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RESEARCH ARTICLE

BIOMOLECULE-PROTECTIVE ACTIVITY OF THE BACOSIDE FRACTION FROM *BACOPA MONNIERI*

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ABSTRACT

Bacopa monnieri is an important Ayurvedic drug and, traditionally, it is reported to be used for skin diseases, fever, inflammation, anaemia, urinary disorder and psychiatric disorders. The major therapeutically important chemical constituents of this plant are the triterpenoid saponins, bacosides. In the present study, bacoside fraction was prepared from the aerial parts of *Bacopa monnieri.* The extent of oxidative damage to cellular biomolecules like membrane lipids, DNA and proteins, and its protection by bacoside fraction, was studied in cell-free systems and intact cells. From the results, it was clear that the bacoside fraction possessed significant biomolecular protection against oxidative stress, both in cell-free systems and in intact cells.

INTRODUCTION

Traditional medicine worldwide is being re-evaluated by extensive research on different plant species and their therapeutic principles. Since plants produce antioxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with antioxidant activity (Biswas *et al*., 2010). Plants are a rich source of natural products used for centuries to cure various diseases. The plant-derived medicines are based upon the premise that they contain natural substances that can promote health and alleviate illness. So, a retrospection of the healing power of plants and a return to natural substances are an absolute need of our time. The demonstration of the presence of natural products, such as polyphenols, alkaloids, flavonoids, coumarins and other secondary metabolites in medicinal plants will provide a scientific validation for the popular use of these plants (Boubaker *et al.,* 2012). Medicinal plants are also in high demand for application of functional food or biopharmaceuticals because of consumer preferences (Khan *et al*., 2012). Herbal and natural products have been used in folk medicine for centuries throughout the world. There has been renewed interest in screening higher plants for novel biologically active compounds, particularly those that effectively intervene in human ailments in the field of chronic diseases (Selvam *et al*., 2012). With this background, the plant chosen for the present study is *Bacopa monnieri. Bacopa monnieri* (L.) Pennell (water hyssop), known locally in India as Neer Brahmi or Jalanimba, is one of the most important plants in the traditional Hindu system of medicine, Ayurveda. The name, Brahmi, comes from the word Brahma, one of the main gods of Hinduism. *B. monnieri* is being used in India for over 5,000 years to treat epilepsy and insomnia, as a sedative and for abolishing raw anxiety. Indian materia medica (Bhavprakasha Nighantu 1,500 years AD) recommends this resource as a means of improving memory and concentration (Lojewski *et al*., 2014). *Bacopa monnieri* is a creeping perennial with small oblong leaves and purple flowers, found in warm wetlands, and native to Australia and India. Commonly found as a weed in rice fields, *Bacopa monnieri* grows throughout East Asia and the United States. The entire plant is used medicinally (Aguiar and Borowski, 2013).Many studies have been conducted on the memory enhancing activity and anticancer property of *Bacopa monnieri.* The methanolic extract of *Bacopa monnieri* exhibited good antioxidant and anticancer property in Hep2 cell line (laryngeal carcinoma) (Radha, 2010).

Oxidative stress is a biological phenomenon that results from a biochemical imbalance between the formation and clearance of free radicals. Mitochondria are the major source of cellular ROS. The accumulation of ROS induces oxidative damage of mitochondrial DNA (mtDNA), proteins, and lipids, that leads to decline in physiological function of cells resulting in a

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variety of diseases and accelerated aging (Pervin *et al*., 2014). Therefore, it was important to study the extent of oxidative damage to biomolecules and its protection by the bacoside fraction. The extent of oxidative damage to cellular biomolecules like membrane lipids, DNA and proteins, and its protection by bacoside fraction, was studied in cell-free systems and intact cells.

MATERIALS AND METHODS

Preparation of bacoside fraction

The bacoside fraction was prepared from the shade dried aerial parts of *Bacopa monnieri* (Kahol *et al*., 2004).

Evaluation of the effects of bacoside fraction on membrane lipids

ROS are generated by redox reactions and the Fenton reaction of H_2O_2, as well as iron that generates the hydroxyl radical, which causes severe damage to DNA, proteins and lipids (Nicolaou *et al*., 2013). Thus, it was felt imperative to study the effects of the bacoside fraction on oxidant-induced damage to lipids, DNA and proteins. As the first step, different concentrations (10µg, 25µg, 50µg, 75µg, 100µg and 200µg) of the bacoside fraction were used to assess the protective effect against oxidant (H_2O_2) induced damage to lipids *in vitro*. Three different membrane models namely, goat RBC ghosts (plasma membrane lipids), goat liver homogenate (plasma membrane and intracellular lipids) and liver slices (intact cells) were used to assess the extent of lipid peroxidation and the protection rendered by the bacoside fraction against induced oxidative stress.

Evaluation of LPO in RBC ghosts

The extent of formation of thiobarbituric acid reactive substances (TBARS) from the lipids damaged by oxidizing agents can be used as a measure of damage of membrane lipids. The RBC ghosts were prepared by the method of Dodge *et al.* (1963).

Estimation of LPO in goat liver homogenate

Goat liver was procured fresh from a slaughterhouse and washed thoroughly using Tris HCl buffer (40mM, pH 7.0). A 20% homogenate of the liver was prepared in the same buffer by using a motorized Teflon homogenizer. The homogenate was clarified by low speed centrifugation and used as the membrane lipid source for the LPO assay, according to the method of Okhawa *et al.* (1979).

Estimation of LPO in goat liver slices

The extent of inhibition of LPO in goat liver slices was estimated by the method proposed by Niehaus and Samuelsson (1968).

Effect of the bacoside fraction on oxidant induced DNA damage

The effect of the bacoside fraction in counteracting oxidant-mediated DNA damage was assessed in different hierarchies of DNA in the order of evolutionary development. The damage was assessed both in commercially available preparations of DNA and intact cells *in vitro*. The commercially available preparations namely λ DNA (viral DNA), pUC18 (bacterial plasmid), herring sperm and calf thymus DNA (animal origin) were used. Human peripheral blood cells constituted the source of intact cells for studying DNA damage.

Estimation of DNA damage in λ DNA and pUC18 DNA

The DNA damage in λ DNA and pUC18 DNA was determined by the method proposed by Chang *et al*. (2002).

Estimation of damage in herring sperm DNA and calf thymus DNA

The extent of DNA damage caused to herring sperm DNA and calf thymus DNA by hydrogen peroxide and the protective effect of bacoside fraction was studied according to the method proposed by Aeschlach *et al*. (1994).

Evaluation of the extent of DNA damage in intact cells

The comet assay was used to quantify the extent of DNA damage in intact cells. It was carried out under alkaline conditions, as described by Singh *et al.* (1988).

Effect of bacoside fraction against protein oxidation

Protein carbonyl assay

Protein carbonyl modification is one of the forms of oxidative damage and the protein carbonyl level is a sensitive biomarker of protein oxidative modification (Kobayashi *et al*., 2014). The method outlined by Jean *et al*. (2010) was used to analyze the protein carbonyl.

1-D gel electrophoresis to analyze protein oxidation

Proteins are the early targets of ROS. However, the ability to identify specific proteins that are most susceptible to oxidative modifications is difficult. Separation of proteins using polyacrylamide gel electrophoresis (PAGE) offers the analytical potential for the recovery. The ability of this method was used in the present study to visualize the *in vitro* damage to proteins.

RESULTS

Preparation of bacoside fraction

The bacoside fraction obtained was 0.6%.

Effect of the bacoside fraction against *in vitro* lipid peroxidation

The per cent inhibition of *in vitro* lipid peroxidation by the bacoside fraction in all the three membrane systems is presented in Figure 1. The maximum inhibition of LPO was observed in the goat liver slices, followed by the liver homogenate and then the RBC ghosts. The extent of *in vitro* lipid peroxidation was inhibited in a dose-dependent manner by the bacoside fraction. There was an increase in the extent of

inhibition in all the three membrane preparations from 10µg to 50µg. Thereafter, a near-linear activity was observed with higher concentrations. Therefore, this concentration (50µg) was chosen as the optimal dose for further analysis. The results showed that the bacoside fraction rendered protection to lipid molecules against oxidant-induced *in vitro* lipid peroxidation.

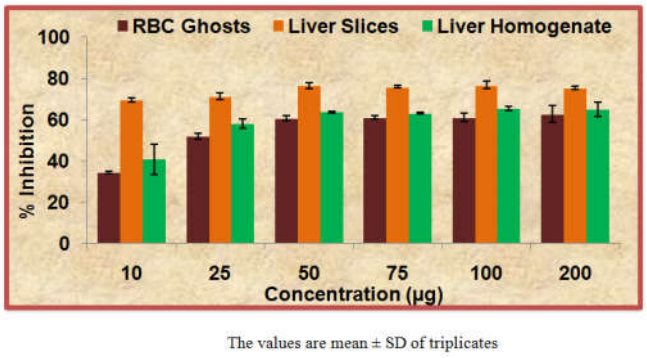


Figure 1. Inhibition of lipid peroxidation in different membrane preparations by the bacoside fraction

Protective effects of the bacoside fraction from *Bacopa monnieri* on oxidative damage to DNA

Protective effects of the bacoside fraction to λ DNA and pUC18 DNA

The extent of damage induced by H_2O_2 to DNA from these sources and the protective effects of the bacoside fraction were studied by viewing the migration pattern of the DNA in agarose gels. The results are presented in Figure 2. In both the DNA types, H_2O_2 caused a significant extent of damage. This was evident by a decrease in the intensity of specific bands in lane 2, wherein the DNA was treated with oxidant alone. Bacoside fraction reversed this damage, which could be seen in lane 4, as indicated by the intact bands. The bacoside fraction, by itself, did not cause any DNA damage. This observation was reiterated by the Integrated Density Values (IDV) of the bands, recorded using a digital gel documentation software (Alpha Ease FC of Alpha Digidoc 1201), the values of which are presented in Table 1.

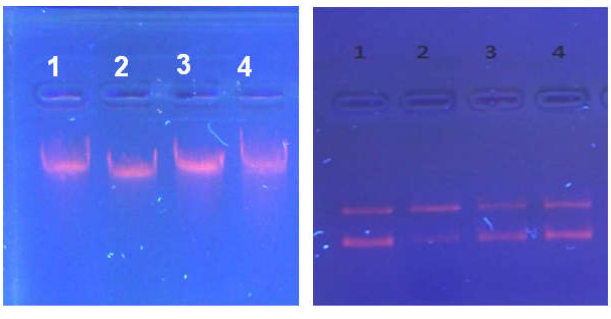


Figure 2. Migration patterns of λ DNA and pUC18 DNA treated with H_2O_2 with and without bacoside fraction

Table 1. IDV of the bands in the agarose gel with λ DNA and pUC18 DNA

Sample	IDV of the bands			
	λ DNA		pUC18 DNA	
	Without H_2O_2	With H_2O_2	Without H_2O_2	With H_2O_2
No extract	909039	595733	43731	5932
Bacoside fraction	906337	563141	41583	39823

Among the two DNA preparations from the lower organisms, the bacterial plasmid DNA was more susceptible to oxidative damage and was also more receptive to the protective effect by the bacoside fraction. The extent of damage by H_2O_2 in the DNA from the viral source was lower; the extent of protection was also lower in λ DNA. The IDV of the bands clearly proved this observation.

Protective effects of the bacoside fraction on H_2O_2 induced damage to herring sperm and calf thymus DNA

The results of the quantification of oxidative damage to herring sperm DNA is schematically presented in Figure 3.

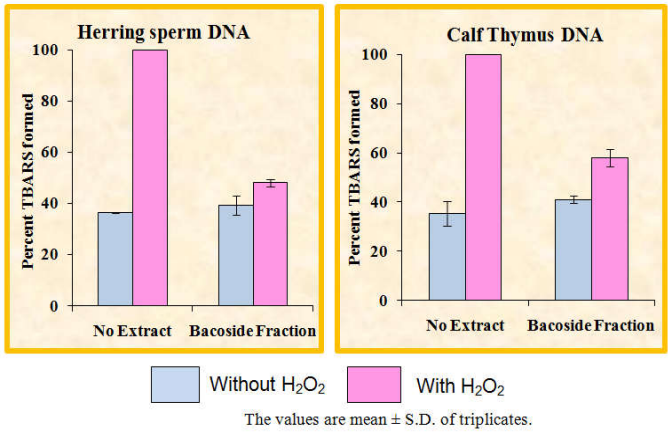


Figure 3. Inhibition of oxidant-induced damage to herring sperm and calf thymus DNA by the bacoside fraction

The value of H_2O_2-treated group was fixed as 100 per cent and the relative values in percentage were calculated for the other groups. It was found that H_2O_2 caused an increased extent of damage to herring sperm DNA. The extent of damage decreased markedly in the presence of the bacoside fraction. This indicated the protective effect rendered by the bacoside fraction against the oxidant. Similar results were also observed with calf thymus DNA (Figure 3). This proved that the bacoside fraction possesses good protective effect against oxidative damage to DNA.

Effect of the bacoside fraction on the damage induced by H_2O_2 to DNA in intact cells

The DNA damaging effect of H_2O_2 in intact cells was studied by following the formation of comets in human peripheral blood cells exposed to the oxidant *in vitro*. The effect of the bacoside fraction is presented in Table 2. The photographic record of the comets in each of the treatment groups is depicted in Figure 4.

Table 2. Effect of the bacoside fraction on DNA damage induced by H_2O_2 in human peripheral blood cells

Treatment Groups	No. of cells with comet/100 cells	
	Without H_2O_2	With H_2O_2
Control	2 ± 1	35 ± 2 [a]
Bacoside fraction	7 ± 2 [a]	20 ± 3 [a,b,c]

The values are mean ± SD of triplicates
a – Statistically significant (P<0.05) compared to untreated control
b – Statistically significant (P<0.05) compared to H_2O_2 alone treated group
c – Statistically significant (P<0.05) compared to the respective bacoside fraction treated group

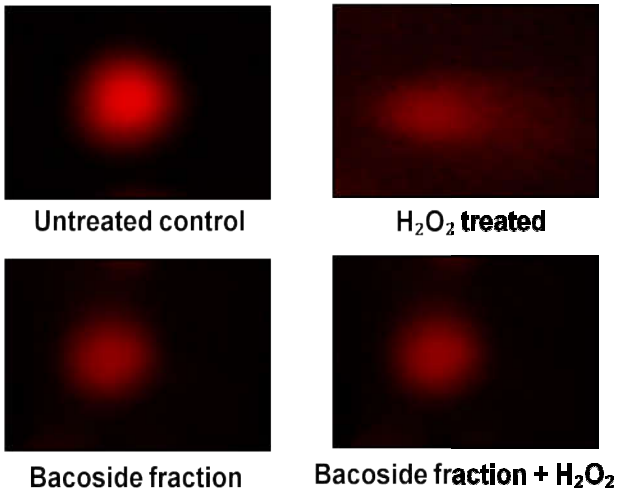


Figure 4. Comet bearing peripheral blood lymphocytes

H_2O_2 exposure caused a steep increase in the number of cells with comets. In the positive control (cells treated only with H_2O_2), the DNA was severely damaged. The co-treatment with the bacoside fraction and H_2O_2 significantly decreased the number of cells expressing the DNA damage. Thus, the results indicated that the bacoside fraction was able to protect the DNA in peripheral blood cells against oxidative damage (Figure 4). These observations suggested that the bacoside fraction is effective in counteracting the DNA damage.

Effect of the bacoside fraction on protein carbonyl formation

The effect of bacoside fraction on protein oxidation is depicted in Table 3. The levels of protein carbonyl significantly increased in the presence of the oxidant. On co-treatment with the bacoside fraction, a significant decrease in the oxidation of proteins was observed when compared to that of the group treated with the oxidant alone. This showed the protective effect of the bacoside fraction against protein oxidation**.**

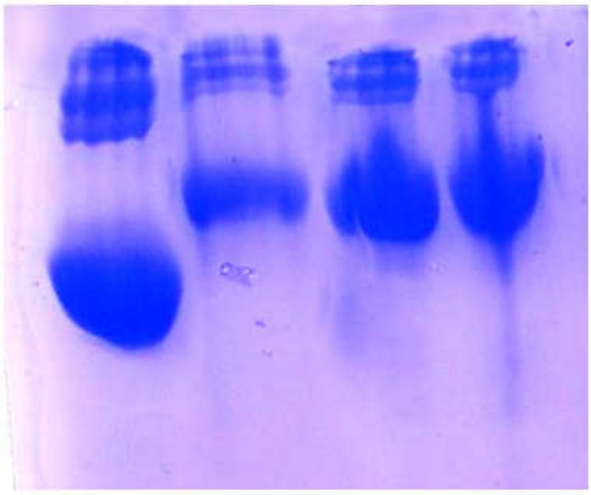
Table 3. Effect of the bacoside fraction on protein carbonyl formation

Sample	Protein carbonyl (nmol/mg protein)	
	Without H_2O_2	With H_2O_2
No Extract	8.44 ± 1.11	35.97 ± 3.17[a]
Bacoside fraction	5.25 ± 0.57[a]	18.30 ± 0.91[a,b,c]

The values are mean ± S.D. of triplicates
a – statistically significant (p<0.05) compared to untreated control
b – statistically significant (p<0.05) compared to H_2O_2 control
c – statistically significant (p<0.05) compared to the respective bacoside fraction control

Effect of the bacoside fraction on protein migration on 1D Gel

The effect of the bacoside fraction on protein oxidation *in vitro* was evaluated by 1D gel electrophoresis. It is evident from the results of the SDS-PAGE depicted in Figure 5, that the intensity of the bands in the H_2O_2-treated group (lane 2) showed a significant decrease when compared to that of the untreated control (lane 1). This effect was counteracted by the co-treatment with the bacoside fraction (lane 4). The integrated density values of the bands obtained are shown in Table 4.



Lane 1: Untreated control
Lane 2: BSA + H_2O_2
Lane 3: BSA + Bacoside fraction
Lane 4: BSA + Bacoside fraction + H_2O_2

Figure 5. Effect of the bacoside fraction on the migration of proteins subjected to oxidative stress

Table 4. IDV of the bands in the polyacrylamide gel of proteins subjected to oxidative stress

Sample	IDV of the bands			
	Band 1	Band 2	Band 3	Band 4
Control	115920	104244	107310	450775
H_2O_2	56355	26622	57525	248100
Bacoside fraction	80730	87318	97565	385140
Bacoside fraction+ H_2O_2	74562	70210	73059	328233

The results showed that 50µg of the bacoside fraction exhibited significant biomolecular protection against oxidative stress, both in cell-free systems and in intact cells.

DISCUSSION

When ROS are generated in the biological systems, the first group of molecules to take their brunt are the lipid molecules in the membrane barriers. If the ROS production is extracellular, their encounter happens with the plasma membrane lipids, and if intracellular, with the lipids in the organelles like mitochondrial and nuclear membranes. Taking this into consideration, in the present study, lipid preparations representing the plasma membrane, intracellular membranes and the intact cells were studied. Aqueous, aqueous-methanol, methanol and acetone extracts of mature pods (fruits) of *Helicteres isora* L. with varying concentrations showed

inhibition of lipid peroxidation in a dose-dependent manner (Kumar *et al*., 2013). Palaniswamy and Padma (2011) reported that the methanolic extract of *Majorana hortensis* leaves substantially declined the extent of LPO in RBC ghosts, goat liver homogenate and goat liver slices to a significant extent in comparison with the chloroform and the aqueous extracts. DNA molecules constitute the ultimate targets of oxidative damage, which, when not repaired, can lead to devastating consequences, resulting in several diseases, including cancer. Therefore, in the present study, DNA, as a target of oxidative assault, and the effect of the bacoside fraction on it, was also studied. In order to test whether the composition of DNA had any influence on the extent of damage and its protection, DNA from different sources, representing low to high hierarchies of evolutionary development, were used.

Sumathi *et al.* (2010) reported that the leaf and herbal extracts of *Withania somnifera* rendered a significant protection against H_2O_2-induced oxidative damage to pUC18, λ and herring sperm DNA. A methanolic extract of the leaves of *Rhinacanthus nasutus* exhibited maximum protection to herring sperm DNA in comparison to the aqueous and chloroform extracts to the oxidative stress imposed *in vitro* (Nirmaladevi and Padma, 2011). Native lactoferrin and holo-lactoferrin clearly protected calf thymus DNA from fragmentation due to ultraviolet irradiation in the presence of H_2O_2 (Ogasawara *et al*., 2014). Peroxynitrite-induced DNA damage was significantly inhibited by a water extract of Chinese bayberry in a concentration-dependent manner in primary astrocytes (Chen *et al*., 2015). Proteins constitute the workhorse molecules of the cell, involved in the most diverse activities that result in the execution and regulation of every activity in the cell. Damage to one group of proteins can cascade into affecting a series of activities, resulting in loss of functional control and cellular death.

Therefore, protein damage was also assessed in the presence and the absence of oxidant and / or bacoside fraction. Aqueous extract of green tea was effective in reducing the protein carbonyl formation enhanced by cyclophosphamide in the reproductive system of male mice (Zanchi *et al*., 2015). A methanolic extract of *Vitex doniana* fruits significantly reduced the formation of protein carbonyls in a dose-dependent manner in mice (Ajiboye, 2015). Makri *et al.* (2013) proved the protective effect *Crocus sativus* stigmas (saffron) extract against selenium-induced crystalline proteolysis of rat lens proteins, using SDS-PAGE.

From the results of the present study, it became evident that the bacoside fraction exhibits strong biomolecule-protecting activities.

Conclusion

Since oxidative stress-mediated biomolecular damage is strongly implied to be linked to the process of carcinogenesis, these results bore significance in suggesting strong anticancer activity to be associated with the bacoside fraction. Therefore, further studies are needed in order to determine the anticancer activity of the bioactive compounds present in the bacoside fraction isolated from *Bacopa monnieri*. The effects of the bacoside fraction can be evaluated *in vivo* using experimental animals.

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