Protective Effect of Bacoside-A against Morphine-Induced Oxidative Stress in Rats

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Sumathi, *et al.*: Bacoside-A and Morphine Neurotoxicity

In the present study, we investigated the protective effect of bacoside-A the active principle isolated from the plant *Bacopa monniera* against oxidative damage induced by morphine in rat brain. Morphine intoxicated rats received 10-160 mg/kg b.w. of morphine hydrochloride intraperitoneally for 21 days. Bacoside-A pretreated rats were administered with bacoside-A (10 mg/kg b.w/day) orally, 2 h before the injection of morphine for 21 days. Pretreatment with bacoside-A has shown to possess a significant protective role against morphine induced brain oxidative damage in the antioxidant status (total reduced glutathione, superoxide dismutase, catalase, glutathione peroxidase and lipid peroxidation) and membrane bound ATP-ases(Na$^+$/K$^+$ ATPase. Ca^{2+} and Mg^{2+} ATPases) activities in rat. The results of the present study indicate that bacoside-A protects the brain from oxidative stress induced by morphine.

Key words: Bacoside-A, brain damage, morphine, membrane-bound ATPases, oxidative stress

The opioid analgesics commonly exemplified by morphine, represent the best option for the treatment of severe pain and for the management of chronic pain states. Morphine abuse is a worldwide health problem that provoked many investigations to study its mechanism of action. Focal glomerulosclerosis[1], hepatotoxicity with a reduced glutathione superoxide dismutase, catalase, glutathione peroxidase levels[2], histamine liberation from mast cells[3], neurotoxicity with spongiform leucoencephalopathy[4], neuroteratogenicity[5] and oxidative damage of DNA[6] are some of the pathological features in chronic morphine toxicity generated by local rearrangement of anti-oxidant homeostasis.

Recently, the development of a systemic oxidative stress was noted as a result of opioid–stimulated free radicals production. An increased lipid peroxidation was found *in vitro* and *in vivo* in heroin consumption[7,8]. Chronic morphine abuse resulted in the decreased level of antioxidants and antioxidases status[9,10]. Measurement of the levels of free radical or ROS production, metabolites of lipid peroxidation such as malondialdehyde and antioxidant capacity may give information about an antioxidant homeostasis under morphine stimulation and morphine-induced toxicity.

Morphine enhances neuronal Na$^+$/K$^+$ ATPase activity, enzyme responsible for ionic exchange across the cellular membrane[11]. This enzyme of membrane bound may be used as a metabolic probe to study the effects of morphine in rat brain[12], due to mechanism involved in this effect are not well known. Previous studies have shown that significant depletion of reduce glutathione (GSH) in peripheral organs following acute systemic or central administration of opioids[13]. ROS or free radicals may be involved in the morphine and opiate abuse[2,14]. It is well recognized that the use of morphine is associated with degeneration in brain cells[2]. Hence, administration of a compound that has membrane-stabilizing as well as antioxidant properties would therefore be expected to prevent morphine induced oxidative stress in rat brain.

Bacoside-A is the dammarene type triterpenoid saponin isolated from the plant *Bacopa monniera*[15], which is held in high repute as a potent nerve tonic[16]. *Bacopa monniera Linn.* is used in the indigenous systems of medicine for the treatment of various nervous system ailments such as insomnia, anxiety, epilepsy, hysteria etc[17]. The plant has been shown as a potent free radical scavenger and

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antioxidant[18]. Besides it also exhibits vasodilatory[19], calcium antagonistic[20], muscle relaxant[21], mast cell stabilizing[22], and antiulcer[23] properties. Recently, protective effect of bacoside-A against chronic exposure to cigarette smoke has been reported by Anbarasi *et al.*[24] Thus, the aim of the present study was designed to explore the protective effect of bacoside-A on morphine induced oxidative stress in terms of antioxidant status and membrane bound ATPases in rat brain.

Earlier we reported, several papers with respect to protective effect of *Bacopa monniera* extract against morphine toxicity, but this is the first report with respect to the bioactive compound level. Hence the present study is undertaken to evaluate the effect of bacoside-A against morphine induced oxidative stress in rat brain.

MATERIALS AND METHODS

Morphine hydrochloride used in the present study was obtained from MOTI and Company, Lakshmipuram, Royapettah, Chennai, Tamil Nadu, India. All other reagents used were of analytical grade and obtained from Himedia, India.

Isolation of bacoside-A:

The plant *Bacopa monniera* was collected in and around Chennai, India and authenticated by Dr. P. Brindha, Central Research Institute (Siddha), Chennai, India. The dammarene type triterpenoid saponin bacoside-A was isolated from the plant by the procedure carried out by Deepak *et al.*[25]. The activity-guided isolation of Bacoside A was carried out using the brine shrimp lethality assay to direct the fractionation of a methanolic proportion of methanol. The fractions eluted with chloroform:methanol 85:15 were combined and concentrated under vacuum (7 g). This fraction was subjected to flash chromatography on silica gel (100–200 mesh) and the fractions eluted with chloroform:methanol 85:15 were combined, concentrated under vacuum and crystallised from 70% methanol in water to obtain bacoside-A (2.5 g). Aqueous suspension of bacoside-A was given orally to the experimental animals at a dosage of 10 mg/kg b.w/day for 21 days.

Animals:

Adult male albino rats of Wistar strain (150-200 g) were used for the present study. The rats were provided with standard pelleted rat feed and water ad libitum. They were acclimatized to the laboratory conditions and maintained less than 12 h light/dark cycle at 25±2°. The experiments were carried out in accordance with the guidelines provided by the Institutional Animal Ethics Committee.

Experimental design:

The animals were divided into four groups of six animals each. Group I–control, Group II - rats received morphine hydrochloride (10-160 mg/kg b.w, i.p)[26], for 21 days, Group III - rats received bacoside-A (10 mg/kg b.w/day, p.o)[24], 2 h before the administration of morphine hydrochloride for 21 days, Group IV - rats received bacoside-A alone orally for 21 days. At the end of the experimental period the animals were sacrificed by cervical decapitation. The brain tissues were collected and homogenized with motor driven Teflon coated homogenizer in ice-cold 0.1M Tris-HCl buffer pH 7.4 to get 10% homogenate and used for biochemical studies.

HPLC-finger print analysis of *Bacopa monniera* extract:

HPLC-Finger Print analysis of *Bacopa monniera* was performed using the conditions given in Table 1.

TABLE 1: PARAMETERS FOR HPLC ANALYSIS

Parameters	Condition used		
HPLC system	Shimadzu HT2010 chromatographic system with in combination with Class LC 10A software and UV detection		
Column	RP C-18 Luna phenomenex (250x4.6 mm)		
Column oven temperature	25°		
Mobile phase	A-0.25% Orthophosphoric acid in water B-Acetonitrile		
Flow rate	1.5 ml		
Injection volume	25.0 µl		
Gradient	Time	Concentration of A	Concentration of B
	0	75	25
	25	60	40
	35	40	60
	38	75	25
	45	75	25
Detection wavelength	205 nm		
Run time	45 min		
Sample preparation	Weigh accurately 500 mg Extract to a 100 ml volumetric flask dissolve in 50 ml methanol, sonicate for 10-15 min. Cool then make up to 100 ml with methanol. Filter through 0.45 microns membrane filter paper		

Biochemical analysis:

The activity of SOD was determined by the method of Marklund and Marklund[27]. Activity of catalase was assayed by the method of Sinha[28]. Glutathione peroxidase was assayed by the method of Rotruck et al.[29]. The level of reduced glutathione was measured by the method of Moron et al[30]. Lipid peroxidation was determined by the method of Hogberg et al.[31]. Ferrous sulphate and ascorbate induced lipid peroxidation was carried out by the method of Devasagayam and Tarachand[32]. The activity of Na^+/K^+-ATPase was assayed by the method of Bonting[33]. Ca^{2+}-ATPase activity was assayed according to the method of Hjerten and Pan[34]. The activity of Mg^{2+}-ATPase was assayed by the method of Ohnishi et al[35]. Inorganic phosphorous was estimated by the method of Fiske and Subbarow[36]. The protein carbonyl content was quantified by the method of Levine et al.[37]. Protein was estimated by Lowry et al.[38] method.

Statistical analysis:

Results were expressed as mean±SD (n=6), analysed by oneway ANOVA, followed by Tukey's post-hoc test by SPSS version 7.0. Student's t-test was used for the statistical evaluation of the data obtained. Statistical comparisons were made between Group II Vs Group I and Group III Vs Group II. A value of P<0.001 was considered to be statistically significant.
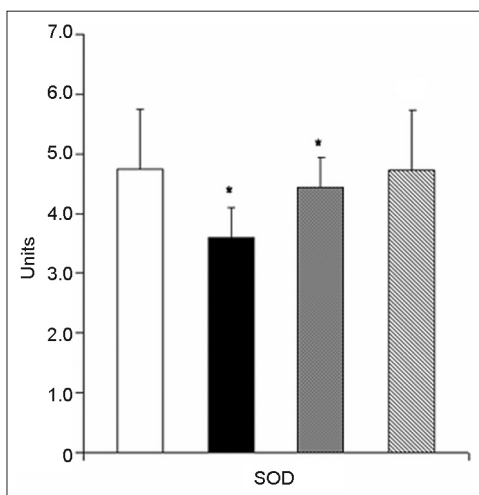
RESULTS

Figs. 1 and 2 represent the effect of bacoside-A on the activities of SOD, CAT, GPx enzymes and the level of glutathione (GSH) in the brain of control and experimental animals. The activities of all these enzymes along with GSH level (P<0.001) were found to be significantly decreased viz; SOD (P<0.001), CAT (P<0.001), GPx (P<0.001) in morphine-induced rats when compared to Group I rats. Whereas pretreatment with bacoside-A significantly alters the changes caused by morphine and found to increase the activities of all these antioxidant enzymes to near normal level when compared to Group II animals. Bacoside-A alone treated rats (Group IV) had no effect on the activities of these enzymes when compared with control rats.

Fig. 3 illustrates the effect of bacoside-A on the levels of Basal, $FeSO_4$, Ascorbate and H_2O_2 induced lipid peroxides in the brain of control and experimental rats. Group II morphine induced rats registered a significant (P<0.001) increase in the levels of lipid peroxides when compared to group I control rats. A significant decrease (P<0.001) in the levels of lipid peroxide was noted in group-III rats when compared to Group-II rats. Bacoside-A alone treated rats (group IV) did not show any changes in the level of lipid peroxides when compared to control rats.

Fig. 1: Effect of bacoside-A on the activity of SOD in the brain of control and experimental animals.
Values are expressed as mean±SD; n=6. Morphine group was compared with control group. Bacoside-A pretreated group was compared with morphine group. *P<0.001; SOD – units/min/mg protein. One unit of the enzyme is defined as the amount of enzyme, which inhibits the rate of pyrogallol autoxidation by 50%. □ Control; ■ Morphine; ▨ BAC-A+MR; ▨ BCA-A
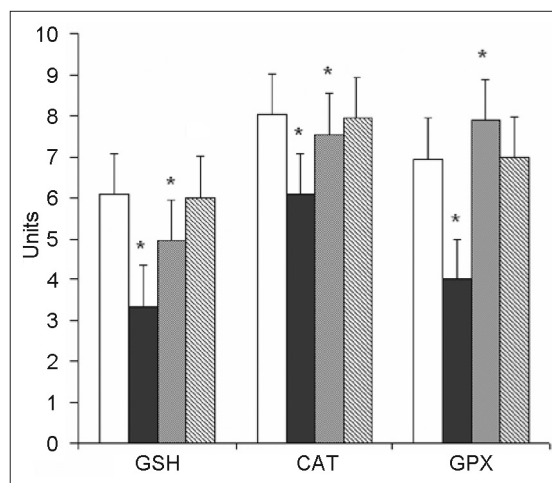

Fig. 2: Effect of bacoside-A on the level of reduced glutathione (GSH) and the activities of CAT, and GPx in the brain of control and experimental animals.
Values are expressed as mean±SD; n=6. Morphine group was compared with control group. Bacoside-A pretreated group was compared with morphine group. *P<0.001; GSH - μg/mg protein; CAT - μmole of H_2O_2 consumed/min/mg protein. GPx - μmoles of glutathione oxidized/min/mg protein. □ Control; ■ Morphine; ▨ BAC-A+MR; ▨ BCA-A
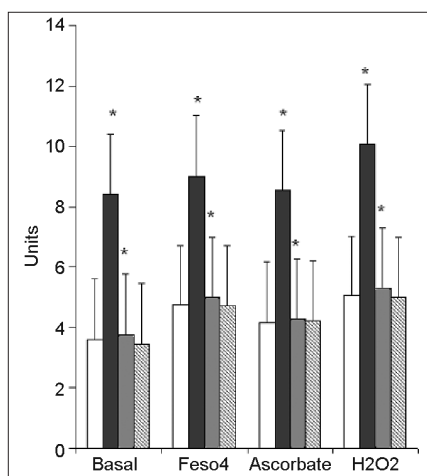
Fig. 3: Effect of bacoside-A on the levels of basal, FeSo₄, ascorbate and H₂O₂-induced lipid peroxides in the brain of control and experimental animals.
Values are expressed as mean±SD; n=6. Morphine group was compared with control group. Bacoside-A pretreated group was compared with morphine group. *$P<0.001$; LPO - The level of lipid peroxides was expressed as n moles of MDA formed/mg protein. □ Control; ■ Morphine; ▨ BAC-A+MR; ▨ BCA-A

Fig. 4 shows the overlaid chromatogram of *Bacopa monniera* extract by HPLC along with bacosides and other flavonoids (luteolin and apigenin).

Table 2 shows the effect of bacoside-A on the level of protein carbonyl content in the brain of control and experimental rats. The level of protein carbonyl content was significantly ($P<0.001$) increased in morphine-induced group II rats when compared to control animals. Whereas the level of protein carbonyl content was maintained at near normal in bacoside-A pretreated rats when compared to group II rats. Group-IV rats showed no significant changes in the level of protein carbonyl when compared to control rats.

Table 3 represents the effect of bacoside-A on the activities of Na⁺/K⁺-ATPase, Ca²⁺-ATPase and Mg²⁺-ATPase in the brain of control and experimental rats. A significant decrease in the activities of all the membrane bound enzymes viz., Na⁺/K⁺-ATPase ($P<0.001$), Ca²⁺-ATPase ($P<0.001$) and Mg²⁺-ATPase ($P<0.001$), was noted in morphine-induced rats when compared to control rats. Pretreatment with bacoside-A (group-III) prevented the morphine-induced changes in the activities of all these ATP-ases when compared to group II rats. Bacoside-A alone treated rats resembled the control rats.
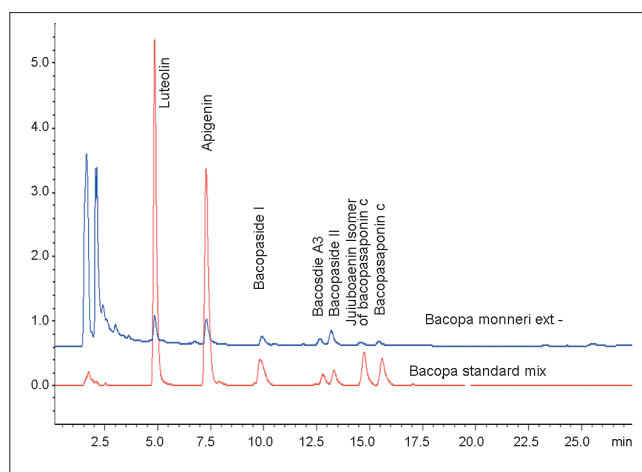

Fig. 4: HPLC – chromatogram of *Bacopa monniera* extract.
HPLC – chromatogram of *Bacopa monniera* extract, showing bacosides and the other flavonoids (Luteolin and apigenin)

TABLE 2: EFFECT OF BACOSIDE-A ON THE LEVEL OF PROTEIN CARBONYL CONTENT IN THE BRAIN OF CONTROL AND EXPERIMENTAL ANIMALS

Groups	Protein carbonyl content (n mole/mg protein)
Control	3.00±0.15
Morphine	4.06±0.18*
Bacoside-A + MR	2.09±0.14*
Bacoside-A	2.94±0.22

Values are expressed as mean±SD; *n*=6. Morphine group was compared with control group. Bacoside-A pretreated group was compared with Morphine group. *$P<0.001$; Protein carbonyl content was expressed as n mole/ mg protein.

DISCUSSION

Brain is highly susceptible to peroxidative damage, since it contains a relatively high degree of unsaturated fatty acids in relation to its level of antioxidants and consumes more oxygen[39]. Morphine is able to induce oxidative cell injury in neuronal cells and neurodegeneration[40]. It has been reported that single dose of morphine administration increased dopamine and xanthine oxidative metabolism with a consequent increase in ROS production[2]. In addition, morphine could also be metabolized into free radicals[3] and the over production of ROS could lead to oxidative damage.

We have found that the level of GSH in the brain were lower in morphine administered rats than in the control animals. This decrease in the GSH level in the brain suggests that morphine unprotected the brain from oxidative stress. These results could be in accordance with the report of Goudas *et al.*[41] who observed acute decreases of GSH levels in

TABLE 3: EFFECT OF BACOSIDE-A ON THE ACTIVITIES OF NA⁺/K⁺-ATPASE, CA²⁺-ATPASE AND MG²⁺-ATPASE IN THE BRAIN OF CONTROL AND EXPERIMENTAL ANIMALS

Groups	Na^+/K^+-ATPase (mmol of phosphorous liberated/ min/mg protein)	Ca^{2+}-ATPase (mmol of phosphorous liberated/ min/mg protein)	Mg^{2+}-ATPase (mmol of phosphorous liberated/ min/mg protein)
Control	0.58±0.05	0.56±0.01	0.67±0.01
Morphine	0.25±0.04*	0.32±0.02*	0.31±0.02*
Bacoside-A + MR	0.54±0.03*	0.53±0.01*	0.64±0.03*
Bacoside-A	0.56±0.02	0.55±0.02	0.66±0.01

Values are expressed as mean±SD; n=6. Morphine group was compared with control group. Bacoside-A pretreated group was compared with morphine group. *P<0.001; The enzyme activity was expressed as mmoles of phosphorous liberated/min/mg protein under incubation conditions.

cerebrospinal fluid samples taken from patients after intracerebroventricular doses of morphine for intractable cancer pain. Such doses of morphine may, by depleting the GSH, render the central nervous system vulnerable to damage from oxidative stress. It has also been reported that the oxidative stress is induced by NO production synthesized in select neuron populations during the opiate syndrome[42], which alters GSH metabolism in the brain.

It has been observed from our study that the protein carbonyl contents and lipid peroxide levels in the brain were significantly higher with the concomitant decrease in antioxidant status in morphine-administered animals than in the control.

Furthermore, it has been reported that oxidative damage in the brain is often observed in association with morphine, which has pro-oxidative properties[14]. Morphine mediated oxidative damage in the brain is compounded by the fact that brain ROS distribution is non-uniform, being particularly high unsaturated fatty acids contents in areas sensitive to neurodegeneration[2,40]. It has been reported that the supplementation of exogenous antioxidants were able to abate the oxidative damages of DNA, protein and lipid, caused by morphine[2].

It has been reported that after administration with heroin, the mice were not only showed, decrease in the total antioxidant status in serum and also there were significant decrease in the antioxidant enzymes such as SOD, CAT, GSH-Px in brain and also exhibited oxidative damages to DNA, protein and lipid[40].

In our present study, we observed that there was a significant decrease in Na^+/K^+-ATPases, Ca^{2+}ATPase and Mg^{2+} ATPase activities in morphine induced rat brain. Masocha et al.[43] indicated from their findings that the activation of Na^+/K^+-ATPases play a vital role in the analgesic effect of morphine and the Na^+/K^+-ATPases activity were greatly decreased in adult morpine induced rats. These results agree with Brase et al.[44], who suggest that morphine induction increases the intracellular sodium in brain and decreases Na^+, K^+-ATPases activity, indicating that the development of morphine tolerance may be accompanied by changes in the disposition of sodium. These results agree with Pillai et al.[45], stating that, morphine produced a significant decrease in Ca^{2+}, Mg^{2+} ATPase activity in synaptosomal fractions, as consequence of containing a high density of opiate receptors (morphine's μ-opioid receptor).

Several authors have shown that *Bacopa monniera* was able to prevent lipid peroxidation *in vitro* and *in vivo*[46,47,18], and quench superoxide, hydroxyl and nitric oxide radicals effectively *in vitro*[48,49]. In our study, a decrease in lipid peroxide level was observed in group-III, bacoside-A pretreated rats when compared to group-II rats, thus establishing its antilipid peroxidative property. Earlier it has been reported by many authors that *Bacopa monniera* has a potential to prevent lipid peroxidation *in vitro* and *in vivo*. This property of the *Bacopa monniera* extract could have been possible due to the presence of one of its major active component namely bacoside-A.

Earlier we reported that *Bacopa monniera* prevented morphine induced rat brain mitochondrial damage[50] that might have allowed the availability of ATP synthesis. This energy promoting action may be responsible for the improved mitochondrial function and maintenance of ATPases in our study, upon administration of bacoside-A. The results of the present study clearly imply that antioxidant and antilipid peroxidative properties of bacoside-A could be attributed to the improved activity of ATPases and maintenance of Na^+/K^+, Ca^{2+}, Mg^{2+} ionic equilibrium

in morphine induced oxidative stress in rat brain. By eliciting a facilitatory effect, bacoside-A offered protection to the brain by stabilizing the membrane and thereby retaining the structural and functional integrity of the membrane. Further studies are needed to study the possible mechanism of action of bacoside-A on morphine-induced oxidative stress in rat brain, in terms of antioxidants and membrane bound enzymes.

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