_2O_2, and B[a]P. The maximum aberrations observed in the CA assay were obtained with 0.29 µmol/L MMC (49% aberrations), 20 mmol/L H_2O_2 (40% aberrations) and 30 µmol/L B[a]P (48% aberrations). BM demonstrated a protective effect against H_2O_2, MMC, and B[a]P induced chromosomal aberrations. In the presence of 31.1 µg/mL BM, the percentage protection observed was 40% against H_2O_2 induced damage and 49% against MMC induced damage. The maximum protection observed with BM was at the highest dose of 125 µg/mL. At this dose, BM showed 60% and 65% protection from H_2O_2 and MMC induced CA, respectively. The above experiments were carried out in the absence of metabolic activation (without S-9 mix).

On challenging the cells with B[a]P (with S-9 mix), BM at dose of 31.1 µg/mL demonstrated 60% protection in the CA assay. The maximum protection observed with BM was at the highest dose of BM used (125 µg/mL) in these studies. At this dose, BM showed 72% protection from B[a]P induced CA. Interestingly, better protection was observed with BM in metabolic activation experiments in comparison with experiments performed without metabolic activation.

Tables 4 and 5 show the number of MN per 2000

TABLE 2

Effect of Simultaneous Treatment With BacoMind™ on Mitomycin C and Hydrogen Peroxide Induced Chromosomal Aberrations Within Human Lymphocytes Without Metabolic Activation

Test Substance	No. of Metaphases	Percentage of Aberrated Metaphases ($\bar{x} \pm s$)	No. of Aberrated Metaphases	Types of Aberration								Mitotic Index ($\bar{x} \pm s$)	Percentage Protection
				G	Ctb	Chb	R	M	F	Dic	Exc		
Cell Control	200	4.7 ± 1.5	5	2	1	ND	ND	ND	2	ND	ND	5.5 ± 0.2	----
Cell Control (0.1% DMSO)	200	7.3 ± 1.8	8	3	2	ND	ND	1	2	ND	ND	5.3 ± 0.2	---
Mitomycin C (0.1 µg/mL)	200	$46.5 \pm 1.6^{+}$	49	6	12	6	2	8	8	3	4	$2.4 \pm 0.2^{+}$	---
Hydrogen Peroxide (20 mmol/L)	200	$39.3 \pm 1.5^{+}$	40	7	12	7	2	5	4	1	2	$2.6 \pm 0.1^{+}$	---
BacoMind™ (31.1 µg/mL)	200	7.2 ± 1.6	8	3	3	ND	ND	ND	2	ND	ND	5.3 ± 0.2	---
BacoMind™ (62.4 µg/mL)	200	9.7 ± 1.9	10	2	3	1	ND	2	2	ND	ND	5.0 ± 0.2	---
BacoMind™ (125 µg/mL)	200	$13.2 \pm 1.3^{++}$	13	2	4	2	ND	2	2	1	ND	$4.9 \pm 0.2^{++}$	---
BacoMind™ (31.1 µg/mL) +MMC (0.1 µg/mL)	200	$24.2 \pm 1.5^{*}$	25	4	5	2	ND	5	7	2	ND	$4.7 \pm 0.3^{*}$	49
BacoMind™ (62.4 µg/mL) + MMC (0.1 µg/mL)	200	$19 \pm 1.4^{*}$	19	6	6	3	ND	ND	2	ND	ND	$4.8 \pm 0.2^{*}$	61
BacoMind™ (125 µg/mL) + MMC (0.1 µg/mL)	200	$16.7 \pm 1.0^{*}$	17	5	4	1	ND	3	4	ND	ND	$5.1 \pm 0.2^{*}$	65
BacoMind™ (31.1 µg/mL) + H_2O_2 (20 mmol/L)	200	$23.3 \pm 1.8^{**}$	24	6	6	2	ND	3	5	2	ND	$4.6 \pm 0.2^{**}$	40
BacoMind™ (62.4 µg\mL) + H_2O_2 (20 mmol/L)	200	$19.2 \pm 1.6^{**}$	20	4	5	ND	ND	5	4	1	1	$4.8 \pm 0.2^{**}$	50
BacoMind™ (125 µg\mL) + H_2O_2 (20 mmol/L)	200	$16 \pm 2.2^{**}$	16	7	5	ND	ND	2	2	ND	ND	$4.9 \pm 0.2^{**}$	60

Note. The results are average of three sets of experiments in duplicate. ND: not detected; G: Gap; Ctb: chromatid break; Chb: Chromosome break; R: Ring; M: Minute; Exc: Exchange; F: Fragment; Dic: dicentric; $^{+}P<0.001$ comparing to cell control, $^{++}P < 0.01$ comparing to cell control, $^{*}P < 0.001$ comparing to MMC, $^{**}P < 0.001$ comparing to H_2O_2.

TABLE 3

Effect of Simultaneous Treatment With BacoMindTM on Benzo[a]pyrene Induced Chromosomal Aberrations Within Human Lymphocytes After Metabolic Activation

Test Substance	No. of Metaphases	Percentage of Aberrated Metaphases ($\bar{x} \pm s$)	No. of Aberrated Metaphases	Types of Aberration								Mitotic Index ($\bar{x} \pm s$)	Percentage Protection
				G	Ctb	Chb	R	M	F	Dic	Exc		
Cell Control (With S-9 mix)	200	3.5 ± 1.0	5	2	1	ND	ND	ND	2	ND	ND	5.6 ± 0.1	---
Cell Control (0.1% DMSO With S-9 mix)	200	4.8 ± 1.2	7	3	2	ND	ND	1	1	ND	ND	5.5 ± 0.2	---
Benzo[a]pyrene (30 µmol/L)	200	$43.7 \pm 1.6^{+}$	48	5	13	8	2	6	7	3	4	$2.6 \pm 0.2^{+}$	---
BacoMindTM (31.1 µg/mL) (With S-9 mix)	200	5.5 ± 1.0	6	1	2	ND	ND	1	2	ND	ND	5.4 ± 0.2	---
BacoMindTM (62.4 µg/mL) (With S-9 mix)	200	6.5 ± 1.0	7	2	3	ND	ND	ND	2	ND	ND	5.3 ± 0.2	---
BacoMindTM (125 µg/mL) (With S-9 mix)	200	8.8 ± 1.2	9	3	3	1	ND	1	1	ND	ND	5.2 ± 0.2	---
BacoMindTM (31.1 µg/mL)+ Benzo[a]pyrene (30 µmol/L)	200	$18.7 \pm 2.1^{*}$	19	6	5	3	ND	2	1	ND	ND	$4.8 \pm 0.2^{*}$	60
BacoMindTM (62.4 µg/mL)+ Benzo[a]pyrene (30 µmol/L)	200	$14.7 \pm 2.9^{*}$	15	5	4	1	ND	1	2	2	ND	$5.1 \pm 0.2^{*}$	69
BacoMindTM (125 µg/mL)+ Benzo[a]pyrene (30 µmol/L)	200	$12.7 \pm 4.0^{*}$	13	4	3	ND	ND	2	2	2	ND	$5.4 \pm 0.3^{*}$	73

Note. The results are average of three sets of experiments in duplicate. ND: not detected; G: Gap; Ctb: chromatid break; Chb: Chromosome break; R: Ring; M: Minute; Exc: Exchange; F: Fragment; Dic: dicentric; $^{+}P < 0.001$ comparing to cell control, $^{*}P < 0.001$ comparing to B[a]P.

TABLE 4

Effect of Simultaneous Treatment With BacoMind™ on Mitomycin C and Hydrogen Peroxide Induced Micronucleus Formation Within Human Lymphocytes Without Metabolic Activation

Treatment	Total Number of Binucleated Cells	Total Number of Micronucleated Cells: Without S9-mix Metabolic Activation ($\bar{x} \pm s$)	Total Number of Micronucleated Cells: CBPI ($\bar{x} \pm s$)	Percentage Protection
Cell Control	2000	5.5 ± 1.4	2.0 ± 0.1	---
Cell Control (0.1% DMSO)	2000	6.5 ± 1.0	1.9 ± 0.1	---
Mitomycin C (0.51 μg/mL)	2000	$46.3 \pm 2.1^{+}$	$1.3 \pm 0.1^{+}$	---
Hydrogen Peroxide (1024 μmol/L)	2000	$36.5 \pm 3.0^{+}$	$1.3 \pm 0.1^{+}$	---
BacoMind™ (31.1 μg/mL)	2000	7.0 ± 0.9	1.9 ± 0.1	---
BacoMind™ (62.4 μg/mL)	2000	8.0 ± 1.2	1.8 ± 0.1	---
BacoMind™ (125 μg/mL)	2000	10.0 ± 1.6	1.8 ± 0.1	---
BacoMind™ (31.1 μg/mL) +MMC (0.51 μg/mL)	2000	$19.2 \pm 2.0^{*}$	$1.9 \pm 0.1^{*}$	59
BacoMind™ (62.4 μg/mL) + MMC (0.51 μg/mL)	2000	$18.3 \pm 2.1^{*}$	$1.9 \pm 0.1^{*}$	60
BacoMind™ (125 μg/mL) + MMC (0.51 μg/mL)	2000	$14.5 \pm 2.3^{*}$	$1.8 \pm 0.2^{*}$	69
BacoMind™ (31.1 μg/mL) + H_2O_2 (1024 μmol/L)	2000	$20.3 \pm 2.8^{**}$	$1.9 \pm 0.1^{**}$	44
BacoMind™ (62.4 μg/mL) +H_2O_2 (1024 μmol/L)	2000	$16.8 \pm 1.9^{**}$	$1.8 \pm 0.2^{**}$	54
BacoMind™ (125 μg/mL) +H_2O_2 (1024 μmol/L)	2000	$13.8 \pm 1.5^{**}$	$1.8 \pm 0.1^{**}$	62

Note. The results are average of three sets of experiments in duplicate. ^{+}P <0.001 comparing to cell control, $^{*}P$ <0.001 comparing to MMC, $^{**}P$ <0.001 when comparing to H_2O_2.

TABLE 5

Effect of Simultaneous Treatment With BacoMind™ on Benzo[a]Pyrene Induced Micronucleus Formation Within Human Lymphocytes After Metabolic Activation

Treatment	Total Number of Binucleated Cells	Total Number of Micronucleated Cells		Percentage Protection
		With S9-mix Metabolic Activation ($\bar{x} \pm s$)	CBPI ($\bar{x} \pm s$)	
Cell Control (With S-9 Mix)	2000	4.8 ± 1.5	2.0 ± 0.1	---
Cell Control (0.1% DMSO and (With S-9 Mix)	2000	6.0 ± 0.7	1.9 ± 0.1	---
Benzo[a]pyrene (30 µmol/L)	2000	37.2 ± 1.5^{+}	1.3 ± 0.1^{+}	---
BacoMind™ (31.1 µg/mL) (With S-9 Mix)	2000	6.7 ± 2.2	1.9 ± 0.1	---
BacoMind™ (62.4 µg/mL) (With S-9 Mix)	2000	7.7 ± 2.7	1.9 ± 0.2	---
BacoMind™ (125 µg/mL) (With S-9 Mix)	2000	7.7 ± 1.4	1.8 ± 0.1	---
BacoMind™ (31.1 µg/mL) + Benzo[a]pyrene (30 µmol/L)	2000	17.5 ± 1.0*	1.8 ± 0.1*	53
BacoMind™ (62.4 µg/mL) + Benzo[a]pyrene (30 µmol/L)	2000	15.3 ± 1.5*	1.8 ± 0.2*	59
BacoMind™ (125 µg/mL) + Benzo[a]pyrene (30 µmol/L)	2000	9.2 ± 2.4*	1.8 ± 0.2*	75

Note. The results are average of three sets of experiments in duplicate. ^{+}P<0.001 comparing to cell control. $^{*}P$<0.001 comparing to B[a]P.

binucleated cells on treatment of human lymphocytes with BM both with and without metabolic activation and also depict the protective effect of BM against clastogen mediated damage. MMC, H_2O_2, and B[a]P at concentrations of 0.51 µg/mL, 1024 µmol/L, and 30 µmol/L respectively, showed statistically significant increases in the number of micronuclei observed in human lymphocytes when compared with cell controls. BM, on the other hand, had no significant effect on the number of micronuclei induced at the concentrations used in the study (31.1-125 µg/mL) as compared with the cell control.

The protective effects of BM against micronucleus formation induced by MMC, H_2O_2, and B[a]P were also studied. The maximum number of micronuclei per 2000 binucleated cells obtained with 0.51 µmol/L MMC was 46, with 1024 µmol/L H_2O_2 was 37 and with 30 µmol/L B[a]P was 36. BM demonstrated a protective effect against H_2O_2, MMC, and B[a]P induced micronucleus formation. At 31.1 µg/mL BM, the percentage protection was observed to be 44% against H_2O_2 and 59% against MMC induced micronuclei. The maximum protection observed with BM in was at 125 µg/mL. At this dose, BM showed 62% and 69% protection from H_2O_2 and MMC induced MN, respectively. The above experiments were carried out in the absence of metabolic activation (without S-9 mix).

On challenging the cells with B[a]P (with S-9 mix), BM at dose of 31.1 µg/mL demonstrated 53% protection. However, the maximum protection observed with BM in MN assays was at the highest dose of BM used (125 µg/mL). At this dose, BM showed 75% protection from B[a]P induced MN.

Table 6 depicts the effect of BM on mutagenicity using the *Salmonella* Ames test. The mean numbers of histidine revertants at all concentrations of BM (5000, 1666.67, 555.55, 185.18, and 61.72 µg/plate), with and without metabolic activation and in all the strains, as compared with the positive controls, were studied. BM did not induce any significant number of histidine revertants at any concentration level in any of the five test strains.

TABLE 6

Mutagenicity of BacoMind™ in Ames Test With *S. typhimurium* Strains TA 1535, TA 97a, TA98, TA100, and TA102

Treatment	Dose µg/plate	S-9	Revertants									
			TA 1535		TA 97a		TA 98		TA 100		TA 102	
			$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$
BacoMind™	5000	–	5.67	1.53	88.00	3.46	37.00	1.00	116.33	1.53	230.00	6.93
		+	7.00	1.00	93.33	3.06	37.67	1.53	120.33	1.53	242.00	3.46
	1666.67	–	10.00	1.00	96.67	3.06	38.67	1.53	120.00	2.00	258.00	2.00
		+	10.33	0.58	99.33	3.06	40.33	1.53	123.67	3.21	265.00	4.36
	555.55	–	9.33	0.58	102.00	4.00	39.67	1.53	125.00	3.00	274.67	3.06
		+	11.00	1.00	112	3.46	43.33	1.53	129.00	1.73	284.67	5.03
	185.18	–	11.00	1.00	109.33	3.06	42.67	2.08	136.33	0.58	294.33	2.52
		+	13.33	1.15	111.33	3.06	45.00	2.65	140.33	1.53	295.67	2.52
	61.72	–	12.00	1.00	114.00	4.00	47.67	1.53	142.33	2.52	302.00	4.0
		+	14.33	0.58	121.33	4.16	52.67	2.08	145.00	4.36	307.33	3.06
DMSO (Solvent Control)	100 µL	–	10.67	1.15	116.33	3.51	42.67	2.08	139.67	2.08	288.33	4.04
		+	12.00	1.00	122.67	6.43	43.33	1.53	144.33	4.04	292.00	5.29
MMS (µL/plate)	1.0	–	–	–		–	–	–	2271.33	33.72	4969.33	70.78
4NQNO	0.5	–	–	–	512.67	15.01	264.00	7.21	–	–	–	–
Sodium Azide	0.5	–	917.33	6.11	–	–	–	–	–	–	–	–
2 – AF	10.0	+	–	–	1142.00	7.21	1124.67	16.04	1162.00	22.27	–	–
DANTHRON	30.0	+	–	–	–	–	–	–	–	–	1186.00	30.27
2 – AA (Positive Control)	0.5	+	1146.00	8.72	–	–	–	–	–	–	–	–

Note. The results in all positive controls are significantly different from other groups.–S-9: in the absence of S-9: mix; +S-9: in the presence of S9 mix. DMSO-dimethyl sulfoxide; MMS-Methyl methane sulphonate; 2-AF-2-aminofluorene and 2-AA-2-Aminoanthracene.

DISCUSSION

In recent years much attention has been focused on the use of medicinal plants in the treatment of various health conditions. This necessitates studies on the potential toxicity of these compounds and on their safety levels. Moreover, governmental regulatory policies worldwide are now making it an imperative that all newly produced natural and synthetic substances, with or without anti-tumor properties, be subjected to genotoxic/mutagenic, carcinogenic/ teratogenic screenings, in at least two end-point models. We therefore carried out a battery of *in vitro* genotoxicity studies to estimate the safety profile of the formulated product BacoMind^{TM}.

Literature survey reveals that subchronic administration of a standardized bioactive-rich extract of *B. monnieri* is capable of reversing the cognitive deficits induced by colchicine and ibotenic acid[25]. One of the mechanisms by which it appears to reverse colchicine-induced reduction in frontal cortex is *via* reductions in hippocampal acetylcholine concentrations, choline acetyltransferase activity and muscarinic cholinergic receptor binding. An extract of *B. monnieri* has also shown potent cognitive enhancing activity by attenuating the dementia effect of scopolamine in rat passive avoidance test[26].

While going through the published scientific information on *Bacopa monnieri*, our group found serious discrepancies in the identification of reported bioactive constituents, *viz*., bacosides A and B. We raised apprehension on the published standardization data and the consequent problem in deriving meaningful conclusions[27]. Subsequent work in our laboratory indicated that 'bacoside A' was a mixture of four saponins as revealed by spectroscopic and HPLC methods[28]. In this scenario there was a need to develop and standardized extract of *Bacopa monnieri* with true claims on the content of active constituents. Based on these observations, a bio-active rich unique phytochemical composition (BacoMind^{TM}) was developed and standardized.

In order to establish the safety and genotoxic profile of this phytochemical composition, a series of short term *in vitro* toxicological studies were undertaken. The three end point models employed for screening the genotoxic potential of BM were chromosomal aberration assay, micronucleus assay and Ames *salmonella* reverse mutation assay. The doses of BM for the CA and MN assays were selected on the basis of MI and CBPI values using human lymphocytes; since those values are a measure of the cytotoxicity profile of a compound.

The treatment of human lymphocytes with BM alone demonstrated a statistically significant reduction in MI and CBPI at concentrations above 150 µg/mL. Thus, CA and MN studies were performed at lower doses of 31.1 µg/mL, 62.4 µg/mL, and 125 µg/mL of BM.

BM was found to exert a mildly clastogenic effect at a dose of 125 µg/mL in the absence of metabolic activation (S-9 mix). However, in the presence of metabolic activation, no sign of clastogenicity was observed in the CA assay at the above dose. In the MN assay, no clastogenicity was observed up to 125 µg/mL both in the absence and presence of metabolic activation. The lack of difference in the clastogenic potential of BM in the absence and presence of S-9 mix within the CA assay suggests that this compound is not transformed into toxic metabolites. Moreover, the differences observed amongst the CA and MN assays at a dose of 125 µg/mL could be due to the fact that the MN assay detects only acentric fragments (chromosome fragments lacking a centromere) while CA detects all structural aberrations. This seems to suggest that while BM may lead to minor chromosomal aberrations at 125 µg/mL in the absence of metabolic activation, this clastogenic effect is lost upon metabolic activation of BM. Since most compounds are transformed/metabolized by the liver enzymes after oral consumption, this study further suggests that BM is non-genotoxic and therefore safe for human consumption at non-cytotoxic doses.

BM at doses of 31.1-125 µg/mL was also found to protect human lymphocytes against the clastogenic effects of MMC, H_2O_2 and B[a]P in both CA and MN studies, suggesting that it possesses protective activity against clastogens at the doses used in this study. However, better protection was observed with BM in presence of metabolic activation compared with experiments performed in absence of metabolic activation. This seems to suggest that a metabolite of BM might be responsible for the protective effect observed against these clastogens. It is also possible that the toxic substances in BM are detoxified by the antioxidant enzymes present in the S-9 mix (e.g. catalase, superoxide dismutase *etc.*).

The clastogens used in these studies were MMC, H_2O_2, and B[a]P, which have been previously shown in the literature to cause chromosomal aberrations both *in vitro* and *in vivo*[29-30]. MMC and H_2O_2 exert their clastogenic effects in the absence of metabolic activation[31]. Well-established studies have demonstrated that these compounds mediate their effects on DNA or protein-DNA cross-linking *via* production of reactive oxygen species (ROS), antioxidant (e.g., GSH) depletion *etc.*

B[a]P, on the other hand, exerts its clastogenic effects on cells after metabolic activation. B[a]P is

metabolized by cytochrome P450 and peroxidases in the S-9 mix into numerous oxidized byproducts[32]. Although several of the B[a]P metabolites thus obtained (i.e. after S-9 treatment) induce mutations, (±)-anti-benzo(a) pyrene-7,8-dihydrodiol-9, 10-epoxide (BPDE) is considered to be the most carcinogenic of these[33]. BPDE causes single and double strand DNA breaks[34] and the chromosomal damage probably results from oxygen radical species. Thus all three clastogens appear to affect cells via production of free radicals. Our results on HPLC demonstrates the high levels of phytoflavonoids (luteolin and apigenin) and saponins (bacoside A_3, bacopaside I, bacopaside II, jujubogenin isomer of bacopa saponin C, bacopa saponin C) in BM. Our studies have also demonstrated scavenging activity of BM against hydroxyl, superoxide, DPPH, and ABTS radicals. We therefore hypothesize that the protective effect of BM against clastogen (MMC, H_2O_2, and B[a]P) mediated damage may be attributed to its radical scavenging capacity.

In the present study we also evaluated the possible mutagenic effects of BM using a short term genotoxicity assay i.e. the Ames *Salmonella* reverse mutation assay. The Ames *Salmonella* reverse mutation assay detects reverse point mutations in the histidine operon. The histidine dependent bacteria become histidine$^+$ when a chemical induces mutation. This assay is capable of detecting both frame shift mutations (using strains TA97a, TA98) as well as base pair substitution mutations (using strains TA100, TA1535, and TA102). Our studies demonstrate that BM has no mutagenic effect on these strains.

Therefore we conclude that BacoMind^{TM} can be used as a natural dietary supplement for supporting optimal health and mental functioning and is safe for oral consumption. It also has the capacity to be used as an anti-oxidant and an anti-carcinogenic agent particularly against those carcinogens that mediate their effects *via* formation of oxygen free radicals.

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REFERENCES

1. Russo A, Borrellib F (2005). *Bacopa monniera*, a reputed nootropic plant: an overview. *Phytomedicine* **12**, 305-317.
2. Murthy P B, Raju V R, Ramakrisana T, *et al.* (2006). Estimation of Twelve Bacopa Saponins in Bacopa monnieri Extracts and Formulations by High-Performance Liquid Chromatography. *Chem Pharm Bull* (Tokyo) **54**(6), 907-911.
3. Ernst E (2006). Herbal remedies for anxiety–a systematic review of controlled clinical trials. *Phytomedicine* **13**(3), 205-208.
4. Sairam K, Dorababu M, Goel R K, *et al.* (2002). Antidepressant activity of standardized extract of *Bacopa monniera* in experimental models of depression in rats. *Phytomedicine* **9**(3), 207-211.
5. Bhattacharya S K, Bhattacharya A, Kumar A, *et al.* (2000). Antioxidant activity of *Bacopa monniera* in rat frontal cortex, striatum and hippocampus. *Phytother Res* **14**, 174-179.
6. Rao Ch V, Sairam K, Goel R K (2000). Experimental evaluation of *Bacopa monniera* on rat gastric ulceration and secretion. *Indian J Physiol Pharmacol* **44**, 35-41.
7. Goel R K, Sairam K, Dora Babu M, *et al.* (2003). *In vitro* evaluation of *Bacopa monniera* on anti-*Helicobacter pylori* activity and accumulation of prostaglandins. *Phytomedicine* **5**, 523-527.
8. Samiulla D S, Prashanth D, Amit A (2001). Mast cell stabilizing activity of *Bacopa monnieri. Fitoterapia* **72**, 284-285.
9. Stough C, Lloyd J, Clarke J, *et al.* (2001). The chronic effects of an extract of *Bacopa monniera* (Brahmi) on cognitive function in healthy human subjects. *Psychopharmacology* **156**(4), 481-484.
10. Singh H K, Dhawan B N (1982). Effect of Bacopa monniera Linn. (brahmi) extract on avoidance responses in rat. *J Ethnopharmacol*. **5**(2), 205-214.
11. Singh R H, Singh L (1980). Studies on the anti-anxiety effect of the medyha rasayana drug, Brahmi (Bacopa monniera Wettst). *J Res Ayur Siddha* **1**, 133-148.
12. Singh H K, Dhawan B N (1992). Drugs affecting learning and memory. In: Tandon, P. N., Bijiani, V., Wadhwa, S. (Eds.), Lectures in Neurobiology, vol. 1. Wiley Eastern, New Delhi, pp. 189-207.
13. Halliwell B, Gutteridge J M, Aruoma O I (1987). The deoxyribose method: A simple "test tube" assay determination of rate constants for reactions for hydroxyl radicals. *Anal. Biochem* **165**, 215.
14. Yen G C, Chen H Y (1995). Antioxidant activity of various tea extracts in relation top their antimutagenicity. *J Agric Food Chem* **43**, 27.
15. Vani T, Rajani M, Sarkar S, *et al.* (1997). Antioxidant properties of the Ayurvedic formulations triphala and its constituents. *Int J Pharmacog* **35**, 313.
16. Auddy B, Ferreira M, Blasina F (2003). Screening of antioxidant activity of three Indian medicinal plants traditionally used for the management of neurodegenerative diseases. *J Ethnopharmacol* **4**, 131.
17. Sreejayan, Rao M N (1997). Nitric oxide scavenging by curcuminoids. *J Pharm Pharmacol* **49**, 105.
18. OECD-Guideline for Testing of Chemicals, 21 July (1997). *In vitro* Mammalian Chromosomal Aberration Test, No. **473**.
19. OECD-Guideline for Testing of Chemicals, draft proposal for a new guideline, June 14 (2004). (1st version). *In vitro* Micronucleus Test, No. **487**.
20. Garner R C, Miller E C, Miller J A *et al.* (1972). Liver microsomal metabolism of aflatoxin B1 to a reactive derivative toxic to *Salmonella typhimurium* TA 1530. *Cancer Res* **32**, 2058-2066.
21. Ames B N, Durston W E, Yamasaki E, *et al.* (1973). Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. *Proceedings of the National Academy of Science USA* **70**, 2081-2285.
22. Api A M, San R H C (1999). Genotoxicity tests with 6-acetyl-1,1,2,4,4,7-hexamethylcyclopenta-y-2-benzopyran. *Mutat Res* **446**, 67-81.
23. Ozkul Y, Silici S, Eroğlu E (2005). The anticarcinogenic effect

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of propolis in human lymphocytes culture. *Phytomedicine* **12**, 742-747.

24. Surralles J, Xamena N, Creus A, *et al.* (1995). Induction of micronuclei by five pyrethroid insecticides in whole-blood and isolated human lymphocyte cultures. *Mutat Res* **341**(3), 169-184.
25. Bhattacharya S K, Kumar A, Ghosal S (1999). Effect of Bacopa monniera on animal models of Alzheimer's disease and perturbed central cholinergic markers of cognition in rats. In: Siva Sankar, D.V. (Ed.), Molecular Aspects of Asian Medicines. PJD Publications, New York.
26. Shanker G, Singh H K (2000). Anxiolytic profile of standardized Brahmi extract. *Indian J Pharmacol* **32**, 152.
27. Deepak M, Amit A (2004). The need for establishing identities of 'bacoside A and B', the putative major bioactive saponins of Indian medicinal plant *Bacopa monnieri. Phytomedicine* **11**, 264-268.
28. Deepak M, Sangli G K, Arun P C, Amit A (2005). Quantative determination of the major saponin mixture Bacoside A and *Bacopa monnieri* by HPLC. *Phytochem Anal* **16**, 24-29.
29. Roneada T, Vicentini V E P, Mantovani M S (2004). Possible modulating actions of plant extracts on the chromosome breaking activity of MMC and Area-C in human lymphocytes *in vitro*. *Toxicology* **5**, 617-622.
30. Stella M A, Montaldi R, Rossi G, *et al.* (1982). Clastogenic effects of chromium on human lymphocytes *in vitro* and *in vivo*. *Mutat Res* **101**, 151-164.
31. Gregory L Erexson, Periago M V, Carol S Spicer (2001). Differential sensitivity of Chinese hamster V79 and Chinese hamster ovary (CHO) cells in the *in vitro* micronucleus screening assay. *Mutat Res* **495**, 75-80.
32. Wei Q, Gu J, Cheng L, *et al.* (1996). Benzo[*a*]pyrene diol epoxide-induced chromosomal aberrations and risk of lung cancer. *Cancer Res* **56**, 3975-3979.
33. Mauthe R J, Cook V M, Coffing S L, *et al.* (1995). Exposure of mammalian cell culture to benzo[*a*]pyrene and light results in oxidative DNA damage as measured by 8-hydroxydeoxyguanosine formation. *Carcinogenesis* **16**, 133-137.
34. Wei Q, Spitz M R, Gu J, *et al.* (1996). DNA repair capacity correlates with mutagen sensitivity in lymphoblastoid cell lines. *Biomarkers Prev* **5**, 199-204.

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