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ORIGINAL RESEARCH

Anti-apoptotic mechanism of Bacoside rich extract against reactive nitrogen species induced activation of iNOS/Bax/caspase 3 mediated apoptosis in L132 cell line

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Abstract Nitric oxide is a highly reactive free radical gas that reacts with a wide range of biomolecules to produce reactive nitrogen species and exerts nitrative stress. *Bacopa monniera* is a traditional folk and ayurvedic medicine known to alleviate a variety of disorders. Aim of the present study is to evaluate the protective propensity of *Bacopa monniera* extract (BME) through its oxido-nitrosative and anti-apoptotic mechanism to attenuate sodium nitroprusside (SNP)-induced apoptosis in a human embryonic lung epithelial cell line (L132). Our results elucidate that pre-treatment of L132 cells with BME ameliorates the mitochondrial and plasma membrane damage induced by SNP as evidenced by MTT and LDH leakage assays. BME pre-treatment inhibited NO generation by down-regulating inducible nitric oxide synthase expression. BME exhibited potent antioxidant activity by up-regulating the antioxidant enzymes. SNP-induced damage to cellular, nuclear and mitochondrial integrity was also restored by BME, which was confirmed by ROS estimation, comet assay and mitochondrial membrane potential assays respectively. BME pre-treatment efficiently attenuated the SNP-induced apoptotic biomarkers such as Bax, cytochrome-c and caspase-3, which orchestrate the proteolytic damage of the cell. By considering all these findings, we report that BME protects L132 cells against SNP-induced toxicity via its free radical scavenging and anti-apoptotic mechanism.

Keywords Sodium nitroprusside · *Bacopa monniera* · Human embryonic lung epithelial cells (L132) · Reactive nitrogen species · Inducible nitric oxide synthase (iNOS) · Apoptosis

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Introduction

Nitric oxide (NO) is an important bio-regulatory molecule involved in numerous physiological and pathological processes. It reacts with molecular oxygen, superoxide anion and transition metal ions to produce reactive nitrogen species (RNS) such as nitrosonium (NO^+), nitroxyl (NO^-), N_2O_3, NO_2, NO_2^-, NO_3^-, peroxynitrite ($OONO^-$) and metal-nitrosyl adducts (Wink et al. 1993; Davis et al. 2001). Among these reactive nitrogen species, peroxynitrite stands out as a strong oxidizing and nitrating species that causes molecular damage leading to cellular dysfunction. It causes nitration of amino acid (such as tyrosine) and oxidizes various cellular constituents

(such as lipids, amino acids, proteins and nucleotides) (Briviba et al. 1999). Peroxynitrite is potent enough to induce cell damage by its oxidative and apoptotic stimulus and it has been implicated in several pathological conditions viz., Alzheimer's and Parkinson's diseases (Aoyama et al. 2000; Chung et al. 2005).

Bacopa monniera belong to Scrophulariaceae family is being used as a traditional folk and ayurvedic medicine, known to alleviate variety of disorders including nitrative stress (Dash and Kashyap, 1980; Singh and Dhawan, 1997). The major constituents of *B. monniera* were found to be bacoside-A. Bacoside A is a mixture of bacosaponin isomers. *B. monniera* possesses anti-fatigue (Anand et al. 2012), anti-amnesic (Bhattacharya et al. 2000), anti-stress (Chowdhuri et al. 2002), memory enhancing, anxiolytic (Ernst 2006), anti-ulcerogenic, anti-arthritis, anti-inflammatory (Channa et al. 2006) and neuroprotective properties (Dhanasekharan et al. 2007). In addition, the herb is known to possess good amount of flavonoids and phenolic compounds, which has strong antioxidant property. In our previous studies we have demonstrated the antioxidant and plasmid DNA damage preventive properties of *B. monniera* (Anand et al. 2011); these results are in support with earlier reports (Tripathi et al. 1996; Bhattacharya et al. 2000; Sairam et al. 2001; Sumathy et al. 2002; Russo et al. 2003). These bioactive molecules isolated from the herbs promises to restrain antioxidant property and protect the cell from various injury caused by reactive oxygen and nitrogen species. Peroxynitrite is one such oxidizing and nitrating species that causes molecular damage via activating oxidative and apoptotic biomarkers in several pathological conditions (Aoyama et al. 2000; Chung et al. 2005). Several studies have shown that peroxynitrite generated by the SNP induction causes nitrative and oxidative stress by depleting antioxidant status in vivo and in vitro (Lipton et al. 1993; Messmer et al. 1996).

Lungs are continuously exposed to oxidants either generated endogenously by metabolic reactions (e.g. from mitochondrial electron transport during respiration, during activation of phagocytes) or exogenously (by air pollutants or cigarette smoke). Cigarette smoke and the exhausts released from the various other sources contain lot of RNS that causes lung damage in humans. Excessive generation of ROS may alter remodelling of extracellular matrix, mitochondrial respiration, cell proliferation, effective alveolar repair response and immune modulation within the lungs (Rahman and MacNee 1999, 2000). Human embryonic lung epithelial cells (L132) are one of the better human lung epithelial cell lines used to study the cellular toxicity, mitochondrial dysfunction and apoptosis in relation to lung toxicity (Dagher et al. 2006; Mehrotra et al. 2011; Panngom et al. 2013). In addition to these investigators, Dagher et al. (2007) and Abbas et al. (2013) have used L132 cell lines to study adverse health effects induced by particulate matter (PM) air pollution. Hence the present study was aimed to rescue oxido-nitrative stress and apoptotic effect induced by SNP in L132 cells.

In the present study we have used SNP as the generator of RNS and Nω-nitro-L-arginine methyl ester (L-NAME) as an inhibitor. Molecular mechanism of SNP-induced cytotoxicity and ameliorative effect of *B. monniera* was aimed to be elucidated.

Materials and methods

Reagents and chemicals

Dulbecco's modified Eagle's medium with high glucose, Penicillin–Streptomycin antibiotic solution, Sodium nitroprusside, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), 2′,7′-dichlorfluorescein-diacetate, rhodamine 123, sodium dodecyl sulfate (SDS), 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), glutathione standard, acetylthiocholine iodide, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), bovine serum albumin, protease cocktail inhibitor and sodium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide, thiobarbituric Acid, butylated hydroxytoluene, trichloroacetic acid, ferric chloride, glycine, hydrochloric acid (HCl), dimethyl sulfoxide (DMSO), sodium chloride, disodium hydrogen phosphate, sodium dihydrogen phosphate were purchased from S.D fine chemicals (Mumbai, India). Fetal bovine serum was procured from Hyclone (Logan, UT, USA).

Purification and quantification of Bacoside A

Aerial parts of *B. monniera* were collected from the foothills of Tirumala, Tirupati, and identified with the help of Prof. N Yasodamma, Head, Department of Botany, Sri Venkateswara University, Tirupati,

Andhra Pradesh, India (Herbarium collection Voucher No. DA-112). The dammarane type triterpenoid saponin, bacoside-rich extract was isolated from the plant by the standard procedure and the amount of bacosides present was quantified by using a Jasco LC-NetII/ADC HPLC system as reported in our previous study. The amount of bacoside A was found to be ~16 % (Anand et al. 2013).

Estimation of total phenolic and flavonoid contents

The total phenolic and total flavonoid contents were determined according to the methods of Anand et al. (2011) and Zou et al. (2004) respectively.

Cell culture and treatments

The L132 cells used in current study were supplied by the National Centre for Cell Science, Pune, India. The cells were grown in Dulbecco's modified Eagle's medium with high glucose and supplemented with 5 % fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humid atmosphere of 5 % CO_2 and 95 % air at 37 °C. For all the experiments 80 % confluence with more than 95 % cell viability was considered as optimum. All experiments were conducted in serum free medium. Freshly prepared sodium nitroprusside was added as NO inducer for 24 h to the cells with or without pre-treatment with BME/L-NAME as NO inhibitor for 1 h before any experiment.

Cell viability assay

Cell viability was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Pandareesh and Anand 2013). The principle of the assay is based on the cleavage of tetrazolium salts by mitochondrial succinate reductase in viable cells to form formazan dye. The L132 cells were seeded in 96-well plates at a density of 5×10^3 cells/well and grown for 24 h and then subjected to the treatments of interest. After treatments, MTT (0.5 mg/ml) was added to each well and incubated for 2 h at 37 °C and the formed formazan crystals were dissolved in DMSO. The absorbance was then measured at 540 nm using Multi-technology plate reader (Plate Chameleon, Type 425-106 s/n 2090137, Finland). Cell viability was expressed as a percentage of the value against the control group.

Lactate dehydrogenase (LDH) leakage assay

Cytotoxicity was quantified by measuring the extent of plasma membrane damage by means of a LDH-estimation kit (Agappe-11407002, Mysore, India) following the manufacturer's instructions. The assay is based on leakage of cytosolic LDH into medium due to plasma membrane damage. The enzyme activity was measured through the oxidation of lactate to pyruvate with simultaneous reduction of nicotinamide adenine dinucleotide (NAD+) to reduced nicotinamide adenine dinucleotide (NADH) read at a wavelength of 340 nm. The rate of increase in formation of reduced NADH is directly proportional to the LDH activity in the sample. The L132 cells were plated at a density of 5×10^4 cells/well on 12-well plates and after 24 h of adherence the cells were subjected to the treatments of interest. After the treatment period, 10 µl of cell lysis solution (2 % Triton X-100) was added to the untreated cells. The cells were separated by centrifugation at $3000 \times g$ for 5 min at 4 °C and the supernatant was estimated for total LDH activity (Pandareesh and Anand 2013).

Observation of morphological changes

The cells were seeded in 75 cm^2 flasks (1×10^6 cells) and then treated with SNP for 24 h with or without pre-treatment of BME/L-NAME for 1 h. The cellular morphology was observed and photographed using phase contrast microscope (Olympus, Tokyo, Japan) equipped with Cool SNAP® Pro color digital camera.

Estimation of NO levels

Nitric oxide produced during SNP treatment was estimated spectrophotometrically as nitrite. After 24 h SNP treatment, 1 ml of culture medium was taken from each well. To measure the nitrite content, 100 µl of the culture medium was incubated with 100 µl of Griess reagent (1 % sulphanilamide in 2 % H_3PO_4 and 0.1 % N-(1-naphthyl) ethylenediamine dihydrochloride) at room temperature for 10 min. Then the absorbance was measured at 540 nm using a Multi-technology plate reader (Plate Chameleon, Type

425-106 s/n 2090137). The nitrite content was calculated from a standard curve constructed with $NaNO_2$.

Estimation of intracellular ROS

The L132 cells were cultured in 24 well plates for fluorimetric analysis and treated as mentioned earlier. After the treatments, the oxidation-sensitive dye DCFH-DA (0.5 mg/ml) was added to the cells and incubated for 30 min. The cells were then collected after washing twice with PBS and the intracellular ROS formation was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using Multi-technology plate reader (Plate Chameleon, Type 425-106 s/n 2090137).

Measurement of lipid peroxidation

Malondialdehyde (MDA), a lipid peroxidation product, was measured following the method of Ohkawa et al. (1979) with minor modifications. The L132 cells were seeded in 75 cm^2 flasks at a concentration of 1×10^6 cells/ml and incubated at 37 °C. After reaching 80 % confluency, the cells were treated as described earlier. The cells were collected, washed twice with PBS and lysed in ice-cold 1.15 % KCl with 1 % Triton X-100 by sonication for 5 min. Aliquots (100 µl) of the cell lysates were mixed with 0.2 ml of 8.1 % SDS, 1.5 ml of 20 % acetic acid (pH 3.5), 1.5 ml of 0.8 % thiobarbituric acid and the volume was brought up to 4 ml using distilled water. The contents were boiled for 2 h to develop the colour, followed by cooling. The content was centrifuged at $3000 \times g$ for 10 min and absorbance of the supernatants was measured at 532 nm. The MDA content was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} (Buege and Aust 1978).

Single cell gel electrophoresis (SCGE) assay

DNA damage preventing efficacy of BME against SNP induced nitrosative stress was assessed by alkaline comet assay with slight modifications (Singh et al. 1988). The cells (1×10^6 cells) were seeded in 75 cm^2 flasks and treated as described previously. The cells were collected and equal volume of cell suspension (4×10^5) was mixed with 0.5 % (w/v) low melting agarose (LMA) in 0.01 M of PBS. The mixture was pipetted on to frosted slides pre-coated with 1 % (w/v) normal melting agarose. After solidification of agarose, the slides were covered with another 100 µl of 0.5 % (w/v) LMA and immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl buffer, 0.1 % SDS and 1 % Triton X-100 and 10 % DMSO; pH 10.0) for 120 min in dark at 4 °C to lyse the cellular and nuclear membranes. The slides were rinsed with unwinding buffer and transferred into an electrophoresis tank containing unwinding buffer (3 M NaOH, 10 mM EDTA; pH 13.0) for denaturing the DNA followed by electrophoresis for 30 min with an electric current of 25 V. The slides were washed twice with neutralizing buffer (0.4 M Tris–HCl; pH 7.5) for 10 min and treated with ethanol for another 5 min. The slides were stained with 40 µl of ethidium bromide (20 mg/ml) and DNA damage was visualized by using fluorescence microscope (Olympus, equipped with Cool SNAP® Pro color digital camera). The damage appeared as a 'comet' with fragmented DNA (tail) being separated from undamaged nuclear DNA (head) and measurements were made by Comet Assay IV software to determine the tail movement (%). The results were expressed as percent tail movement.

Measurement of mitochondrial membrane potential (MMP)

The protective effect of BME on mitochondrial damage induced by SNP was determined by measuring the MMP using fluorescent dye rhodamine 123. The cells were cultured in 24 well plates for fluorimetric analysis. After the treatments, rhodamine 123 (10 mg/ml) was added to the cells and incubated for 1 h at 37 °C. After washing twice with PBS, the cells were collected and the fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using multi-technology plate reader (Plate Chameleon, Type 425-106 s/n 2090137).

Estimation of antioxidant status

The L132 cells (1×10^6 cells) were seeded in 75 cm^2 flasks and treated as described previously. Cells were collected and lysed by sonication in ice-cold 50 mM sodium phosphate buffer, pH 7.4 containing 2 mM EDTA and 0.1 % Triton X-100. The cell homogenate was centrifuged at $13{,}000 \times g$ for 10 min at 4 °C to

remove cell debris. The resulting supernatants were analyzed for protein contents by the method published by Lowry (1951). Activity of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) were estimated according to the manufacturer's instructions (Randox, Cat no. SD. 125, RS 504, GR 2368, Canada). Catalase (CAT) activity was estimated by measuring the decay of 6 mM H_2O_2 solution at 240 nm by the spectrophotometric degradation method (Aebi 1984). An extinction coefficient of 43.6 $M^{-1}cm^{-1}$ was used to determine the enzyme activity and values were expressed as mmol H_2O_2 degraded/min/mg of protein.

Estimation of reduced GSH levels and total antioxidant status by ABTS radical scavenging method

Reduced glutathione was estimated according to our previous method (Pandareesh and Anand 2013). L132 cells grown in 75 cm^2 flask received various treatments for 24 h, washed with ice-cold PBS. Cells were homogenized in 0.1 M sodium phosphate buffer (pH 8.4) and then the protein was precipitated by adding 5 % trichloroacetic acid. To 0.1 ml supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5′5′ dithiobis (2-nitrobenzoic acid) (DTNB) and 0.4 ml of double distilled water were added, vortexed and the absorbance was measured at 412 nm within 15 min. ABTS radical scavenging method was followed as per our previous studies (Pandareesh and Anand 2013). Briefly, L132 cell homogenate (10 µl) was added as source of antioxidants to the pre-formed radical cation, $ABTS^{\bullet+}$. The decrease in the absorbance was monitored at 734 nm for 3 min at an interval of 1 min. $ABTS^{\bullet+}$ radical cation was determined as a function of concentration and time by molar extinction coefficient of $ABTS^{\bullet+}$ 1.5 × 10^4 M^{-1} cm^{-1}.

Western blot analysis

Total cellular protein was separated on SDS-PAGE and transferred onto a nitrocellulose membrane using an electro blotting apparatus (Cleaver Scientific Ltd, Rugby, UK) as per earlier method (Anand et al. 2012). After transfer, the membranes were probed with α-tubulin B-7 (sc-5286), anti HSP-70 C92F3A (sc-66048), Bcl2 (sc-7382), Bax (sc-70408), cytochrome c (sc-13156) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and iNOS (N9657), caspase 3 (C8487, Sigma, St.Louis, MO, USA) at 1:500 dilution and incubated at 37° C for 3 h. The membranes were then washed four times with TBST for 15 min followed by incubation at room temperature for 2 h in horseradish peroxidase conjugated rabbit anti-goat, goat anti-mouse and goat anti-rabbit secondary antibodies (DAKO, Glostrup, Denmark) at 1:10,000 dilutions. The membranes were washed again and developed using an enhanced chemiluminescence detection system (ProteoQwest®, Sigma). Developed membranes were exposed to x-ray film and the developed band intensity was captured. The western blot band intensity was measured using NIH image analysis software.

Statistical analysis

The data are expressed as mean ± standard error of the mean (SEM). Data were analysed using one-way ANOVA followed by Tukey's post hoc test using SPSS version 15 software. Differences at $p < 0.05$ were considered to be significant.

Results

Estimation of total phenolic and total flavonoid content in BME

The total phenolic content in BME was found to be 54.60 ± 1.1 µg GAE/mg extract and the total flavonoids present were found to be 51.96 ± 0.9 µg CE/mg extract.

Cytotoxicity of SNP and protective effect of BME against cell death

To evaluate SNP induced cytotoxicity, cells were treated with various concentrations of SNP (100–600 µM) for 24 h. The SNP induced toxicity by decreasing cell survival in a dose-dependent manner. The cell survival rate of 49.10 % was observed at 500 µM SNP insult and the same concentration was used in subsequent experiments (Fig. 1A). Further, to determine the protective effects of BME/L-NAME against SNP induced cytotoxicity, the cells were pre-treated with 25–100 µg/ml of BME or with 50–200 µM of L-NAME for a period of 1 h, followed by treatment with SNP (500 µM) for 24 h. As shown

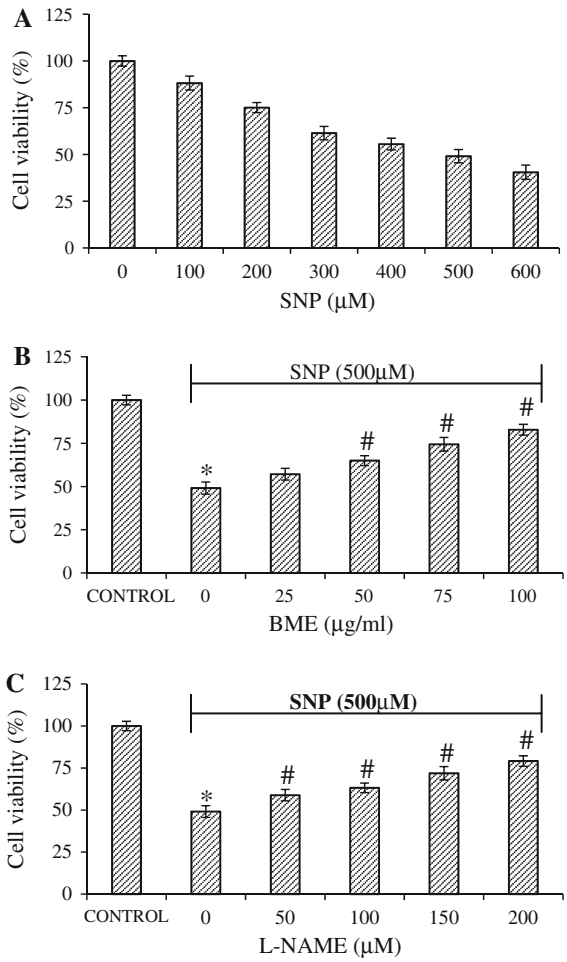

Fig. 1 **A** Dose-dependent effects of SNP on L132 cell viability. **B** Dose-dependent protective effect of BME on SNP-induced cytotoxicity in L132 cells. **C** Dose-dependent protective effect of L-NAME on SNP-induced cytotoxicity in L132 cells. The data presented are mean ± SEM of three independent experiments. $^{*}p < 0.05$ versus control group and $^{\#}p < 0.05$ versus SNP treated group

in Fig. 1B and C, cells were significantly protected by BME (100 μg/ml) and L-NAME (200 μM) pre-treatment from SNP induced cell death to the extent of 82.86 and 79.15 %, respectively. These results were further confirmed by LDH leakage assay. The L132 cells were treated with increasing concentrations of SNP (100–600 μM) for 24 h. SNP induced LDH leakage in a dose-dependent manner. An LDH leakage of 515.72 % was observed in SNP (500 μM) treated cells, in comparison with control group (100 %) (Fig. 2A). In contrast, 100 μg/ml BME or 200 μM L-NAME pre-treated cells showed a decreased release of LDH up to 317.09 and 353.00 % respectively. Results of the study indicate that BME and L-NAME rescued SNP induced cytotoxicity suggesting its cytoprotective efficacy (Fig. 2B). The protective effect of BME was confirmed by morphological observation using phase-contrast microscope. Exposure of cells to toxicant leads to cell membrane damage and shrinkage of cells which were protected by BME and L-NAME pre-treatment (Fig. 2C).

Effect of BME on NO production via down-regulation of iNOS expression

Pre-treatment with BME and L-NAME has significantly reduced the nitrosative stress by inhibiting the generation of RNS with SNP treatment. Expression of iNOS and subsequent production of NO were measured by immunoblotting analysis (Fig. 7) and Griess assay (Fig. 3), respectively. The expression of iNOS was found to increase with SNP treatment and also elevated the amount of nitrite released into the medium. The pre-treatment of BME or L-NAME could effectively down-regulate iNOS expression which led to decreased nitrite levels.

Effect of BME on reactive oxygen species (ROS) generation and lipid peroxidation

Exposure of L132 cells to SNP (500 μM) elicited ~2.5 fold increase in ROS production as compared to the control group. ROS generation was attenuated significantly with BME (~155 %) and L-NAME (~175 %) pre-treatment indicating the potent antioxidant activity of BME and NO inhibitory activity of L-NAME (Fig. 4A) which is further confirmed by fluorescence imaging (Fig. 4B). SNP exposure leads to lipid peroxidation, which is measured as MDA levels. L132 cells exposed to SNP (500 μM) for 24 h had a significantly increased the MDA levels by 259.26 % relative to the control group. Pre-treatment with BME (154.94 %) and L-NAME (195.66 %) significantly decreased the rate of lipid peroxidation as compared to the SNP treated group ($p < 0.05$) (Fig. 5).

Protective role of BME on SNP induced DNA damage

SNP-induced DNA damage was quantified by SCGE assay. SNP treated cells showed a definite tail with a

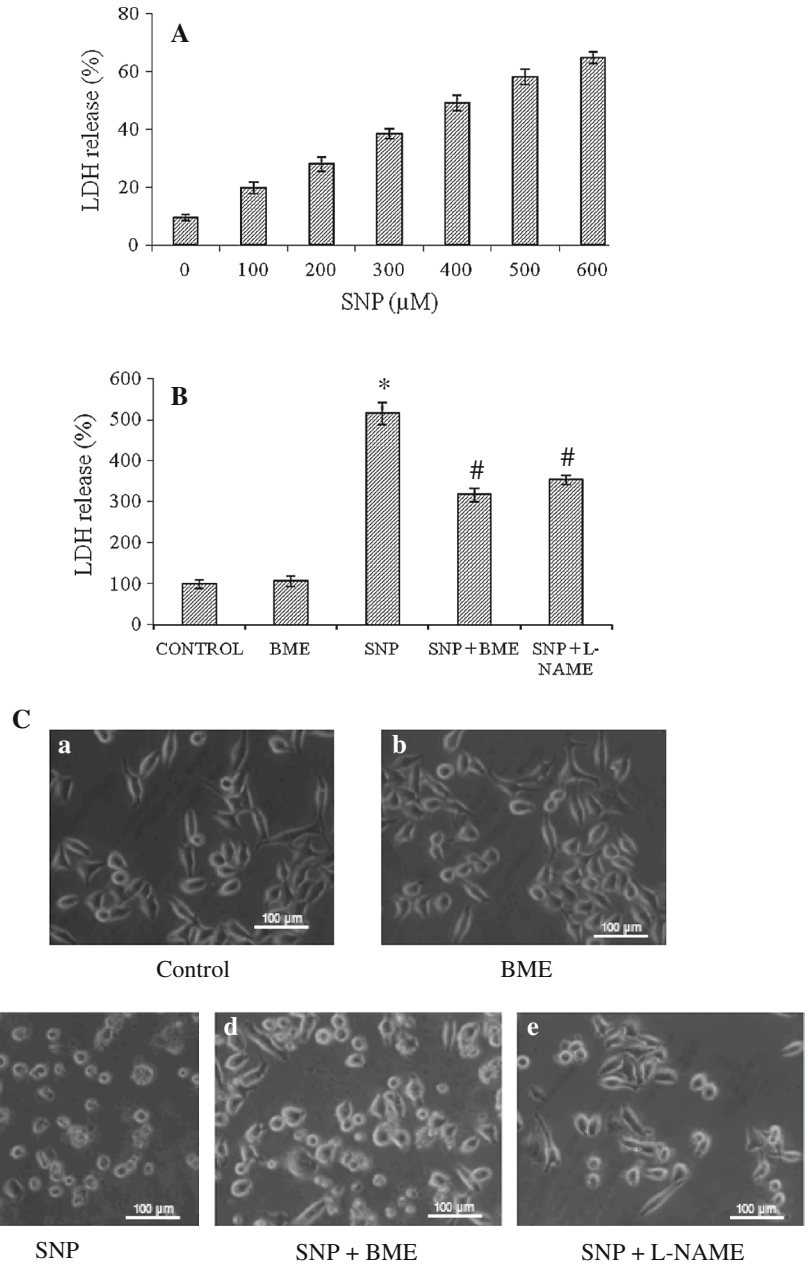

Fig. 2 **A** Dose-dependent effects of SNP on LDH release. **B** Protective effect of BME and L-NAME on SNP-induced LDH release in L132 cells. The data presented are mean ± SEM of three independent experiments. $^{*}p < 0.05$ versus control group and $^{\#}p < 0.05$ versus SNP treated group. **C** Effects of BME on SNP-induced morphological alterations in L132 cells by phase-contrast microscopy. **a** Control cells with no treatment, **b** BME (100 μg/ml), **c** SNP (500 μM) **d** cells pre-treated with BME (100 μg/ml) for 1 h and then treated with SNP (500 μM) and **e** cells pre-treated with L-NAME (200 μM) for 1 h and then treated with SNP (500 μM)

consistent amount of fragments indicating genetic damage (Fig. 6A). The tail movement of SNP exposed cells was found to be 24 %. BME and L-NAME pre-treatment has decreased the tail movement by ∼14 and ∼18 %, respectively (Fig. 6B). These pre-treated cells appeared with a small tail, little migration of fragments of DNA indicate the protective efficacy of BME against SNP induced DNA damage.

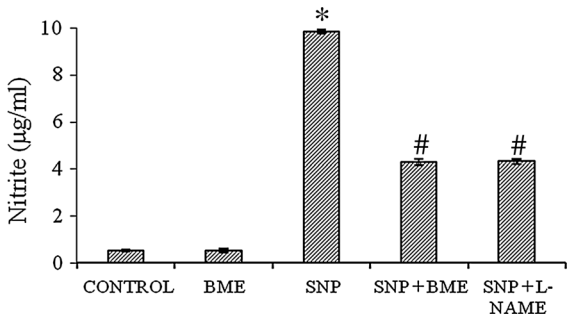

Fig. 3 Protective effects of BME against SNP induced NO production in L132 cells. The data presented are mean ± SEM of three independent experiments. $^{*}p < 0.05$ versus control group and $^{\#}p < 0.05$ versus SNP treated group

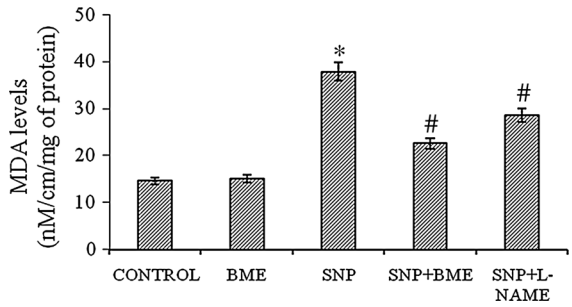

Fig. 5 Protective effect of BME against SNP induced lipid peroxidation in L132 cells. The data presented are mean ± SEM of three independent experiments. $^{*}p < 0.05$ versus control group and $^{\#}p < 0.05$ versus SNP treated group

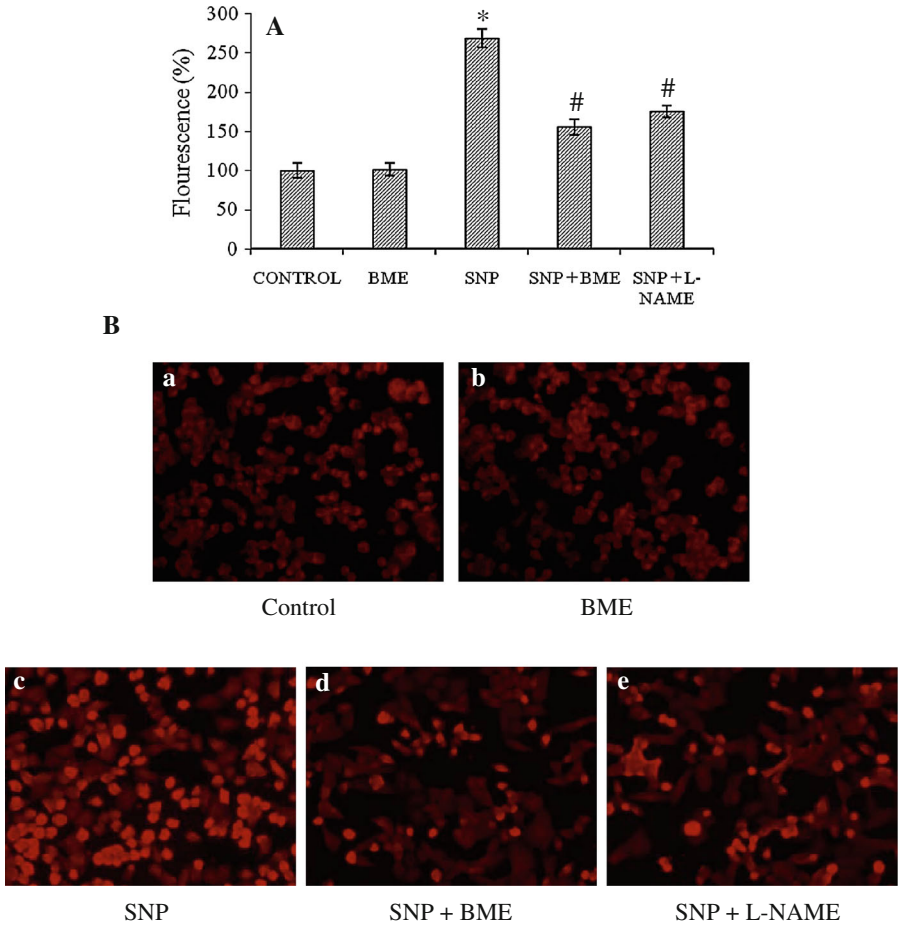

Fig. 4 **A** Estimation of ROS production by 2′,7′ DCFH-DA using a spectrofluorimeter. **B** The ROS production in L132 cells was monitored by fluorescence microscopy (Olympus) **a** Control cells with no treatment, **b** BME (100 μg/ml), **c** SNP (500 μM) **d** cells pre-treated with BME (100 μg/ml) for 1 h and then treated with SNP (500 μM) and **e** cells pre-treated with L-NAME (200 μM) for 1 h and then treated with SNP (500 μM)

Protective role of BME on SNP induced MMP reduction

Lipid peroxidation caused by the free radicals attack leads to a loss of mitochondrial membrane potential. This was analyzed by the accumulation of rhodamine 123, a membrane permeant, cationic fluorescent dye. The rhodamine 123 fluorescence was decreased by ~69 % with SNP exposure compared to control indicating the depolarization of mitochondrial membrane. However, the cells pre-treated with BME and L-NAME prior to SNP exposure, showed a significant regain in the florescence intensity to an extent of ~90 and ~92 %, respectively, compared to control (Fig. 6C).

Effect of BME on HSP-70 expression and apoptosis markers

Heat shock protein −70 (HSP-70) is a stress induced molecular chaperon. In the present study, we observed the significant up-regulation of HSP-70 protein expression in L132 cells treated with SNP. Pre-treatment with BME/L-NAME significantly brought down the HSP-70 expression levels to normal, indicating the cytoprotective role of BME (Fig. 7A and B). Bax, Bcl2, cytochrome c, caspase 3 are the biomarkers of apoptosis. SNP-induced a significant up-regulation of Bax, cytochrome c and caspase 3 and down-regulation of Bcl2 whereas pre-treatment with BME and L-NAME significantly brought the expression levels to normal indicating the anti-apoptotic role of BME (Fig. 8A and B).

Effect of SNP on antioxidant status of cells

The cellular antioxidant enzymes including SOD, CAT, GPx, GR activity was reduced by SNP exposure by 50.06, 33.35, 47.37 and 53.91 %, respectively, compared to that of control cells. Significant protection was achieved by pre-treatment with BME (89.48, 87.29, 81.69 and 80.58 %, respectively) and L-NAME (88.24, 77.92, 79.42 and 80.11 %, respectively). SNP induced a 41.33 and 50.35 % decrease in the levels of reduced GSH and total antioxidant status, respectively. The decrease in GSH levels and total antioxidant status was improved by pre-treatment with BME (81.00 and 90.85 %, respectively) and L-NAME (73.05 and 79.61 %, respectively) (Table 1).

Discussion

Sodium nitroprusside generates NO in the form of a nitrosonium cation co-ordinated to an iron-cyanide complex. The release of NO in aqueous solution has been reported to occur by spontaneous oxidation (Peranovich et al. 1995), photochemical degradation (Hurst et al. 1996) and also by bio-reduction (Verovski et al. 1996). SNP decomposition releases CN^-, Fe^{2+} and $[Fe(CN)_6]^{4-}$ in addition to NO, but the toxicity of these molecules is negligible. When NO levels exceed physiological levels by its continuous generation and accumulation, it initiates a series of toxic cascade reactions that lead to cell death. Several other studies have identified NO as a causative agent for apoptotic cell death and have warranted providing data for SNP-induced apoptotic death (Albina et al. 1993; Okuda et al. 1996). BME has been used in the present study to attenuate various impairments induced by SNP. The extract contains good amount of bacoside-A (~16 %) which has been established in our previous studies (Anand et al. 2013; Pandareesh and Anand. 2013). In addition, the extract contains ample amount of polyphenols and flavonoids. Polyphenols and flavonoids are plant secondary metabolites known to possess a strong antioxidant property and contribute to the antioxidant and anti-apoptotic properties of the extract. In the current study, exposure of L132 cells to SNP-induced cell mortality, which is confirmed by MTT and LDH leakage assay (Figs. 1, 2). These results were supported by earlier studies by Kroncke et al. (1993) and Babich et al. (1998), who demonstrated the plasma membrane damage and LDH leakage upon SNP exposure. SNP is also known to induce cell death by apoptosis in human epithelial cancer cell line (Sumitani et al. 1997), murine neuroblastoma N1E − 115 cells (Yamada et al. 1996). Pre-treatment with BME has increased the cell viability with decreased plasma membrane damage. This is due to the NO scavenging activity of BME followed by down-regulation of iNOS expression that was induced upon SNP exposure. Further the morphological observation supports the protective effect of BME against SNP-induced morphological changes (Fig. 2C). Our results corroborate with earlier reports by Babich et al. (1998) who demonstrated in vitro toxicity of SNP in human endothelial cells (ECV304) and also with Choi et al. (2002) who observed similar morphological changes in amiodarone induced apoptosis in L132 cells.

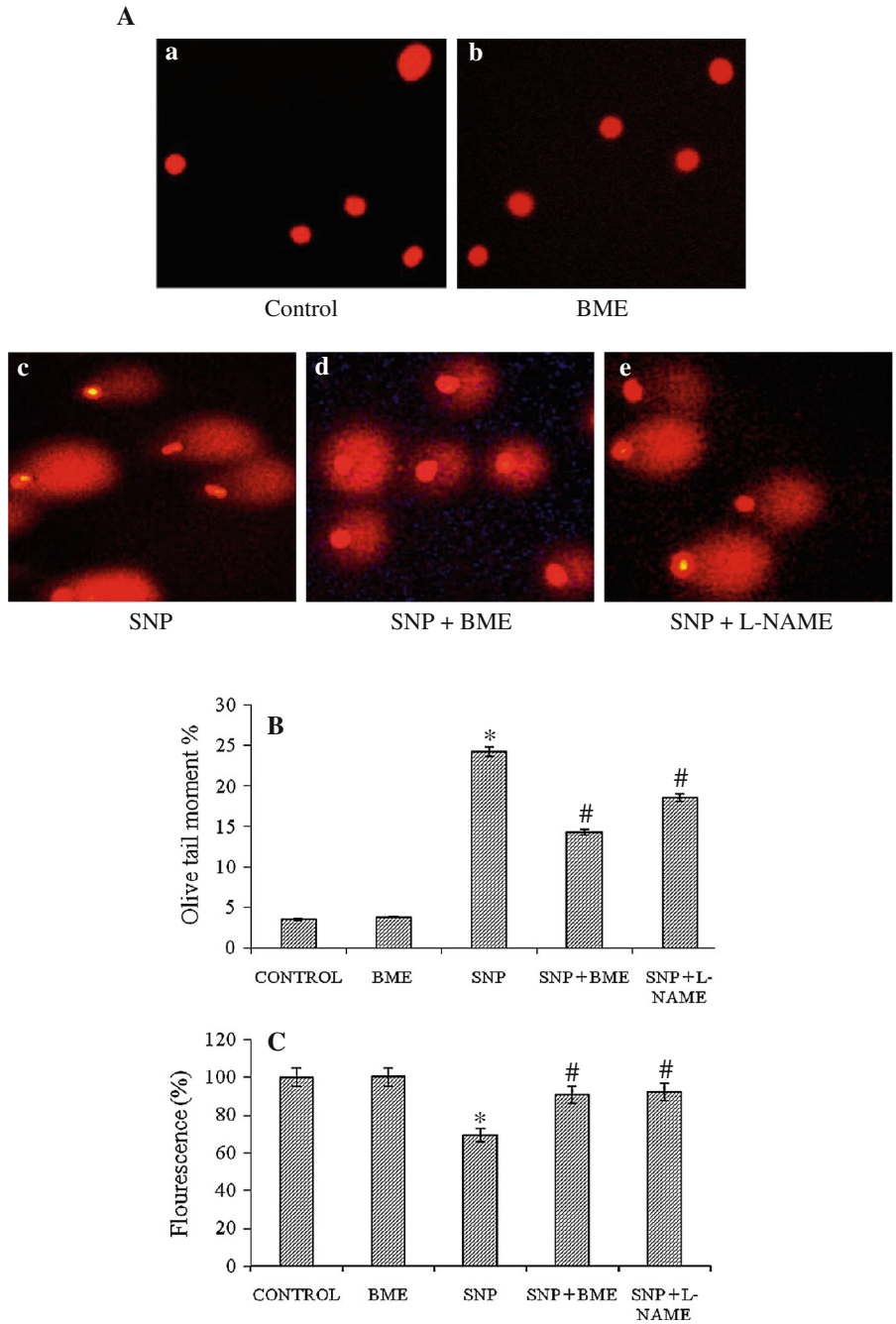

Fig. 6 **A** Effect of BME on DNA damage induced by SNP in L132 cells. **a** Control cells with no treatment, **b** BME (100 μg/ml), **c** SNP (500 μM) **d** cells pre-treated with BME (100 μg/ml) for 1 h and then treated with SNP (500 μM) and **e** cells pre-treated with L-NAME (200 μM) for 1 h and then treated with SNP (500 μM). **B** Tail movement of COMET (%) **C** Effect of BME on SNP-induced decrease of mitochondrial membrane potential determined by spectrofluorimetric method. The data presented are mean ± SEM of three independent experiments. $^{*}p < 0.05$ versus control group and $^{\#}p < 0.05$ versus SNP treated group

The oxidation–reduction of NO leads to generation of other RNS which has been found to be associated with increased ROS generation. To check the antioxidant propensity of BME total ROS was estimated spectrofluorimetrically using a non-ionic, non-polar fluorescent probe DCFH-DA. The dye DCFH-

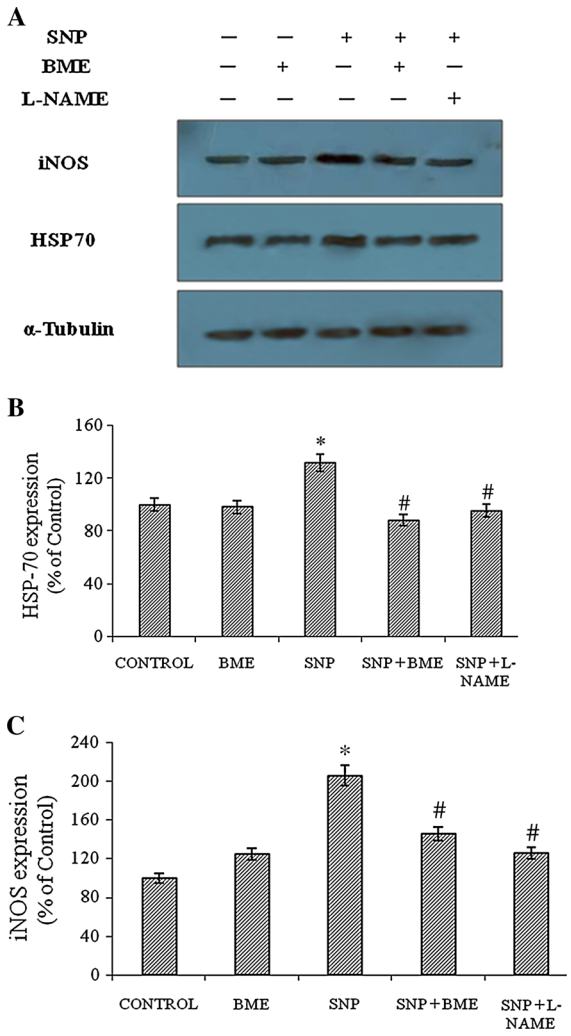

Fig. 7 **A** Protective effect of BME on SNP induced expression of oxidative stress markers HSP-70 and iNOS, a stress marker was analyzed by immunobloting. The band intensity was quantified by Image-J software. **B** and **C** Densitometric analysis of HSP-70 and iNOS expression, respectively. The data presented are mean ± SEM of three independent experiments. $^{*}p < 0.05$ versus control group and $^{\#}p < 0.05$ versus SNP treated group

DA crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to non-fluorescent DCFH. In presence of ROS, DCFH is oxidized to highly fluorescent dichlorofluorescein. The resulting fluorescence is used as an index to quantify the ROS levels (LeBel et al. 1992). The emitted fluorescence is directly proportional to the ROS generation induced by SNP. Our results elucidated BME pre-treatment decreases fluorescence, indicating the ROS inhibitory effect of BME (Fig. 4A, B). The generated RNS and ROS reacts with the glycophospholipids, fatty acids and other essential lipids present in mitochondrial membrane and cell membrane leading to formation of lipid peroxides and other radicals which can propagate end products such as aldehydes, among which MDA is one. In the present study, we found that BME pre-treatment inhibits the SNP-induced lipid peroxidation (Fig. 5). In the current study we have demonstrated the inhibition of lipid peroxidation by BME pre-treatment. These results corroborate with our previous in vivo and in vitro studies (Anand et al. 2012; Pandareesh and Anand. 2013).

Lipid peroxidation triggers the arachidonic-acid cascade, with the production of cell proliferation-stimulating eicosanoids. The by-products of the arachidonic-acid pathway such as malondialdehyde and 4-hydroxynonenol are DNA-damaging agents. In addition, NO and peroxynitrate are potent enough to mediate DNA damage through the formation of nitrosamines, generation of other RNS and by inhibition of DNA damage repair mechanism. In the present study, we observed the protective effect of BME against SNP-induced DNA damage by SCGE assay. In brief, the undamaged chromosomal DNA remains compact within the nucleus and when damaged by SNP exposure, this organization is disrupted. During electrophoresis damaged fragments move faster and are visualized in the form of a tail and appear as 'comet', whereas undamaged DNA remains compact. The length of the comet tail is measured as an index of DNA damage in the cell. These results are corroborating with our earlier in vitro studies showing plasmid DNA damage preventive properties of BME (Anand et al. 2011).

HSP-70, a member of the heat shock protein family, functions as an inducible molecular chaperon to various stress conditions. It has been used as a marker for cell damage, apoptosis and oxidative stress. Studies have shown that exposure of cells to toxic compounds results in the induction of HSP-70 expression (Anand et al. 2012). In the present study, we observed the protective effect of BME by down-regulation of HSP-70 expression induced by SNP exposure, which confirms the cytoprotective role of BME (Fig. 7). Mitochondria are one of the major target sites of free radical generation which causes lipid peroxidation and increases the permeability of the mitochondrial membrane. The increased permeability forms mitochondrial transition pores which is

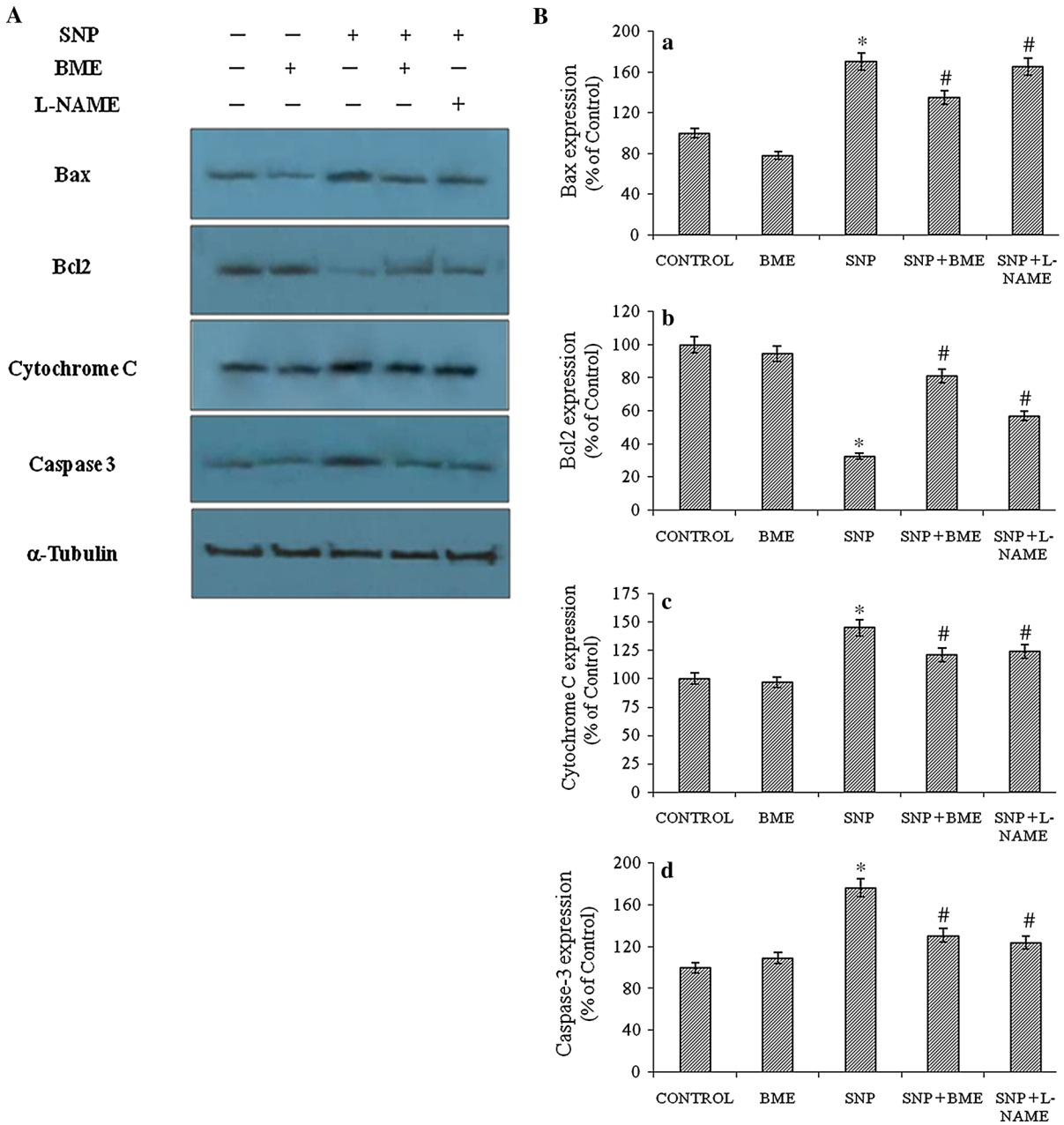

Fig. 8 **A** Protective effect of BME on SNP induced expression of apoptosis marker proteins Bax, Bcl-2, cytochrome c and caspase-3 analyzed by immunobloting. The band intensity was calculated by Image-J software. **B a–d** Densitometric analysis of Bax, Bcl-2, cytochrome c and caspase-3 expression, respectively. The data presented are mean ± SEM of three independent experiments. $^{*}p < 0.05$ versus control group and $^{\#}p < 0.05$ versus SNP treated group

assumed to be one of the early events of apoptosis (Hruszkewycz 1992). Bcl2 appeared to function by inhibiting the mitochondria depolarization. Conversely, Bax induced mitochondria depolarization. The ratio of Bcl2 to Bax has been reported to be correlated with apoptosis in cancer cells. These proteins lead to opening of transition pores and inter membrane proteins, such as cytochrome c, are released out of the mitochondria and leak out to cytosol. The released cytochrome c binds to the

Table 1 Effects of BME pre-treatment on antioxidant status in L132 cells against SNP-induced cytotoxicity

	SOD (U/mg of protein)	CAT (mM H_2O_2 degraded/min/mg of protein)	GPx (U/mg of protein)	GR (U/mg of protein)	GSH (μM/mg of protein)	Total antioxidant capacity (nM/cm/mg of protein)
Control	1.49 ± 0.09	0.94 ± 0.03	758.51 ± 38.84	720.43 ± 35.80	5.13 ± 0.37	37.15 ± 1.62
BME	1.49 ± 0.08	0.99 ± 0.04	801.03 ± 44.20	744.29 ± 31.53	5.70 ± 0.19	38.37 ± 1.23
SNP	0.75 ± 0.09*	0.31 ± 0.03*	359.32 ± 45.94*	388.43 ± 13.42*	2.12 ± 0.12*	18.71 ± 1.51*
SNP + BME	1.34 ± 0.12$^{\#}$	0.82 ± 0.04$^{\#}$	619.60 ± 47.07$^{\#}$	580.52 ± 23.16$^{\#}$	4.15 ± 0.14$^{\#}$	33.75 ± 1.83$^{\#}$
SNP + L-NAME	1.32 ± 0.06$^{\#}$	0.73 ± 0.05$^{\#}$	602.42 ± 44.95$^{\#}$	577.13 ± 25.08$^{\#}$	3.74 ± 0.13$^{\#}$	29.57 ± 1.97$^{\#}$

The data presented are mean ± SEM of three independent experiments. * $p < 0.05$ versus control group and $^{\#}$ $p < 0.05$ versus SNP treated group

protein apoptotic protease activating factor-1. Using the energy provided by ATP, these complexes aggregate to form apoptosomes. These apoptosomes bind to and activate over a dozen of caspases. Caspase-3 is one among them. The activation of these caspases leads to apoptotic cell death. During this process the Bcl2: Bax ratio would be altered along with the activation of poly adenosine ribose polymerase, an executioner of DNA damage and cell death. Our experiments demonstrated that BME pre-treatment regulated SNP induced up-regulation of Bax, cytochrome c and caspase 3 expressions along with a down-regulation of Bcl2 expression (Fig. 8). These results are in agreement with previous reports, that say that NO displays both pro- and anti-apoptotic properties induced by endogenously generated or exogenously supplied NO in hepatocytes (Kim et al. 1995), thymocytes (Fehsel et al. 1995), macrophages (von Knethen et al. 1999), and several other cells (Ankarcrona et al. 1994; Vincent and Maiese 1999; Taimor et al. 2000).

As mentioned earlier, free radicals generation causes lipid peroxidation and increases the permeability of the mitochondrial membrane. This decrease in mitochondrial membrane potential has been found associated with several apoptosis models and its estimation is a biomarker to evaluate stress induced apoptotic cell damage (Ly et al. 2003). Rhodamine 123, a mitochondrial selective, cationic and lipophilic fluorescent dye that partitions into active mitochondria based on the highly negative mitochondrial membrane was used to check MMP. The diffusion and accumulation of this dye in mitochondria is proportional to the degree of MMP (Ubl et al. 1996). Any damage in mitochondrial membrane leads to leakage of rhodamine 123 into cytosol which subsequently moves out of the plasma membrane and exhibits decreased intracellular fluorescence. However, the present study showed the restoration of fluorescence by BME pre-treatment indicating the cytoprotective role of BME by restoring mitochondrial membrane potential against SNP-induced damage.

Oxidative stress can occur either due to the over production of ROS or decrease in cellular antioxidant levels. The pre-dominant enzymatic antioxidant defense systems present within all cells are SOD, CAT, GPx and GR. These enzymes play a crucial role in ameliorating the oxidative damage within the cells (Li et al. 2012). In the present study, SNP-induced significant alleviation in the levels of these antioxidant enzymes. This perturbation within the system makes the cells more vulnerable to oxidant injury. The decreased activity of antioxidant enzymes may be due to decreased cellular antioxidant levels viz GSH levels. Hence, the GSH redox cycle plays a major role in protection against severe oxidative stress (Merad-Boudia et al. 1998; Ibi et al. 1999) and may be considered as one of the most prevalent and important free radical scavenger for the antioxidant defense of the cells. Depletion of GSH leads to peroxidation of lipids, proteins, carbohydrates and DNA damage consequently causing cellular damage. However, the present study delineates anti-apoptotic and NO inhibitory mechanism of action of BME against SNP-induced cell damage. However BME pre-treatment significantly ameliorated the various impairments induced by SNP exposure and protected the L132 cells by elevating the cellular antioxidant status.

Nω-nitro-L-arginine methyl ester (L-NAME) is a non-selective competitive inhibitor of NO synthase (NOS). An in vivo study showed that L-NAME inhibits iNOS and thereby reduced formation of peroxynitrite and other reactive nitrogen and oxygen species (Seif-el-Nasr and Fahim. 2001). In addition, the antioxidant effects of L-NAME on various oxidative stress biomarkers like lipid peroxidation have ameliorated SNP-induced cytotoxicity (Seif-el-Nasr and Fahim 2001; Sayan et al. 2004; Awooda et al. 2013). Decreased mitochondrial lipid peroxidation by L-NAME results in increased mitochondrial membrane potential and decreased cytochrome c release, thereby inhibiting the apoptosis. These findings were supported by studies conducted by Rakesh et al. (1999) and Ding-Zhou et al. (2002) reported that NOS inhibitor L-NAME blocks apoptosis. In the present study, pre-treatment with L-NAME has significantly attenuated SNP-induced cytotoxicity in L132 cells.

Conclusion

In the present study we have clearly demonstrated that *B. monniera* extract exhibit cytoprotective effects by ameliorating the SNP induced cytotoxicity by neutralizing oxido-nitrosative stress. In this study, we report that BME efficiently ameliorated mitochondrial, plasma membrane damage by alleviating the generation of NO via down-regulation of iNOS expression. It also restored the cellular antioxidant status by regulating the oxidative stress biomarkers. Further, BME showed a protective role by modulating bio-markers of apoptosis viz., Bax, cytochrome c and caspase-3. Further studies are warranted to understand the molecular mechanism of action of BME in an animal model.

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Conflict of interest The authors have declared that there is no conflict of interest.

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