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ORIGINAL ARTICLE

Calotropis procera attenuates chronic unpredictable mild stress-induced depression in experimental animals

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Abstract

Calotropis procera (CP; Apocynaceae) is reported to have several neuroprotective activities however it's anti-depressant activity yet to be established. Therefore, the present study was proposed to evaluate the anti-depressant activity of the standardized ethanolic extract of CP (ECP) in chronic unpredictable mild stress (CUMS) paradigm exposed male rats. Animals were exposed to CUMS from day-1 (D-1) to D-28 except control group animals of the experimental schedule. ECP (50, 100 and 200 mg/kg, p.o.) and Imipramin (15.0 mg/kg, p.o.) were administered for seven consecutive days after CUMS paradigm. On D-35, ECP (200 mg/kg) significantly attenuated immobility period of the animals in both forced-swim and tail suspension and improved behavioural parameters in open-field and anhedonia in sucrose feeding tests. ECP (200 mg/kg) attenuated CUMS-induced hyperactivity of HPA-axis function. Further, ECP (200 mg/kg) mitigated CUMS-induced decrease in serotonin (5-HT), increase in 5-hydroxy indole acetic acid (5-HIAA) and increase in the ratio of 5-HIAA/5-HT in hippocampus and pre-frontal cortex. The CUMS-induced decrease in the level of expression of BDNF was significantly reversed with ECP (200 mg/kg) treatment. Moreover, ECP (200 mg/kg) significantly reduced the CUMS-induced decrease in the mitochondrial function and integrity in terms of level of formazan formed and intensity of tetramethyl rhodamine methylester dye in both the brain regions respectively. Therefore, ECP (200 mg/kg) mitigates CUMS-induced alterations in the behaviours, HPA-axis function, serotonergic activity, neurogenesis and mitochondrial function in the rodents. Thus, it can be assumed that ECP could be a potential alternative candidate in the management of depression.

Keywords *Calotropis procera* · Chronic unpredictable mild stress · Corticosterone · Serotonin · Mitochondria · Brain derived neurotrophic factor

Introduction

Depression is a neurological disorder that is characterized by an inability to experience pleasure (anhedonia), general loss of interest and motivation (Mauskopf et al. 2009). Stressful life events are considered as contributing factors in most episodes of major depression (Lavergne and Jay 2010). Therefore, medical intervention is necessary in depression as it is a serious neuropsychiatric condition. The most important concern in the management of depression is that the therapeutic effect of anti-depressant drugs requires several weeks to execute. Moreover, these drugs are often accompanied with several unwanted side effects. In addition, it has been suggested that the response to anti-depressants is not uniform in all the patients (Taylor et al. 2005). Thus, new strategies are imperative in the management of depression.

Medicinal plants are considered as an important source of herbal drugs in the management of depression. They are comparatively better than that of synthetic anti-depressants in terms of safe and lesser unwanted side effects (Schulz 2006; Singh et al. 2009). *Calotropis procera* Linn (Family: Asclepiadaceae) is an Ayurvedic plant with important medicinal properties. *C. procera* Linn is an erect, tall large, highly branched, and perennial shrub or small tree that grows to a height of 5.4 m with milky latex throughout the plant (Sharma et al. 2011). Traditionally, the leaves are used against several diseases including asthma, inflammation, rheumatoid arthritis and neurological disorders (Meena et al. 2011). However, the effect of leave extract of *C. procera* on depression has not yet been evaluated.

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Chronic unpredictable mild stress (CUMS) is widely considered as a animal model of depression. Earlier studies report that many of the deleterious effects of CUMS are ameliorated by anti-depressant agents (Willner et al. 1996; Willner 1997; Pothion et al. 2004). In rodents, CUMS also elicit depression-like symptoms such as lack of sucrose preference (Yalcin et al. 2005) interpreted as anhedonia, a core symptom of depression (American Psychiatric Association 2000). It has been well documented that there is an increase in the level of corticosterone in the plasma which is the ultimate result of the hypothalamic-pituitary-adrenal cortex-axis (HPA-axis) hyperactivity during depression (Cai et al. 2015). Several anti-depressant drugs alleviate depression-induced elevated level of corticosterone in the plasma (Jin et al. 2015; Cai et al. 2015), and indicating the fact that neuroendocrine regulation is one of the plausible anti-depressant activity of such drugs. Thus, this model has a high degree of predictive validity (behavioral changes are reversed with antidepressant drugs), face validity (CUMS induces the behavioral alterations characterizing depressed patients) and construct validity (CUMS decreases sensitivity in the brain reward system).

Literature evidences demonstrate that serotonergic system plays an important role in the pathophysiology of depression (Elhwuegi 2004). Several studies have reported that certain anti-depressants act by inhibiting the uptake of serotonin (5-HT) and thus attenuate the behavioral deficits induced by CUMS (Nestler et al. 2002; Naughton et al. 2000). It has been well suggested that hyperactivity of HPA-axis for long duration can cause mitochondrial dysfunction. Subsequently, it leads to the death of serotonergic neurons through monoamine oxidase (MAO) activated increase in the turnover of 5-HT in several brain regions including hippocampus (HIP) and pre-frontal cortex (PFC; Głombik et al. 2016; Głombik et al. 2015). The brain-derived neurotrophic factor (BDNF) is closely related to neuronal survival and neurogenesis and plays an important role in animal models of depression. Number of stress procedures including CUMS result in decrease of BDNF expression in the brain, while chronic administration of almost all kinds of anti-depressants including imipramine regulates BDNF level during depression (Kalueff et al. 2006; O'Leary et al. 2009; Lee and Kim 2010). Moreover, it has also been reported that the several therapeutic strategies exerts anti-depressant activity through regulation in mitochondrial function and BDNF-mediated activity (Rinwa et al. 2013; Della et al. 2013; Aguiar Jr et al. 2014). These observations clearly illustrate the fact that there is a strong relationship between mitochondrial function and, serotonergic and BDNF-mediated activity in brain during depression.

Therefore, in the present study, the standardized ethanolic leave extract of *C. procera* (ECP) was evaluated for neuroprotection against CUMS subjected animals. Further, the mitochondrial function, serotonergic activity and level of BDNF were estimated to elaborate the plausible mechanism of action of the proposed pharmacological candidate.

Materials and methods

Chemicals and reagents

Tetra methyl rhodamine methyl ester (TMRM) was procured from Sigma Aldrich (St. Louis, MO, USA). All other chemicals and reagents of high-performance liquid chromatography and analytical grade were procured from Merck Pvt. Ltd., New Delhi and Himedia Laboratories Pvt. Ltd., Mumbai, India.

Plant material and extraction procedure

The leaves from *C. procera* were collected from medicinal garden of GLA University, Mathura, Uttar Pradesh, India in September 2015. A voucher specimen was identified and deposited in the National Botanical Research Institute, Lucknow, India (Voucher specimen No.: NBRI/CIF/493/2015). The dried and powered plant material was extracted in ethanol (98%) at 1:7 plant material/solvent (*w*/*v*) for five consecutive times at room temperature using Soxhlet apparatus. Subsequently, the extract was sonicated in ultrasound (30 min/day). The solvent was removed under reduced pressure using rotary evaporator and lyophilized to yield dried ethanolic extract of leaves (ECP; 9%, *w*/w).

Phytochemical analysis of *C. procera* leaves

Preliminary phytochemical screening and estimation of total phenolic and flavonoid content

The ECP was subjected to standard protocols of identification tests for flavonoids, alkaloids, tannins, cardiac glycosides anthraquinones, saponins and phenols (Ajikumaran et al. 2006).

Estimation of total phenolic content

The ECP was subjected to quantify total phenolics using Folinciocalteu reagent with slight modification to assay method of Singleton and Rossi (1965). Absorbance was measured against blank using Double-Beam UV-Vis Spectrophotometer (Simadzu-1800) at λ_{max} 725 nm. Gallic acid was used as a standard and the total phenolic content was expressed as milligrams of gallic acid equivalent/ g extract.

Estimation of total flavonoid content

The ECP was subjected to quantify total flavonoid content using aluminium trichloride ($AlCl_3$) in colorimetric assay procedure of Zhishen et al. (1999). The absorbance was recorded against blank using Double-Beam UV-Vis Spectrophotometer at 420 nm against a blank. Quercetin was used as a standard and the amount of total flavonoid was expressed as quercetin equivalents (mg quercetin/g extract).

Animals

Inbreed adult male Charles-Foster albino rats (150–200 g) were obtained from Central Animal House, Institute of Pharmaceutical Research, GLA University, Mathura. The animals were housed in Poly-acrylic cages under standard condition (24 ± 2 °C temperature, 45–55% relative humidity and 12 h light: 12 h dark cycle). Animals were allowed to freely feed their standard pellet diet (Lipton India, Ltd., Mumbai) and water ad libitum. Fasted animals were deprived with their food for 16–18 h before experimentations but allowed to water ad libitum.

Acute toxicity study

Organization for Environmental Control Development (OECD) guideline 420 (Fixed Dose Method) was considered to evaluate acute oral toxicity of ECP (suspended in 0.5% carboxy methyl cellulose; CMC) in albino male rats ($N = 6$, 150–200 g) those were overnight fasted for 18 h in the experiment. The ECP was administered in increasing dose levels of 5, 50, 300 and maximum dose up to 2000 mg/kg to the experimental animals.

Experimental design

All animals were divided into six groups with six in each group namely; Control, CUMS, IMP, CUMS+ECP-50, CUMS+ECP-100 and CUMS+ECP-200. All animals were exposed to CUMS paradigm starting from Day-1 (D-1) to Day-28 (D-28) except control group rats. On D-1 and D-28, all behavior observations were monitored in all the animals. On D-28, Imipramine (IMP; 15 mg/kg, p.o.) and ECP (50, 100 and 200 mg/kg, p.o.) were administered once daily to IMP, CUMS+ECP-50, CUMS+ECP-100 and CUMS+ECP-200 group rats for 7 consecutive days respectively. All animals were subjected to access the antidepressant activity after 1 h to last dose of ECP (50, 100 and 200 mg/kg) in FST, TST, OFT and sucrose feeding test on D-35. All behavioral observations were monitored with the help of ANY-maze™ video tracking software (Version- 4.96, USA). Immediately after behavioral observation, the animals were killed by decapitation for further study to access the CUMS-induced hyperactivity of HPA-axis function, alterations in serotonergic and BDNF-mediated activity, mitochondrial function, integrity and oxidative stress in HIP and PFC of the rodents.

CUMS paradigm

The standard protocol of CUMS (Papp et al. 1991) with slight modification (Moreau et al. 1992) was employed on the animals to induce depression. Briefly, each week consisted of different stressors such as one period (2 h) of paired caging, one period (3 h) of tilted cage (45°), one period of food deprivation (18 h) immediately followed by 1 h of restricted access to food, two periods of water deprivation (18 h) immediately followed by 1 h exposure to an empty bottle, one 21 h period with wet cage (200 ml water in 100 g sawdust bedding), and one period with 36 h of continuous light. Thus, stressors were presented both during the active (dark) and inactive (light) period of the rats. Randomization of stressors was done in order to minimize the predictability of the stressors. Control rats were left undisturbed in their home cages.

Behavioral observations

Modified FST

The anti-depressant activity of ECP (50, 100 and 200 mg/kg) was evaluated using standard procedures of Porsolt et al. (1978) with slight modifications (Detke et al. 1995). Briefly, climbing behavior (defined as upward directed movements of the forepaws usually along the side of the swim chamber), swimming behavior (defined as horizontal movement throughout the swim chamber which includes crossing across quadrants of the cylinder) and immobility (measured when no additional activity was observed other than that required to keep the rat's head above the water) were measured in the modified FST.

Tail suspension test (TST)

The anti-depressant activity of ECP (50, 100 and 200 mg/kg) was evaluated using standard protocol of Chermat et al. (1986). The total duration of immobility of each animal was recorded when they were suspended by their tail for a period of 5 min. Rats were considered immobile only when they hung passively and completely motionless.

Open field test (OFT)

The neuroprotective activity of ECP (50, 100 and 200 mg/kg) was evaluated in open field test using standard procedure of Bronstein (1972). The ambulation (the number of squares crossed), rearing (the number of time animal stood up on its

hind limb), total time spent in the central square and total distance travelled by the animal were recorded for 5 min.

Sucrose feeding test

The effect of ECP (50, 100 and 200 mg/kg) on the extent of anhedonia was evaluated in the sucrose preference test using standard procedure of Papp et al. (1991). Briefly, the volume of consumption of sucrose solution was recorded as an indicator of anhedonia.

Corticosterone estimation

The plasma was used to estimate the level of corticosterone using HPLC with an ultraviolet (UV) detector system (Waters, USA; Woodward and Emery 1987) with slight modification (Garabadu et al. 2011). The chromatogram was recorded at 250 nm using the UV detector (Model 2487, Waters, USA) and analyzed with Empower software.

Brain dissection

The brain was isolated and microdissected (Paxinos and Watson 1998) into HIP and PFC. Subsequently, the tissues were homogenized in 1 ml of 0.1 M perchloric acid with a homogenizer. The homogenate was put up in the polypropylene tubes for 15 min after which 50 μl of 4 M potassium acetate was mixed to adjust the pH to 4.0 followed by centrifugation for 15 min at 4000 *g* (Muthuraju et al. 2009).

Monoamine estimation

The levels of monoamines such as 5-HT (Serotonin), 5-hydroxy indole acetic acid (5-HIAA), and its ratio were estimated in HIP and PFC using HPLC with electrochemical detector (Kim et al. 1987; Garabadu et al. 2011).

Estimation of mitochondrial function and integrity

Isolation of mitochondria from rat brain

The standard procedure of Pedersen et al. (1978) was adopted to isolate mitochondria from the selected tissues. The mitochondrial protein content was evaluated by using the standard method (Lowry et al. 1951).

Estimation of mitochondrial function

The mitochondrial function in terms of formazan formed as a byproduct of reduction of (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; MTT) was estimate at 595 nm (Kamboj et al. 2008). Results were expressed as mg formazan formed/min/mg protein.

Evaluation of mitochondrial membrane potential (MMP)

The mitochondrial integrity in terms of MMP was evaluated with the help of fluorescent cationic dye TMRM. The fluorescence intensity was recorded at an excitation λ_{max} of 535 ± 10 nm and emission λ_{max} of 580 ± 10 nm using spectrofluorometer (Hitachi, F2500, Japan; Huang 2002). The results were expressed as fluorescence intensity value/mg protein.

Western blot analysis

Briefly, the brain tissues were lysed in buffer containing protease inhibitor cocktail. BDNF concentration was determined according to standard procedure (Bradford 1976). A standard plot was generated by using bovin serum albumin. An aliquot of each sample was electrophoresed in 12% SDS-PAGE gels for BDNF proteins, respectively transferred to polyvinylidene fluoride membranes and probes with specific antibodies. The membrane was incubated overnight anti-BDNF (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) polychlonal primary antibody at a dilution of 1:500. After detection with the desired antibodies against the protein of interest, the membrane was stripped with stripping buffer (25 mM glycine pH 2.0, 2% SDS for 30 min at room temperature) and re-probed overnight with rabbit anti-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) polyclonal primary antibody at a dilution of 1:500 confirm equal loading of protein. Further, membrane was probed with corresponding secondary antibodies. Immunoreactive bands of protein were evaluated by chemiluminescence by using enhance chemiluminescence (ECL) reagent (Amersham Bioscience, USA) quantification of the results was performed by densitometric scan of films. The immunoreactive area was determined by densitometric analysis by using Biovis gel documentation software.

Data analysis

The results were expressed as Mean ± standard error of mean (S.E.M.). Statistical analysis was performed using Graph Pad Prism 4.2® software. Two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was considered to analyze different behavioral observations in FST, TST, OFT and sucrose feeding test. All other data were analyzed using one-way ANOVA followed by Student Newmann Keuls post-hoc test. The significance level for rejection of the null hypothesis was always ≥5% ($p < 0.05$).

Results

Phytochemical analysis of *C. procera* leave

The phytochemicals such as flavonoids, alkaloids, tannins, cardiac glycosides anthraquinones, saponins and phenols were present in ECP as reported from preliminary phytochemical screening. Further, the total phenolic and flavonoid content was found to be 215.3 ± 7.7 (mg gallic acid equivalent/g extract) and 23.1 ± 1.7 (mg quercetin/g extract) in ECP respectively.

Selection of dose of standardized ECP

ECP at 5, 50, 300 and maximum dose up to 2000 mg/kg was found to be tolerable in the experimental animals in the acute oral toxicity study. Low dose: 50 mg/kg (half of the median dose), Median dose: 100 mg/kg (half of the one-tenth of the maximum lethal dose) and High dose: 200 mg/kg (one-tenth of the maximum lethal dose) were selected for evaluation of anti-depressant activity. Moreover, the rats did not exhibit any gross behavioral, neurological or autonomic toxic effects after first 3 h of ECP administration and for any lethality after 24 to 72 h till 14 days.

ECP (50, 100 and 200 mg/kg) ameliorated CUMS-induced depressive-like behaviors during FST

Figure 1 depicts the effect of ECP (50, 100 and 200 mg/kg) on CUMS-induced changes in the immobility (A), swimming (B) and climbing (C) period of rats in the FST paradigm. Statistical analysis revealed that there were no significant differences in immobility, swimming and climbing period among groups during FST on D-1 of the experimental protocol. CUMS caused a significant increase in immobility period and decrease in swimming and climbing periods during FST paradigm on D-28 and this effect was maintained up to D-35. On D-35, ECP (200 mg/kg) and IMP significantly reversed the CUMS-induced increase and decrease in immobility and swimming periods respectively.

ECP (50, 100 and 200 mg/kg) ameliorated CUMS-induced depressive-like behavior during TST

Effect of ECP (50, 100 and 200 mg/kg) on CUMS-induced changes in the immobility period of rats in the TST paradigm is depicted in Fig. 2. Statistical analysis revealed that there were no significant differences in immobility period among groups during TST on D-1 of the experimental protocol. CUMS caused a significant increase in immobility period during TST paradigm on D-28 and this effect was persisted up to D-35. On D-35, ECP (200 mg/kg) and IMP significantly decreased the CUMS-induced increase in immobility period.

ECP (50, 100 and 200 mg/kg) mitigated CUMS-induced behaviors during OFT

Figure 3 depicts the effect of ECP (50, 100 and 200 mg/kg) on CUMS-induced changes in the ambulation (A), rearing (B), time spent in the centre (C) and total distance travelled (D) in the OFT paradigm. Statistical analysis revealed that there were no significant differences in all the behavioural parameters among groups during OFT on D-1 of the experimental protocol. CUMS caused a significant decrease in all the behavioural parameters during OFT paradigm on D-28 and this effect was maintained up to D-35. On D-35, ECP (200 mg/kg) and IMP significantly increased the CUMS-induced decrease in all the behavioural parameters during OFT paradigm.

ECP (50, 100 and 200 mg/kg) ameliorated CUMS-induced anhedonia behavior

Effect of ECP (50, 100 and 200 mg/kg) on CUMS-induced changes in the anhedonic behavior in terms of sucrose consumption is depicted in Fig. 4. Statistical analysis revealed that there were no significant differences in sucrose consumption among groups on D-1 of the experimental protocol. CUMS caused a significant decrease in sucrose consumption on D-28 and this effect was persisted up to D-35. On D-35, ECP (200 mg/kg) and IMP significantly increased the CUMS-induced decrease in sucrose consumption in rats.

ECP (50, 100 and 200 mg/kg) mitigated CUMS-induced changes in plasma corticosterone

Figure 5 illustrates the effect of ECP (50, 100 and 200 mg/kg) on CUMS-induced changes in the level of plasma corticosterone in the animals. Statistical analysis revealed that ECP (200 mg/kg) and IMP significantly attenuated the CUMS-induced increase in plasma corticosterone on D-35 of the experimental schedule in rats.

ECP (50, 100 and 200 mg/kg) ameliorated CUMS-induced altered serotonergic activity in HIP and PFC

Effect of ECP (50, 100 and 200 mg/kg) on CUMS-induced changes in the levels of 5-HT (A) and 5-HIAA (B), and ratio of 5-HIAA/5-HT (C) is depicted in Fig. 6. Statistical analysis revealed that ECP (200 mg/kg) and IMP significantly attenuated the CUMS-induced decrease in the level of 5-HT in both the brain regions of the animals. Interestingly, ECP (200 mg/kg) significantly decreased CUMS-induced increase in 5-HIAA level in both the brain regions. However, IMP significantly further increased the CUMS-induced increase

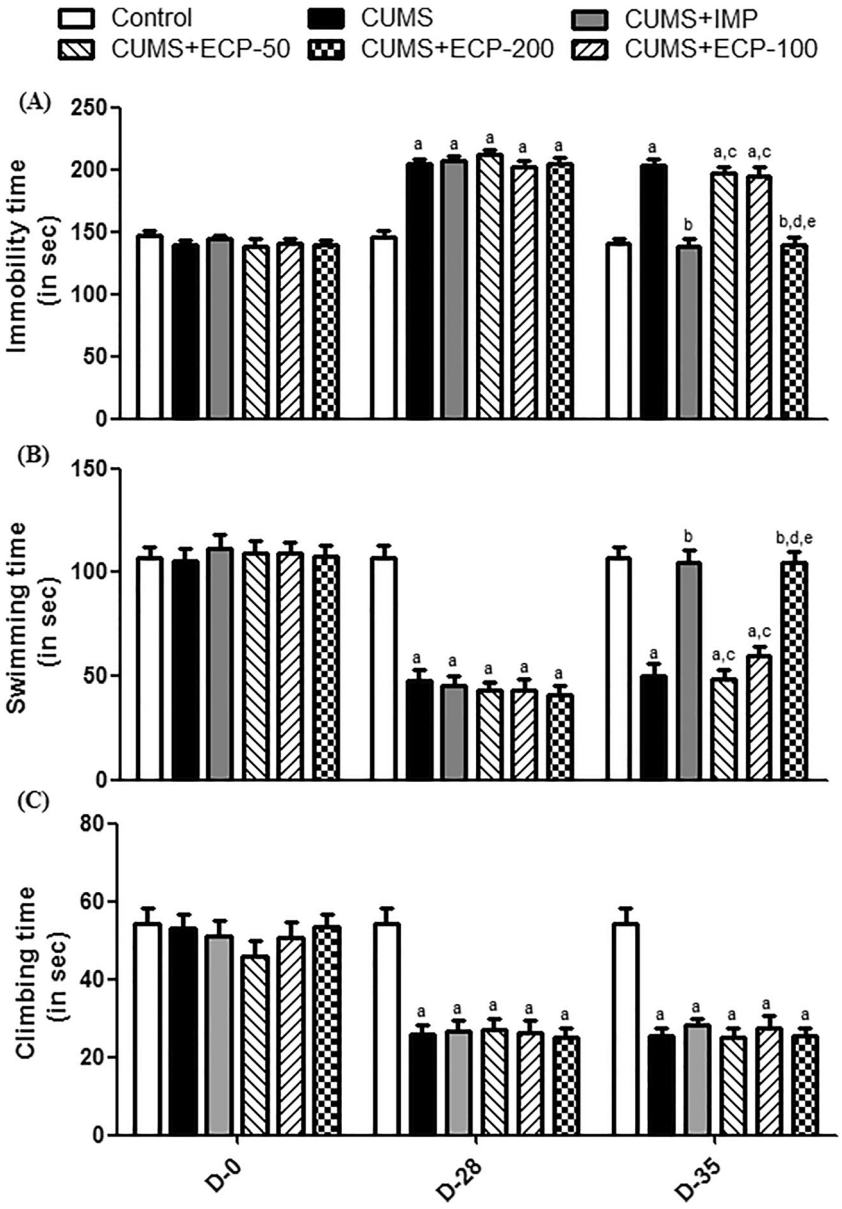

Fig. 1 Effect of ECP (50, 100 and 200 mg/kg) on chronic unpredictable mild stress (CUMS)-induced changes in the immobility (**a**), swimming (**b**) and climbing (**c**) period of rats in the FST paradigm. All values are Mean ± Standard Error of Mean (SEM; $N = 6$). $^{a}P < 0.05$ compared to Control, $^{b}P < 0.05$ compared to CUMS, $^{c}P < 0.05$ compared to CUMS+IMP, $^{d}P < 0.05$ compared to CUMS +ECP-50 and $^{e}P < 0.05$ compared to CUMS +ECP-100 (Two-way ANOVA followed by Bonferroni Post-hoc test)

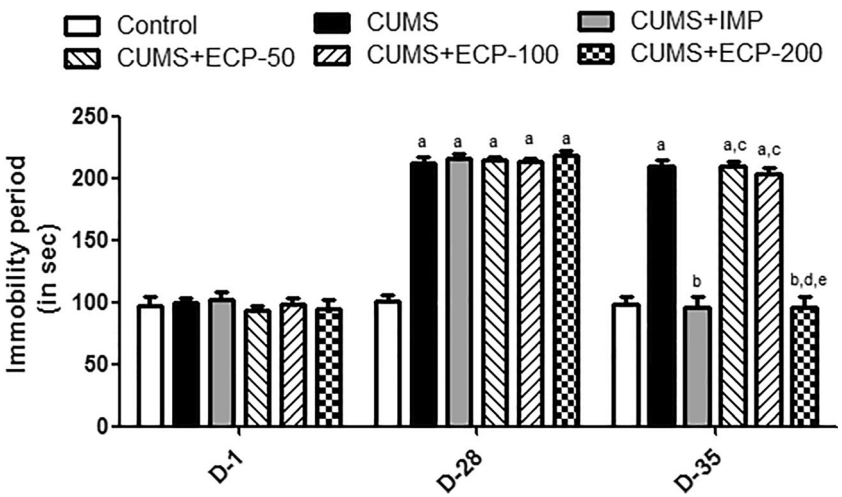

Fig. 2 Effect of ECP (50, 100 and 200 mg/kg) on CUMS-induced changes in the immobility period of rats in the TST paradigm. All values are Mean ± Standard Error of Mean (SEM; $N = 6$). $^{a}P < 0.05$ compared to Control, $^{b}P < 0.05$ compared to CUMS, $^{c}P < 0.05$ compared to CUMS+IMP, $^{d}P < 0.05$ compared to CUMS +ECP-50 and $^{e}P < 0.05$ compared to CUMS +ECP-100 (Two-way ANOVA followed by Bonferroni Post-hoc test)

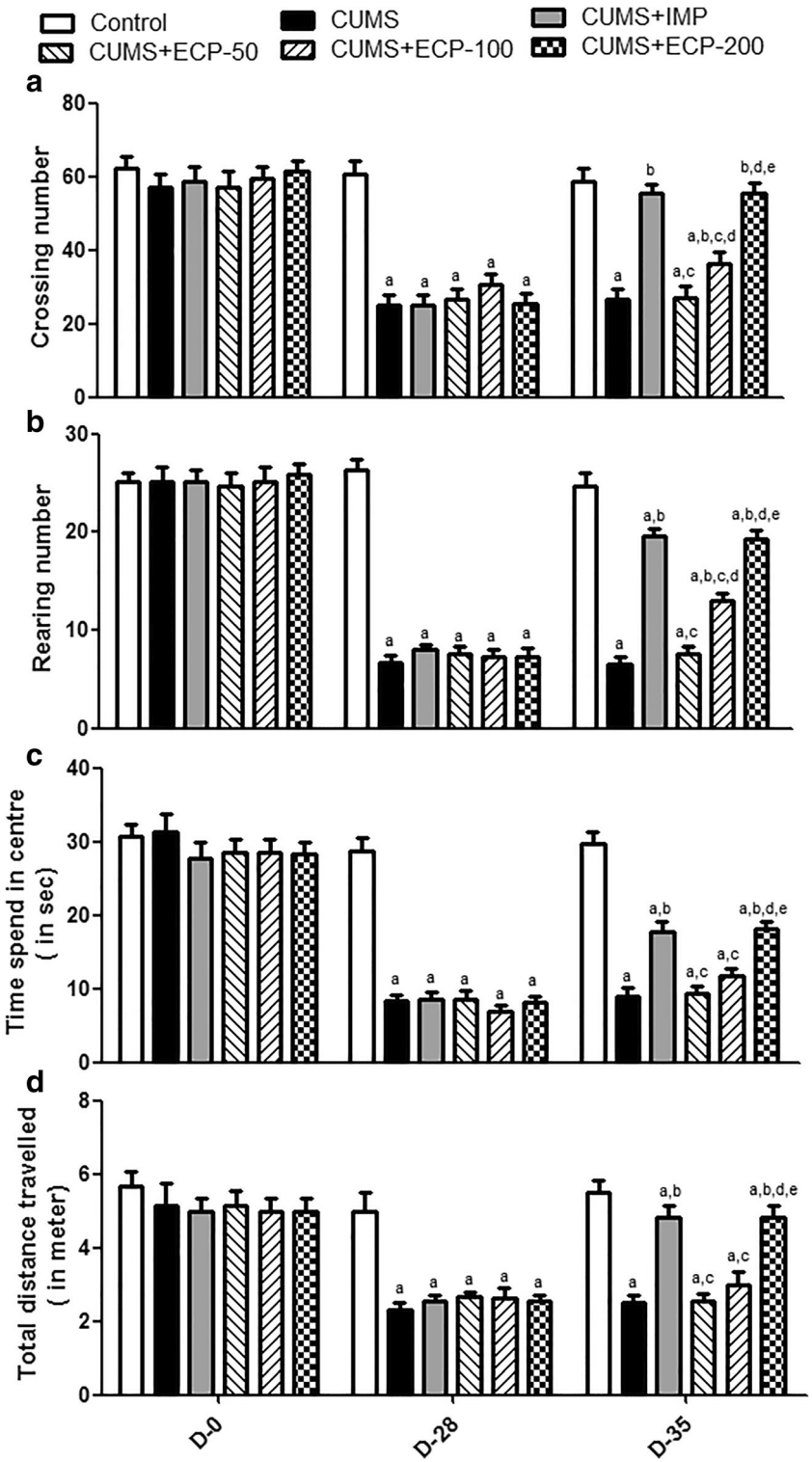

Fig. 3 Effect of ECP (50, 100 and 200 mg/kg) on CUMS-induced changes in the ambulation (**a**), rearing (**b**), time spent in the centre (**c**) and total distance travelled (**d**) in the OFT paradigm. All values are Mean ± Standard Error of Mean (SEM; $N = 6$). $^{a}P < 0.05$ compared to Control, $^{b}P < 0.05$ compared to CUMS, $^{c}P < 0.05$ compared to CUMS+IMP, $^{d}P < 0.05$ compared to CUMS +ECP-50 and $^{e}P < 0.05$ compared to CUMS +ECP-100 (Two-way ANOVA followed by Bonferroni Post-hoc test)

in the level of 5-HIAA in both the brain regions of the rats. Similarly, ECP (200 mg/kg) significantly decreased CUMS-induced increase in ratio of 5-HIAA/5-HT in all the brain regions. On contrary, IMP significantly further increased the CUMS-induced increase in the ratio of 5-HIAA/5-HT in both the brain regions of the rats.

ECP (50, 100 and 200 mg/kg) ameliorated CUMS-induced altered mitochondrial function and integrity in selected brain regions

Effect of ECP (50, 100 and 200 mg/kg) on CUMS-induced changes in the mitochondrial function (A) and integrity (B) is

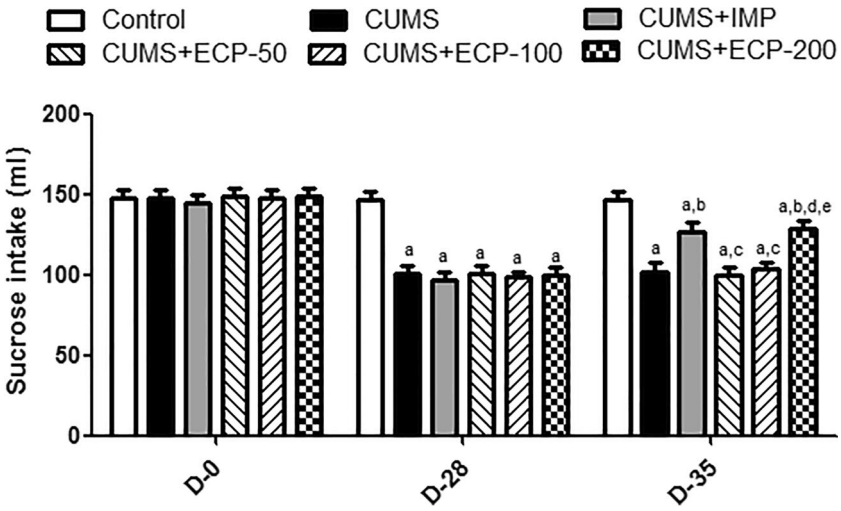

Fig. 4 Effect of ECP (50, 100 and 200 mg/kg) on CUMS-induced changes in the anhedonic behavior in terms of sucrose consumption of the rodents. All values are Mean ± Standard Error of Mean (SEM; $N = 6$). $^{a}P < 0.05$ compared to Control, $^{b}P < 0.05$ compared to CUMS, $^{c}P < 0.05$ compared to CUMS+IMP, $^{d}P < 0.05$ compared to CUMS +ECP-50 and $^{e}P < 0.05$ compared to CUMS +ECP-100 (Two-way ANOVA followed by Bonferroni Post-hoc test)

depicted in Fig. 7. Statistical analysis revealed that ECP (200 mg/kg) significantly attenuated the CUMS-induced decrease in the mitochondrial function and integrity in both the rat brain regions.

ECP (50, 100 and 200 mg/kg) mitigated CUMS-induced altered level of BDNF in HIP and PFC

Figure 8 illustrates the effect of ECP (50, 100 and 200 mg/kg) on CUMS-induced changes in the levels of expression of BDNF in HIP and PFC of the rats. Statistical analysis revealed that ECP (200 mg/kg) and IMP significantly attenuated the CUMS-induced decrease in the level of BDNF in both the rat brain regions.

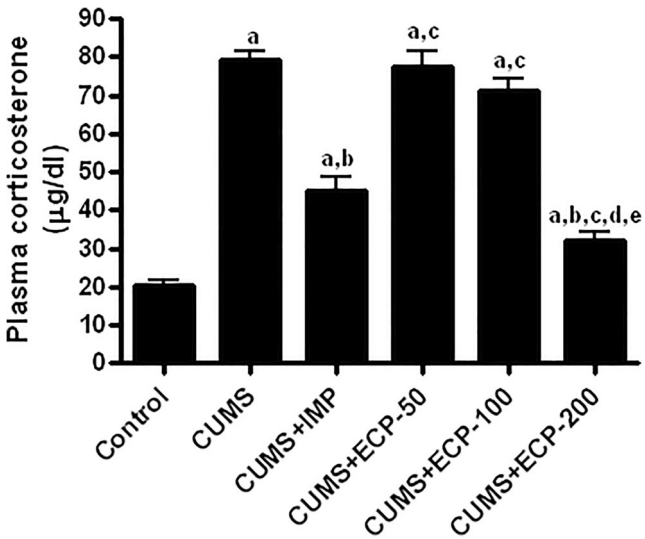

Fig. 5 Effect of ECP (50, 100 and 200 mg/kg) on CUMS-induced changes in the level of plasma corticosterone in the animals. All values are Mean ± Standard Error of Mean (SEM; N = 6). $^{a}P < 0.05$ compared to Control, $^{b}P < 0.05$ compared to CUMS, $^{c}P < 0.05$ compared to CUMS+ IMP, $^{d}P < 0.05$ compared to CUMS+ECP-50 and $^{e}P < 0.05$ compared to CUMS+ECP-100 (One-way ANOVA followed by Student Newmann Keuls Post-hoc test)

Discussion

The present study demonstrates that ECP at 200 mg/kg exhibited anti-depressant-like activities in CUMS challenged animals. ECP (200 mg/kg) attenuated CUMS-induced hyperactivity of HPA-axis function. Further, ECP (200 mg/kg) mitigated CUMS-induced alterations in serotonergic and BDNF-mediated activity, mitochondrial function and integrity in HIP and PFC of the rodents. These observations may implicate the fact that ECP could be a potential candidate in the management of depression.

In the present study, CUMS increased the anhedonia behavior in sucrose feeding test in the rats similar to earlier finding (Tianzhu et al. 2014). CUMS also increased the immobility period in FST and TST, and decreased the swimming and climbing behaviors in the animals in FST similar to that earlier study (Tianzhu et al. 2014). In addition, CUMS decreased the ambulation, rearing, time spent in the center and total distance travelled in rodents in OFT as that of previous report (Tianzhu et al. 2014). These observations indicate that CUMS exposure develops depression-like behaviors in these animals. ECP at 200 mg/kg dose level attenuated all CUMS-induced depression-like behaviors in all the test paradigms for the first time in the present study. The neuroprotective activity of this drug has already been reported to some extent (Meena et al. 2011; Kumar et al. 2013; Malabade and Taranalli 2015). Interestingly, ECP (200 mg/kg) increased the CUMS-induced decrease in the swimming behavior in rats during FST. However, it did not cause any alterations in the CUMS-induced changes in the climbing behavior of the animals during FST. It has been already suggested that the serotonergic activity in the brain mostly influence the swimming behavior

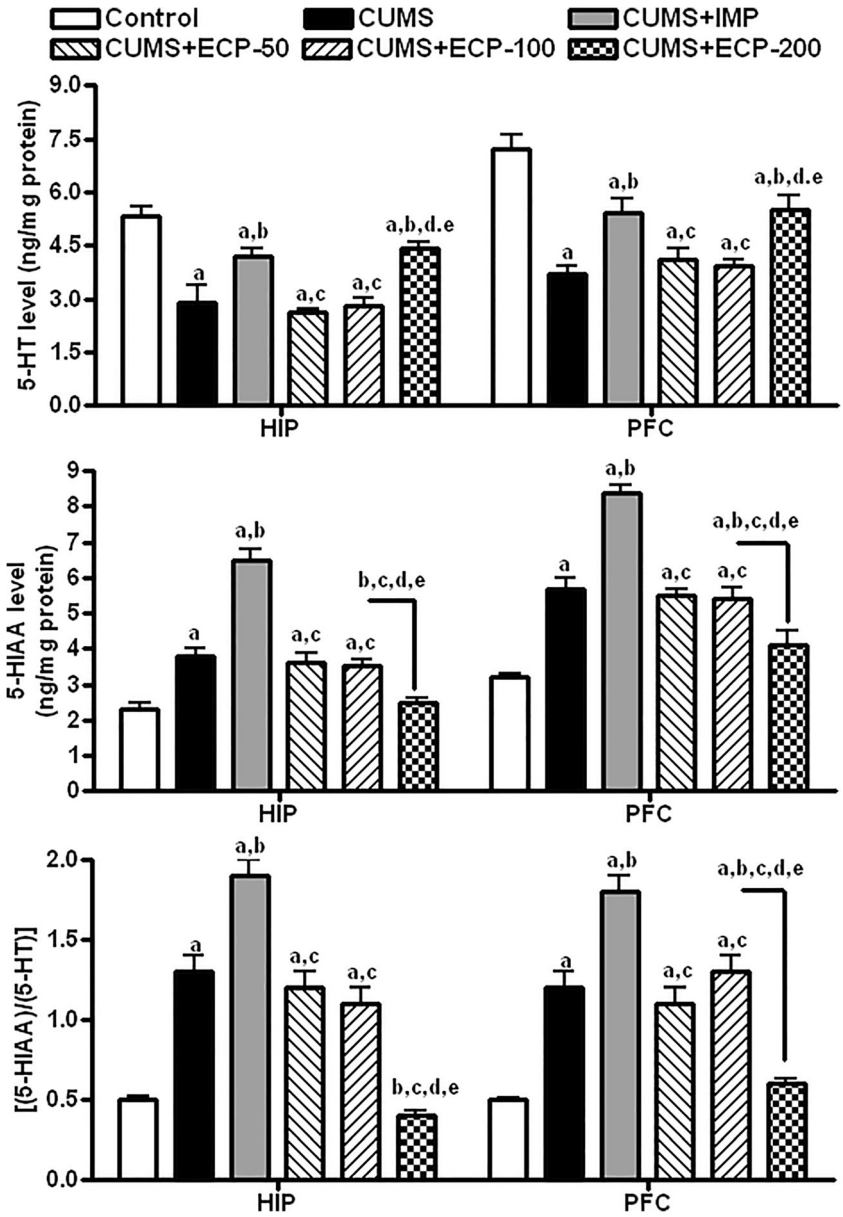

Fig. 6 Effect of ECP (50, 100 and 200 mg/kg) on CUMS-induced changes in the levels of 5-HT (**a**) and 5-HIAA (**b**), and ratio of 5-HIAA/5-HT (**c**) in discrete rat brain regions. All values are Mean ± Standard Error of Mean (SEM; N = 6). $^{a}P < 0.05$ compared to Control, $^{b}P < 0.05$ compared to CUMS, $^{c}P < 0.05$ compared to CUMS+IMP, $^{d}P < 0.05$ compared to CUMS+ECP-50 and $^{e}P < 0.05$ compared to CUMS+ECP-100 (One-way ANOVA followed by Student Newmann Keuls Post-hoc test)

of the animals during FST (Pesarico et al. 2014). Hence, the effect of ECP (200 mg/kg) may influence serotonergic activity in brain of CUMS challenged animals and thus may exhibit anti-depressant-like activity in these rodents.

The present work paid special attention to the HIP and PFC, which are brain regions structurally and functionally affected by stress responses and critically involved in the regulation of depression (Elhwuegi 2004; Mao et al. 2011). In the present study, CUMS caused a significant reduction in the 5-HT and elevation in the 5-HIAA levels, and increase in their turnover (5-HIAA/5-HT) in all brain regions of the rodents similar to that of earlier findings (Song et al. 2015; Ding et al. 2015; Wang et al. 2014). Numberous studies have reported that certain anti-depressant drugs act by inhibiting the uptake of 5-HT (Nestler et al. 2002), facilitating the secretion of 5-HT and reducing the metabolism of 5-HT through MAO inhibition, regulate depression in patients (Naughton et al. 2000), which suggest that the increasing of 5-HT level seems to be connected with the behavioral deficits induced by CUMS. In the present study, ECP (200 mg/kg) and IMP reversed the CUMS-induced decrease in the 5-HT level in both the brain regions. Interestingly, ECP (200 mg/kg) attenuated the CUMS-induced increase in the level of 5-HIAA and 5-HT turnover in both the brain regions of the animals. However, IMP further increased the CUMS-induced increase in the level of 5-HIAA and 5-HT turnover in both the brain regions of the rats. These results indicate the fact that ECP (200 mg/kg) exhibits improvement in serotonergic activity in these brain

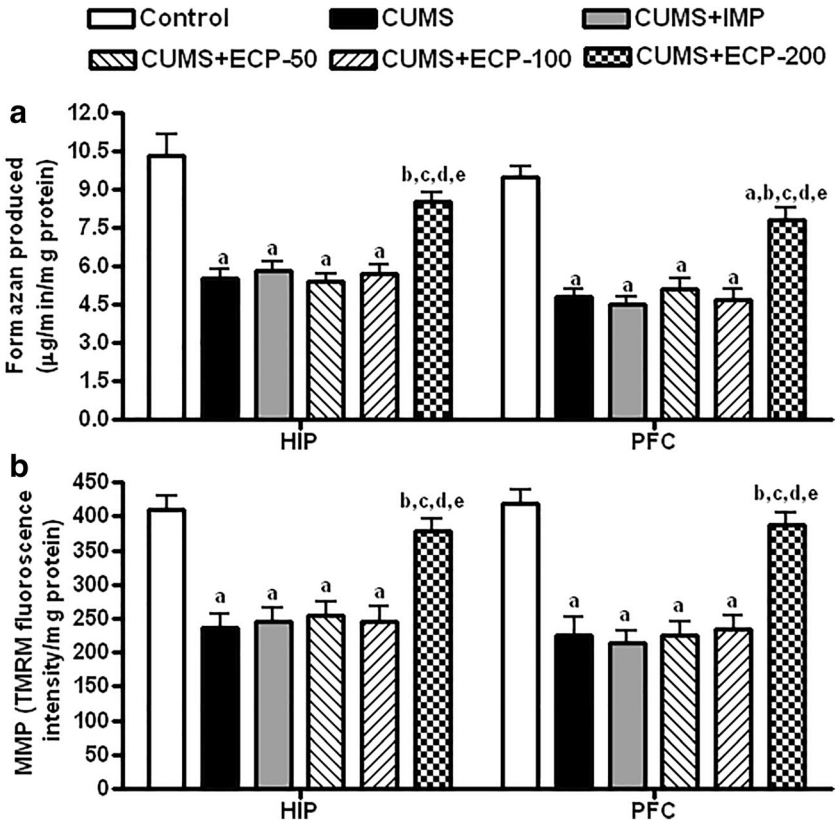

Fig. 7 Effect of ECP (50, 100 and 200 mg/kg) on CUMS-induced changes in the mitochondrial function (**a**) and integrity (**b**) in discrete brain regions. All values are Mean ± Standard Error of Mean (SEM; N = 6). $^{a}P < 0.05$ compared to Control, $^{b}P < 0.05$ compared to CUMS, $^{c}P < 0.05$ compared to CUMS+IMP, $^{d}P < 0.05$ compared to CUMS+ECP-50 and $^{e}P < 0.05$ compared to CUMS+ECP-100 (One-way ANOVA followed by Student Newmann Keuls Post-hoc test)

regions probably through inhibiting 5-HT transporter or facilitating its release and synthesis and/or inhibiting MAO enzyme activity which has to be clarified with future experimentations.

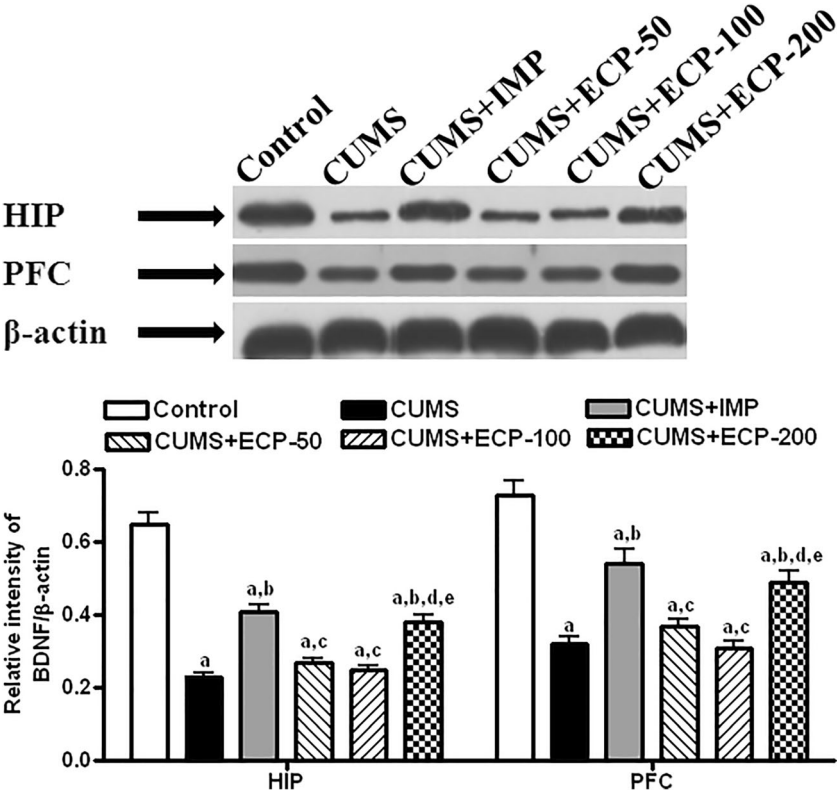

Fig. 8 Effect of ECP (50, 100 and 200 mg/kg) on CUMS-induced changes in the level of expression of BDNF protein in discrete rat brain regions. The blots (**a**) are representative of BDNF protein in rat brain regions. The histogram of BDNF (**b**) protein is expressed as the ratio of relative intensity of level of expression of BDNF to β-actin. All values are Mean ± Standard Error of Mean (SEM; N = 6). $^{a}P < 0.05$ compared to Control, $^{b}P < 0.05$ compared to CUMS, $^{c}P < 0.05$ compared to CUMS+IMP, $^{d}P < 0.05$ compared to CUMS+ECP-50 and $^{e}P < 0.05$ compared to CUMS+ECP-100 (One-way ANOVA followed by Student Newmann Keuls Post-hoc test)

It has been well documented that neurotrophins such as BDNF play a significant role in the pathophysiology of depression and that anti-depressants exert in part their effects through its regulation (Brunoni et al. 2008; Gervasoni et al. 2005). In the present study, CUMS caused a decrease in the level of expression of BDNF in both the brain regions similar to that of earlier report (Wang et al. 2015). ECP (200 mg/kg) and standard drug IMP both attenuated CUMS-induced reduction in the level of expression of BDNF in both the brain regions. This indicates that ECP at this dose level probably influence neurogenesis in part as its anti-depressant activity.

In the present study, ECP (200 mg/kg) attenuated CUMS-induced decrease in the mitochondrial function and integrity in both the brain regions. Interestingly, IMP did not cause any change in the CUMS-induced reduction in the mitochondrial function and integrity in both the brain regions. In contrast to our study, IMP exerts beneficial effect on mitochondria in genetic depressed and olfactory bulbectomy-induced depressed animals (Rinwa et al. 2013; Chen et al. 2013). This effect of IMP could be the variable of mode of induction of depression in the animals. It is well known that the elevated level of corticosterone in the brain tissues can cause mitochondrial dysfunction probably through glucocorticoid receptor and thus may exert several deleterious effects including elevated activity of MAO enzyme and decreased level of BDNF protein in specific brain areas (Głombik et al. 2016; Głombik et al. 2015). In the present study, IMP elevated the turnover of 5-HT while ECP (200 mg/kg) reduced the same in both the brain regions of the CUMS challenged animals. This indicates that ECP perhaps in part exert beneficial effect on the serotonergic system through mitochondrial modulation in such condition. Additionally, the present study revealed that CUMS elevated the level of corticosterone in the plasma of the animals similar to that of earlier report (Wang et al. 2015). Both ECP (200 mg/kg) and IMP attenuated the CUMS-induced increase in the level of corticosterone in rat plasma. The phytochemical screening revealed that ECP contains flavonoids, alkaloids, tannins, cardiac glycosides anthraquinones, saponins and phenols. The phytochemistry of this plant reveals the presence of triterpenoids, flavonoids, cardiac glycosides, cardenolides, anthocyanins, α-amyrin, β-amyrin, lupeol, β-sitosterol, flavanols, mudarine, resins, a powerful bacteriolytic enzyme calactin, a nontoxic proteolytic enzyme calotropin, and a wax (Sharma et al. 2012). These observations emphasize the fact that either one or multiple constituent or their combination of ECP (200 mg/kg) exert anti-depressant activity probably through HPA-axis regulation and thereby modulation of mitochondria-dependent serotonergic activity and neurogenesis which is subject to further investigation in future.

In conclusion, ECP exhibited anti-depressant-like activity in CUMS challenged animals. ECP attenuated CUMS-induced hyperactivity of HPA-axis function, alterations in serotonergic and BDNF-mediated activity, mitochondrial function and integrity in HIP and PFC of the rodents. These observations may implicate the fact that ECP could be a better pharmacological agent in the pharmacotherapy of depression.

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Compliance with ethical standards All the experimental procedures utilized were performed in accordance with the approval of the Institutional Animal Ethics Committee (GLAIPR/CPCSEA/IAEC/2016/P.Col/R7) under strict compliance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for the experimental studies.

Conflict of interest The authors declare that they have no conflict of interests.

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