Neuroprotective effect of *Bacopa monnieri* against morphine-induced histopathological changes in the cerebellum of rats

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Abstract: Opioid addiction is associated with oxidative cell injury in neuronal cells. In this study, *Bacopa monnieri* (L.), a reputed nootropic plant, was evaluated against morphine-induced histopathological changes in the cerebellum of rats. *B. monnieri* methanolic extract (mBME) (40 mg/kg, p.o) and ascorbic acid (50 mg/kg, i.p) were administered two hours before morphine (20 mg/kg, i.p) for 14 and 21 days. The *in vitro* antioxidant activity of mBME was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging assay. Morphine produced vacuolization of basket and stellate cells and reduced the size of Purkinje cells in the cerebellum after 14 days. However, treatment for 21 days was associated with severe shrinkage of Purkinje cells with loss of their characteristic flask-shaped appearance as well as degeneration of basket, stellate and granule cells. Pretreatment with mBME and ascorbic acid for 14 and 21 days attenuated the morphine-induced histopathological changes in the cerebellum. The EC_{50} for the DPPH free-radical scavenging assay of mBME (39.06 µg/mL) as compared to ascorbic acid (30.25 µg/mL) and BHT (34.34 µg/mL) revealed that mBME strongly scavenged the free-radicals and thus possessed an efficient antioxidant propensity. These results concluded that *B. monnieri* having strong antioxidant activity exerted a protective effect against morphine-induced cerebellar toxicity.

Keywords: Opioid addiction, opioid-induced neurotoxicity, cerebellar damage, oxidative stress, brahmi, antioxidant.

INTRODUCTION

Drugs of abuse initiate a cascade of interacting toxic, vascular and hypoxic factors that result in widespread disturbances in the central nervous system (Büttner *et al*., 2000). The neurotoxicity of abuse drugs involves oxidative stress, mitochondrial dysfunction, apoptosis and inhibition of neurogenesis (Cunha-Oliveira *et al*., 2008). Morphine has been associated with oxidative stress in the brain (Guzmán *et al*., 2006) and spinal cord (Ozmen *et al*., 2007). Oxidative stress-induced neuronal apoptosis, associated with morphine and other opioids, is mediated through inactivation of superoxide dismutase, catalase and glutathione peroxidase by reactive oxygen species (Zhou *et al*., 2011; Zhou *et al*., 2001).

The cerebellum plays an important role in the processes of motor coordination, motor control and cognitive functions (Mier and Petersen, 2002). Opioid receptors, particularly the mu-opioid receptors, are widely distributed throughout the cerebellum (Peng *et al*., 2012). Opiate drugs of abuse interfere with cerebellar maturation by disrupting normal opioid signaling and inhibiting the proliferation of granule cell precursors (Hauser *et al*., 2000).

Bacopa monnieri (Linn.) Pennell [Syn. *Bacopa monniera* (L.) Wettst., *Herpestis monniera*, *Gratiola monniera*] (family - *Scrophulariaceae*) known as "Brahmi" in India and "Jal Neem Booti" in Pakistan, is a perennial herb, which is found in marshy places around the world including Pakistan (Qureshi and Raza Bhatti, 2008). It is used in traditional medicine for the management of anxiety, poor cognition and lack of concentration (Russo and Borrelli, 2005). The major chemical constituents isolated from *B. monnieri* are dammarane type triterpenoid saponins with jujubogenin and pseudojujubogenin as the aglycones including bacosides A_1-A_3, bacopasaponins A-G and bacopasides I-V (Murthy *et al*., 2006; Deepak *et al*., 2005). Bacoside-A is the major chemical entity responsible for *B. monnieri* well known nootropic effect as well as other neuromodulatory (Morgan and Stevens, 2010; Calabrese *et al*., 2008) and antioxidant activities (Sumathi and Nongbri, 2008). *B. monnieri* is effective for neurodegenerative disorders involving oxidative stress (Shinomol, 2011). *B. monnieri* inhibits pharmacological effects induced by morphine (Sumathi and Veluchamy, 2007) and is helpful for attenuation of morphine associated withdrawal symptoms (Sumathi *et al*., 2002). The use of *B. monnieri* as adjuvant therapy in the management of opioid addiction may be beneficial (Shahid *et al*., 2016).

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Keeping in view the neuroprotective potential of *B. monnieri*, the present study evaluated the prospective beneficial effect of *B. monnieri* methanolic extract on morphine-induced histopathological changes in the cerebellum of rats.

MATERIALS AND METHODS

Chemicals

Morphine sulphate was obtained through legal channels from Punjab Drug House (Pvt.) Ltd., Lahore, Pakistan. 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany.

Plant material

Bacopa monnieri whole plant was collected in April from Ramali stream near Quaid-e-Azam University, Islamabad, Pakistan. It was authenticated by Prof. Dr. Mohammad Ibrar (Pharmacognosist) of the Department of Botany, University of Peshawar, Peshawar, Pakistan and a specimen was deposited in the herbarium with a voucher number 20016 (PUP). The aerial parts were separated, shade dried and coarsely grinded. It was defatted with *n*-hexane and was further treated with acetone to remove chlorophyll type pigments. Extraction was done with methanol in Soxhlet apparatus and the extract was then filtered and concentrated under reduced pressure at 50°C in a rotary evaporator. A semisolid mass (yield 6.5%) was obtained on drying the concentrated extract on a water bath at 50°C.

Animals

Adult male Sprague-Dawley rats (age range = 7.0 ± 1.0 weeks), weighing 150-200 g and maintained in a 12h/12h light/dark cycle at 22 ± 2°C were used in the experiments. Food and water were provided *ad libitum*. The animals were transferred to grid floor cages to avoid suffocation during cataleptic episodes after dosing with morphine. Experiments on animals were performed in compliance with the UK Animals (Scientific Procedures) Act 1986 and according to the rules and ethics set forth by the Ethical Committee of the Department of Pharmacy, University of Peshawar. Approval for the study was granted with the registration number: Pharm/EC/446.

Treatment groups

All drugs were dissolved in normal saline. *B. monnieri* methanolic extract (mBME) (Sumathi and Devaraj, 2009) and ascorbic acid (Zhang *et al*., 2004) were administered two hours before administration of morphine (Pacifici *et al*., 2000). Treatment was continued for 14 and 21 days. A total of 96 animals were randomly assigned to 12 groups (n = 8 rats per group). Half of those (i.e. 6 groups) were used for 14 days treatment and the other half were used for 21 days treatment. Animals received the following treatment, either for 14 or 21 days.

Group I: Control (Saline) (n = 8)
Group II: Morphine (20 mg/kg/day, i.p) (n = 8)
Group III: mBME (40 mg/kg/day, p.o) + Morphine (20 mg/kg/day, i.p) (n = 8)
Group IV: Ascorbic acid (50 mg/kg/day, i.p) + Morphine (20 mg/kg/day, i.p) (n = 8)
Group V: mBME (40 mg/kg/day, p.o) (n = 8)
Group VI: Ascorbic acid (50 mg/kg/day, i.p) (n = 8)

Histological evaluation

After 14 and 21 days of treatment, brain from each animal was carefully removed and fixed immediately in 10% neutrally buffered formalin. The tissues were dehydrated in graded ethanol solutions (50, 70, 80, 90, two changes each of 100%), cleared in two changes each of 100% xylene and were infiltrated and embedded in paraffin wax. Tissue blocks were sectioned at 4 µm on a rotary microtome (SLEE Mainz CUT 5062, Germany) and were stained with Harris hematoxylin and eosin (H & E) for microscopic observation (Labomed Lx400 with digital camera iVu 3100, USA).

DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical scavenging activity in vitro

The *in vitro* antioxidant activity was evaluated by the DPPH free radical scavenging assay (Lue *et al*., 2010; Shahid and Subhan, 2014). Briefly, 2 mL of methanolic 0.1 mM DPPH free radical solution was mixed with 1 mL of different concentrations (1, 10, 30, 50, 150, 350, 550, 750, 950 and 1150 µg/mL) of mBME or standards in methanol. The solutions were thoroughly mixed and incubated in dark at ambient temperature for 40 min. Absorbance was then measured at 517 nm using a UV/Visible spectrophotometer (Lambda 25, PerkinElmer, USA). Ascorbic acid and butylated hydroxytoluene (BHT) were used as standards. Control was prepared by mixing 2 mL of 0.1 mM DPPH free radical solution with 1 mL of methanol. Blank consisted of methanol alone. The percent scavenging of DPPH free radicals was calculated as follows.

Percent of DPPH scavenging activity = $[(A_I\text{-}A_{II}/A_I) \times 100]$

The absorbance of the control reaction was A_I while the absorbance in the presence of the sample was A_{II}. The EC_{50} value, which is the concentration of the antioxidant at which there is 50% loss of DPPH activity, was calculated from the graph of absorbance versus respective concentrations. The antiradical power and stoichiometry was determined according to Mishra and others (Mishra *et al*., 2012). All experiments were performed in triplicate.

STATISTICAL ANALYSIS

Data were expressed as mean ± standard deviation (SD) or standard error of the mean (SEM). The statistical significance of the differences between sample and

Table 1: $EC_{50,}$ antiradical power and stoichiometry of *Bacopa monnieri* methanolic extract (mBME) and standards [ascorbic acid and butylated hydroxytoluene (BHT)] in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay.

Extract/Standards	EC_{50} (µg/mL)	Antiradical power	Stoichiometry
mBME	39.06 ± 0.7737	0.025 ± 0.0004	78.11 ± 1.5474
Ascorbic acid	30.25 ± 0.5006	0.033 ± 0.0005	60.49 ± 1.0012
BHT	34.34 ± 0.3504	0.029 ± 0.0003	68.68 ± 0.7008

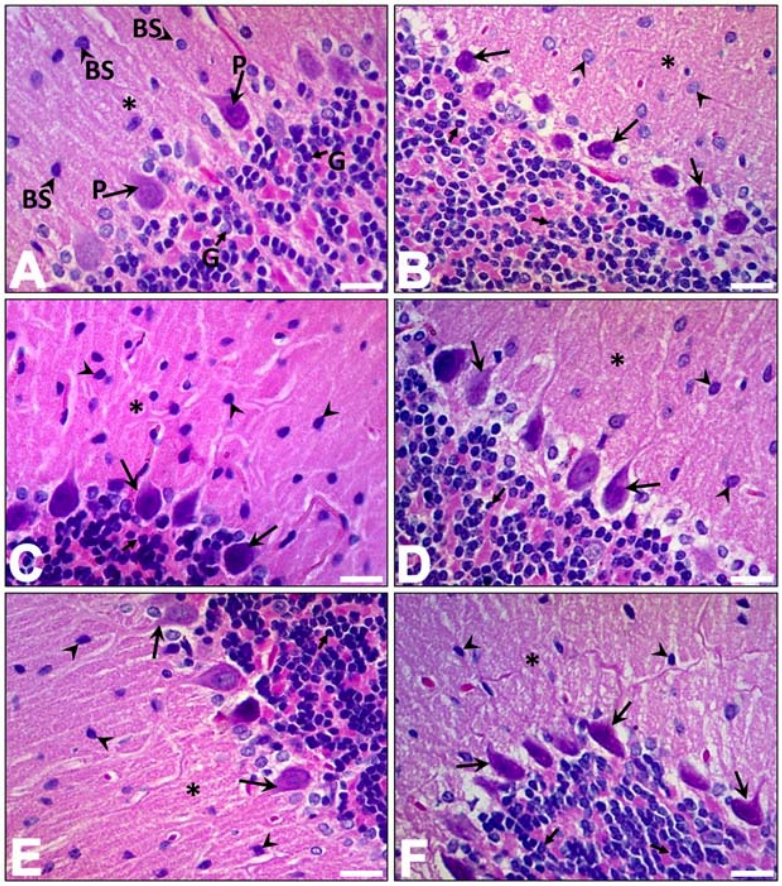

Fig. 1: Histopathological evaluation of morphine-induced cerebellar toxicity pretreated with *Bacopa monnieri* methanolic extract (mBME) and ascorbic acid for 14 days (H & E staining; scale bar = 450 µm) (n = 8 rats per group). (A): Photomicrograph of a section of cerebellum from a rat treated with saline showing the three layers of the cerebellar cortex. The outer molecular layer contains basket and stellate cells (BS, arrow heads) scattered among dendrites (asterisk) of Purkinje cells (P, large arrows). The Purkinje cell layer contains large flask shaped Purkinje cells which give off thick dendrites that branch extensively throughout the molecular layer. The granular layer contains numerous small darkly stained granule cells and large Golgi type-II cells (G, small arrows). (B): Photomicrograph of a section of cerebellum from a rat treated with morphine showing small clear vacuoles in the cytoplasm of stellate and basket cells (arrow heads), scattered among the degenerated dendrites (asterisk) of round-appearing Purkinje cells (large arrows). The granule cells (small arrows) have small clear vacuoles in their cytoplasm. Normal histological appearance of basket and stellate cells (arrow heads) scattered among the dendrites (asterisk) of Purkinje cells (large arrows) along with granule cells (small arrows) are visible in groups of rats treated with (C): mBME two hours before administration of morphine, (D): ascorbic acid two hours before administration of morphine, (E): mBME and, (F): ascorbic acid.

standards in the antioxidant activity was tested by two-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. A value of $P \leq 0.05$ was accepted as significant. The EC_{50} was calculated from the graph of absorbance versus respective concentrations using nonlinear regression analysis (GraphPad Software Inc. San Diego CA, USA).

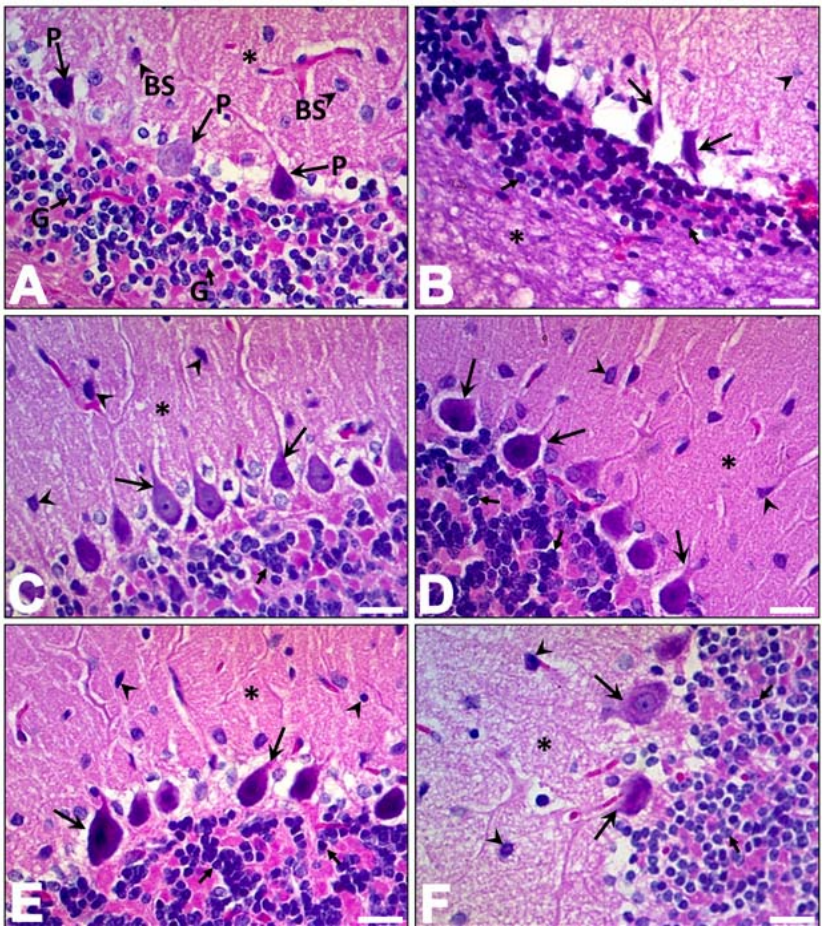

Fig. 2: Histopathological evaluation of morphine-induced cerebellar toxicity pretreated with *Bacopa monnieri* methanolic extract (mBME) and ascorbic acid for 21 days (H & E staining; scale bar = 450 µm) (*n* = 8 rats per group). (A): Photomicrograph of a section of cerebellum from a rat treated with saline showing scattered basket and stellate cells (BS, arrow heads) among dendrites (asterisk) of Purkinje cells (P, large arrows). The granular layer contains small granule and large Golgi type-II cells (G, small arrows). (B): Photomicrograph of a section of cerebellum from a rat treated with morphine showing degenerated basket cells (arrow head) scattered among the dendrites of severely shrunk Purkinje cells (large arrows). The granule cell layer contains granule cells compacted with each other (short arrows). The white matter (asterisk) has small to medium size clear vacuoles. Normal histology of basket and stellate cells (arrow heads) scattered among the dendrites (asterisk) of Purkinje cells (large arrows) along with granule cells (small arrows) are visible in groups of rats treated with (C): mBME two hours before administration of morphine, (D): ascorbic acid two hours before administration of morphine, (E): mBME and, (F): ascorbic acid.

RESULTS

Morphine-induced histopathological changes in the cerebellum after 14 and 21 days

Animals treated with saline for 14 and 21 days showed normal histoarchitecture of the cerebellum. The three layers of the cerebellar cortex were clearly visible. The outer molecular layer contained normal appearing basket and stellate cells interspersed among Purkinje cells dendrites. The Purkinje cell layer contained flask shaped Purkinje cells, whose thick dendrites branched extensively throughout the molecular layer. Numerous small darkly stained granule cells and large Golgi type-II cells were visible in the granular layer (figs. 1A and 2A).

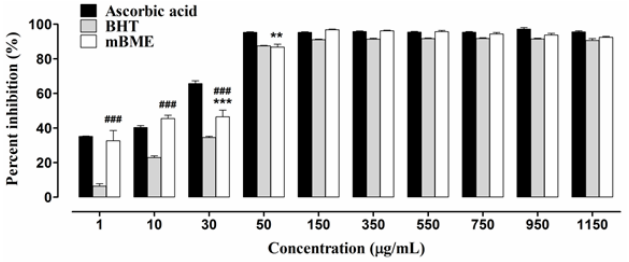

Fig. 3: Percent free radical scavenging activity of *Bacopa monnieri* methanolic extract (mBME) and standards [ascorbic acid and butylated hydroxytoluene (BHT)] in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. Results are mean ± SEM of three

separate experiments. $^{**}P$ ℜ 0.01, $^{***}P$ ℜ 0.001 compared to ascorbic acid, $^{\#\#\#}P$ ℜ 0.001 compared to BHT. Two-way ANOVA followed by Bonferroni *post hoc* test.

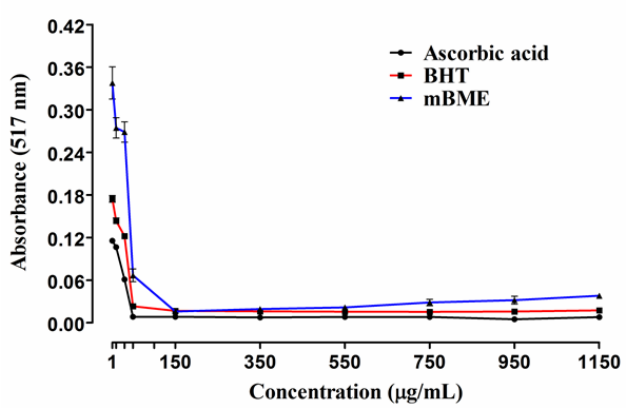

Fig. 4: 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay of *Bacopa monnieri* methanolic extract (mBME) and standards [ascorbic acid and butylated hydroxytoluene (BHT)]. Results are mean ± SD of three separate experiments.

After 14 days of treatment with morphine, the basket and stellate cells of the molecular layer of cerebellum contained small vacuoles in their cytoplasm. The Purkinje cells lost their flask shaped appearance. Some Purkinje cells appeared rounded while others showed mild shrinkage. Their nuclei were not visible. The granule cells of the granular layer had small cytoplasmic vacuoles. The white matter contained small number of medium to large sized clear vacuoles (fig. 1B).

After 21 days of treatment with morphine, the molecular layer contained degenerated basket and stellate cells scattered among the disoriented nerve fibers. The cytoplasm of some basket cells contained small vacuoles. The Purkinje cell layer contained severely shrunk Purkinje cells and their cell boundaries were not visible. The degenerated dendrites emerged from the Purkinje cells and ascended into the molecular layer. The granule cells were compacted together and some of them contained large clear vacuoles in their cytoplasm. In the white matter, large numbers of medium to large sized vacuoles were visible (fig. 2B).

Protective effect of Bacopa monnieri methanolic extract and ascorbic acid against morphine-induced histopathological changes in the cerebellum after 14 and 21 days

Pretreatment with mBME (figs. 1C and 2C) and ascorbic acid (figs. 1D and 2D) for 14 and 21 days provided protection against morphine-induced histopathological changes in the cerebellum as the morphological changes present in the morphine treated groups were not visible. Moreover, animals treated with mBME (figs. 1E and 2E) and ascorbic acid (figs. 1F and 2F) alone showed no significant histopathological changes in the cerebellum.

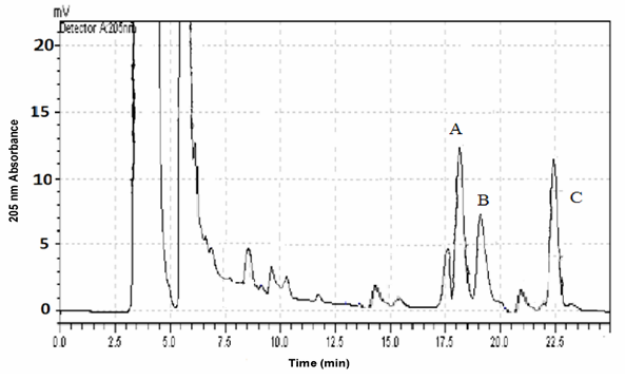

Fig. 5: HPLC chromatogram showing three major components of bacoside-A as bacoside-A_3 (A), bacopaside-II (B) and bacopasaponin-C (C) in the methanolic extract of *Bacopa monnieri*. Adapted from Rauf *et al* (Rauf *et al*., 2012).

In vitro antioxidant activity of Bacopa monnieri methanolic extract

The maximum percent inhibition of DPPH free radicals by mBME was 96.83% at 150 µg/mL while that of ascorbic acid and BHT were 97.16% and 91.76% at 950 and 750 µg/mL respectively. The percent DPPH free radical scavenging activity was in the order of: Ascorbic acid > mBME > BHT. As shown in fig. 3, the percent inhibition by mBME was significantly lower than ascorbic acid at 30 µg/mL (P ℜ 0.001) and 50 µg/mL (P ℜ 0.01). However, it was significantly greater (P ℜ 0.001) than BHT at 1, 10 and 30 µg/mL. No significant difference between mBME and standards (ascorbic acid and BHT) was observed at 150, 350, 550, 750, 950 and 1150 µg/mL. mBME as well as the standards exhibited a concentration dependent decrease in absorption (fig. 4). The EC_{50}, antiradical power and stoichiometry of mBME, ascorbic acid and BHT are shown in table 1. The EC_{50} for the DPPH free radical scavenging activity was in the order of: Ascorbic acid < BHT < mBME.

DISCUSSION

In the present study, the beneficial effect of *Bacopa monnieri* and ascorbic acid was evaluated against morphine-induced histopathological changes in the cerebellum of rats. Morphine has been shown to modulate cell death/ survival in neurons of the central nervous system (Zhang *et al*., 2008). Prolonged exposure to morphine induces apoptotic cell death in spinal cord (Lim *et al*., 2005), dorsal raphe nucleus (Charkhpour *et al*., 2010), striatum (Suzuki *et al*., 2011) and cortical neurons (Hassanzadeh *et al*., 2011). Morphine reduces cortical thickness and number of neurons in the developing frontal cerebral cortex (Sadraie *et al*., 2008), produces Purkinje cell loss and a decrease in the thickness of Purkinje and granular layers of the cerebellar cortex (Ghafari *et al*., 2011). In this study, treatment with morphine caused

degenerative changes in the gray and white matter of the cerebellum. The Purkinje cell layer was mainly affected. Treatment for 14 days produced mild shrinkage and reduction in the size of Purkinje cells. The cytoplasm of basket and stellate cells were vacuolated. Treatment for 21 days however, resulted in severe shrinkage of Purkinje cells with loss of their characteristic flask shaped appearance and cell boundaries. The basket and stellate cells were highly vacuolated and the granule cells were compacted together and showed degeneration. The white matter contained large number of medium- and large-sized clear vacuoles. These results are in accordance with the previously reported studies (Bekheet *et al*., 2010; Golalipour and Ghafari, 2012). Purkinje cells are GABAergic neurons, which serve as the sole output of the cerebellar cortex (Voogd and Glickstein, 1998). Morphine is especially associated with apoptosis of GABAergic neurons (Mao *et al*., 2002). Purkinje cell dysfunction is associated with ataxia, characterized by poor balance, loss of posture and difficulties in performing rapid coordinated movements (Walter *et al*., 2006).

In this study, daily pretreatment with mBME at a dose of 40 mg/kg for 14 and 21 days provided protection against morphine-induced histopathological changes in the cerebellum. The tested dose of *B. monnieri* is considered as safe and is based on its toxicity profile, as previously reported (Abbas *et al*., 2011). *B. monnieri* has been shown to alleviate methyl mercury induced oxidative stress (Sumathi *et al*., 2012) and thus protects the cerebellum against neurochemical and histopathological changes (Christinal and Sumathi, 2013). The neuroprotective effect of *B. monnieri* is due to its strong antioxidant activity that decreases nitrite, nitrate and lipid peroxidation and improves catalase activity (Saraf *et al*., 2010). Scavenging of DPPH free radicals is the basis of antioxidant activity of pure and natural compounds (Sharma and Bhat, 2009). In this study, *B. monnieri* exhibited a concentration dependant increase of percent scavenging of DPPH free radicals and the effect was similar to that of standards used in the assay (ascorbic acid and BHT). The EC_{50} value of *B. monnieri* (39.06 µg/mL) as compared to ascorbic acid (30.25 µg/mL) and BHT (34.34 µg/mL) indicates that the extract possesses strong antioxidant potential. A low EC_{50} value signifies a strong antioxidant activity in an extract (Loo *et al*., 2007). The antioxidant property of *B. monnieri* has been attributed to the presence of bacoside-A (Sumathi and Nongbri, 2008). Bacoside-A occurs as a mixture of bacopasaponin-C, jujubogenin isomer of bacopasaponin-C, bacopaside-II and bacoside-A_3 (Deepak *et al*., 2005). A previous study in our laboratory showed that the methanolic extract of *B. monnieri* have contents of bacopaside-II, bacoside-A_3, and bacopasaponin-C in quantities of 1.3 µg/mg, 4 µg/mg and 1.3 µg/mg, respectively (fig. 5) (Rauf *et al*., 2012).

The neuroprotective effect of *B. monnieri* presented in this study can be attributed to the presence of strong antioxidant components, as antioxidants have been shown to protect brain against oxidative stress (Mori *et al*., 2004; Zhao, 2005; Guerra-Araiza *et al*., 2013). Accordingly, pretreatment with ascorbic acid also provided protection against morphine-induced histopathological changes in the cerebellum. These effects revealed that morphine-induced cerebellar toxicity was associated with oxidative stress and *B. monnieri*, due to its strong antioxidant activity reduced this oxidative stress thus resulting in the attenuation of neurotoxicity. Opioid-induced cytotoxic effects are associated with an increase in reactive oxygen species formation (Oliveira *et al*., 2002). Moreover, the effects of morphine and heroin on brain and spinal tissues involve oxidative stress (Cunha-Oliveira *et al*., 2008). *B. monnieri* by preventing the decrease in the activities of superoxide dismutase, glutathione peroxidase and catalase, protect cerebellar neurons from oxidative damage (Sumathi *et al*., 2012). The resistance against neurotoxicant-induced oxidative damage offered by *B. monnieri* suggests its therapeutic utility in the treatment of oxidative stress-mediated neurodegenerative diseases (Shinomol, 2011).

CONCLUSION

Morphine was associated with degenerative changes in the cerebellum especially affecting the Purkinje cells. The histopathological changes were attenuated by pretreatment with *Bacopa monnieri* and ascorbic acid. *B. monnieri* exerted a protective effect against morphine-induced cerebellar toxicity which may be due to its strong antioxidant proclivity.

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