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Panax quinquefolium involves nitric oxide pathway in olfactory bulbectomy rat model

Puneet Rinwa, Anil Kumar *

Pharmacology Division, University Institute of Pharmaceutical Sciences, UGC Centre of Advanced Study, Panjab University, Chandigarh 160014, India

HIGHLIGHTS

- Depression is associated with decline in mental and functional capacities.
- Olfactory bulbectomy (OBX) is a reliable model to mimic depression like behavior.
- Therapeutic effects of *Panax quinquefolium* (PQ) against OBX induced depression
- Nitric oxide (NO) is an intercellular messenger found in the brain.
- Possible involvement of nitric oxide pathway in the protective effects of PQ

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ABSTRACT

Olfactory bulbectomy (OBX) is a well known screening model for depression. *Panax quinquefolium* (PQ) is known for its therapeutic potential against several psychiatric disorders. Nitric oxide (NO), an intercellular messenger has been suggested to play a crucial role in the pathogenesis of depression. The present study was designed to explore the possible involvement of NO mechanism in the protective effect of PQ against olfactory bulbectomy induced depression. Wistar rats were bulbectomized surgically and kept for a rehabilitation period of two weeks. PQ (50, 100 and 200 mg/kg; p.o.) alone and in combination with NO modulators like L-NAME (10 mg/kg, i.p.) and L-arginine (100 mg/kg; i.p.) were then administered daily for another two weeks. Ablation of olfactory bulbs caused depression-like symptoms as evidenced by increased immobility time in forced swim test, hyperactivity in open field arena, and anhedonic like response in sucrose preference test. Further, OBX caused elevation in serum corticosterone levels and increased oxidative–nitrosative damage. These deficits were integrated with increased levels of neuroinflammatory cytokines (TNF-α), apoptotic factor (caspase-3) and a marked reduction in neurogenesis factor (BDNF) in both cerebral cortex and hippocampal regions of bulbectomized rats. Treatment with PQ significantly and dose-dependently restored these behavioral, biochemical and molecular alterations associated with OBX. Further, pretreatment of L-NAME with subeffective dose of PQ (100 mg/kg) significantly potentiated its protective effects; however L-arginine pretreatment reversed the beneficial effects. The present study suggests that protective effect of *P. quinquefolium* might involve nitric oxide modulatory pathway against olfactory bulbectomy-induced depression in rats.

1. Introduction

Olfactory bulbectomy (OBX) is a well known animal model of depression that leads to a variety of behavioral alterations, many of which are similar to those seen in patients with major depression [1,2]. OBX is a reliable model to evaluate anti-depressant activity since its behavioral changes are normalized following chronic but not acute administration of antidepressants [2]. OBX is associated with a variety of behavioral abnormalities such as hyperactivity in the open-field test [3], increased immobility time [2] and anhedonia like state in sucrose preference test [4]. These behavioral changes are independent of anosmia and result due to retrograde neuronal degeneration after bulbectomy [2]. Further, removal of olfactory bulbs has also been reported to reduce neurogenesis and promote neuronal cell death in different regions of the brain, a putative pathogenic mechanism in depression syndrome [5,6]. Although the current pharmacotherapy of depression includes a battery of drugs, clinicians are still looking for alternative therapies to exploit herbal medications for the treatment of psychiatric disorders. The approach towards development of dietary and medicinal phytochemicals as novel therapeutics may prove to be a useful tool in recognition of natural medicines globally.

Panax quinquefolium; PQ (American ginseng) belonging to the family *Araliaceae* is a native plant of North America and cultivated in many

* Corresponding author. Tel.: +91 172 2534106; fax: +91 172 2543101.
E-mail address: kumaruips@yahoo.com (A. Kumar).

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countries. The active constituents responsible for most of the central nervous system (CNS) related bioactivities of PQ are ginseng saponins, namely ginsenoside [7]. Ginsenosides are well-known for their antioxidant and free radical scavenging properties [8]. Studies have shown antidepressant effects of oral ginsenosides in both forced swimming test and chronic mild stress model of depression [9]. Further, ginsenosides are also well known for their neuroprotective effects in several animal models [10,11]. Earlier, studies from Sheikh et al. [12] showed that stress induced alteration in plasma corticosterone levels in rats was normalized on treatment with PQ. Saponins derived from PQ have shown a significant attenuation effect on apoptotic factor (caspase-12) [13] and are known to protect hippocampal neurons against ischemia [14]. These results clearly show the neuroprotective potential of ginsenosides; however, the exact cellular or molecular pathway in their protective effect has not been reported so far.

Nitric oxide (NO) is an intercellular messenger in the brain, synthesized from L-arginine by nitric oxide synthase (NOS) and plays an important role in synaptic plasticity, learning, memory, aggression and depression [15]. Nitric oxide has an unpaired electron, and therefore acts as reactive free radical species. The generation of reactive nitrogen species is associated with nitration of proteins [16] and lipid peroxidation [17] and promotes carbonylation [18]. Inhibition of NOS is known to produce anxiolytic and antidepressant-like effects in experimental animal models [19]. Inhibition of neuronal or inducible NOS (nNOS) in the rat hippocampus exerts antidepressant-like effects [20]. Moreover, reports have suggested that L-arginine-nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) is an important signaling pathway involved in depression [21]. With this background, the present study attempts to elucidate the behavioral, biochemical and molecular aspects of PQ with respect to its antidepressant activity against olfactory bulbectomy model and to further investigate the involvement of nitric oxide signaling pathway.

2. Materials and methods

2.1. Animals

Twelve week old male Wistar rats (200–240 g) were procured from Central Animal House, Panjab University, Chandigarh and from Animal House of Panacea Biotec Ltd., Lalru (Panjab). Male rats were chosen to avoid the influence of female estrogen hormone on depression like behavior. Animals were housed under standard (25 ± 2 °C, 60–70% humidity) laboratory conditions and maintained on a 12 hour natural day–night cycle, with free access to food and water. Animals were acclimatized to laboratory conditions before the experimental tests. The experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC) of Panjab University (IAEC/282/UIPS/39 dated 30/8/12) and conducted according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines of the Government of India.

2.2. Surgical procedure (olfactory bulb ablation)

After the accommodation period, animals underwent either olfactory bulbectomy or sham surgery. Animals were anesthetized with ketamine (75 mg/kg, i.p) and xylazine (5 mg/kg, i.p) combination prior to surgery. Bilateral olfactory bulb ablation was performed as described by different investigators [22]. The animals were fixed in a stereotactic frame (Stoelting Co., USA), 1 cm rostral–caudal midline incision was made in the skin of the head, and two small burr holes (2 mm in diameter) were drilled into the skull 6 mm rostral of bregma and 1 mm lateral of the midline. Both olfactory bulbs were removed by suction and hemostatic sponge (AbGel, absorbable gelatin sponge USP, Srikrishna Laboratories, India) was inserted into the cavity to control bleeding. The incision was then closed with absorbable sutures (Ethicon 4-0, absorbable surgical sutures USP (Catgut), Johnson and Johnson, India) and animals were injected with sulprim injection® (each ml containing 200 and 40 mg of sulfadiazine and trimethoprim respectively), intramuscularly (0.2 ml/300 g) once a day for 3 days to prevent post surgical infection. Sham animals were given similar treatment as OBX animals except the removal of the olfactory bulbs. The success and validation of the OBX surgery were verified by using two methods: (a) dissection and direct observation of remaining olfactory bulb tissue and by (b) measurements of key behavioral variables altered by OBX, namely hyperactivity behavior during open field test. The OBX/Sham animals were housed singly in cages for two weeks (14 days) of surgical rehabilitation period and drug treatments were started after that. Pictogram of the entire protocol is represented in Fig. 1.

2.3. Drugs and treatment schedule

P. quinquefolium (American ginseng), L-NAME and L-Arginine were purchased from Sigma chemicals Co. (St. Louis, MO, USA). ELISA kit for TNF-α and caspase-3 was purchased from R&D Systems (USA). While ChemiKine™ Brain Derived Neurotrophic Factor (BDNF) kit was procured from Millipore (USA). All other chemicals used for biochemical estimations were of analytical grade. The animals were randomly divided into nine experimental groups with eight animals in each. Out of the total of 72 animals used in the study 6 animals died of surgery (similar to 5–10% mortality reported in olfactory bulbectomy experiments) and were replaced by fresh animals and surgery was performed. The first and second groups were named as sham and OBX (ablation of olfactory bulbs) control groups respectively. *P. quinquefolium* (PQ) (50, 100 and 200 mg/kg) was treated as groups 3–5 respectively. Pretreatment of L-NAME (10 mg/kg) and L-arginine (100 mg/kg) with PQ (100 mg/kg) served as groups 6–7. Treatment of L-NAME (10 mg/kg) and L-arginine (100 mg/kg) per se was categorized as groups 8 and 9 respectively. *P. quinquefolium* (PQ) was prepared in peanut oil where as L-NAME (10 mg/kg) and L-arginine (100 mg/kg) were dissolved in normal saline and administered orally on the basis of body weight (0.5 ml/100 g). The doses of *P. quinquefolium* were selected on the basis of literature [21]. Solutions were made freshly at the beginning of each day of the protocol. Drugs were administered daily once a day for a period of 14 days.

2.4. Behavioral assessment

2.4.1. Sucrose preference test

The rats were tested for sucrose consumption as described earlier [23]. Animals were housed individually throughout the test duration and presented two bottles simultaneously in the home cage, one containing a 1% w/v sucrose solution and the other containing standard drinking water during the 48 h training session. To prevent the preference to position, the location of the two bottles was varied during this period. After an 18 h period of food and water deprivation, an 8 h test session was conducted. The amount of liquid remaining in each bottle was measured at the end of the testing period. The sucrose preference score was expressed as percent of total liquid intake. Sucrose preference (SP) was calculated according to the following equation:

$$SP = \left(\frac{\text{sucrose intake(g)}}{\text{sucrose intake(g)} + \text{water intake(g)}}\right) \times 100.$$

2.4.2. Open field exploration

Open field behavior of rats was recorded in a circular arena of diameter 80 cm, surrounded by a 30 cm high wooden wall [24]. The arena painted white, was divided in to 25 small sections. Each rat was carefully placed in the center of circular arena and allowed to explore the open field for 5 min. During this period, the ambulatory activity, in terms of the number of sections crossed, and the frequency of rearing were

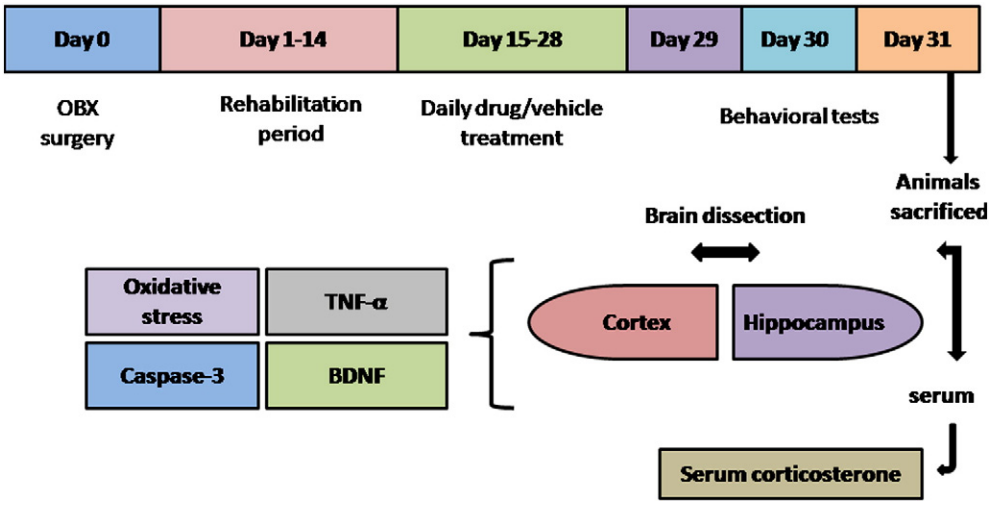

Fig. 1. Diagrammatic representation of the entire study protocol.

recorded along with defecation and licking episodes and values were expressed as counts per 5 min.

2.4.3. Immobility period

Forced swim test was performed as described [25]. One day prior to the test, a rat was placed for conditioning in a clear plastic tank (45 cm × 35 cm × 60 cm) containing 30 cm of water (24 ± 0.5 °C) for 15 min (pretest session). Twenty-four hours later (test session), the total immobility period within a 5-min session was recorded as immobility scores (in seconds). A rat was judged to be immobile when its hind legs were no longer moving and the rat was hunched forward (a floating position). The immobility time was recorded manually by an observer who was blind to the drug treatment.

2.5. Biochemical estimations

Immediately after the last behavioral test (day 31), blood was collected by retro-orbital bleeding for serum corticosterone estimation, the animals were sacrificed by cervical dislocation and brain samples were rapidly removed and placed on dry ice for isolation of cerebral cortex and hippocampus. 10% (w/v) tissue homogenates were prepared in 0.1 M phosphate buffer (pH 7.4). The homogenates were centrifuged at 10,000 ×*g* for 15 min. Aliquots of supernatants were separated and used for biochemical estimations.

2.5.1. Lipid peroxidation

The extent of lipid peroxidation was determined quantitatively by performing the method as described by Wills [26]. The amount of malondialdehyde (MDA) was measured by reaction with thiobarbituric acid at 532 nm using a PerkinElmer Lambda 20 spectrophotometer (Norwalk, CT, USA). The values were calculated using the molar extinction co-efficient of chromophore ($1.56 \times 10\ M^{-1}\ cm^{-1}$).

2.5.2. Nitrite

The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide was determined by a colorimetric assay with Griess reagent (0.1% N-(1-napththyl) ethylene diamine dihydrochloride, 1% sulfanilamide and 5% phosphoric acid) [27]. Equal volumes of the supernatant and the Griess reagent were mixed and the mixture was incubated for 10 min at room temperature in the dark. The absorbance was measured at 540 nm using a PerkinElmer Lambda 20 spectrophotometer (Norwalk, CT, USA). The concentration of nitrite in the supernatant was determined from sodium nitrite standard curve.

2.5.3. Reduced glutathione

Reduced glutathione in the brain was estimated according to the method of Ellman et al. [28]. Homogenate (1 ml) was precipitated with 1.0 ml of 4% sulfosalicylic acid and the samples were immediately centrifuged at 1200 ×*g* for 15 min at 4 °C. The assay mixture contained 0.1 ml of supernatant, 2.7 ml of phosphate buffer of pH 8.0 and 0.2 ml of 0.01 M dithiobisnitrobenzoic acid (DTNB). The yellow color developed was read immediately at 412 nm using a PerkinElmer lambda 20 spectrophotometer (Norwalk, CT, USA). The results were expressed as micromoles of reduced glutathione per milligram of protein.

2.5.4. Superoxide dismutase

Superoxide dismutase (SOD) activity was assayed by the method of Kono [29] wherein the reduction of nitro blue tetrazolium (NBT) was inhibited by the superoxide dismutase and is measured. The assay system consists of EDTA 0.1 mM, sodium carbonate 50 mM and 96 mM of nitro blue tetrazolium (NBT). In the cuvette, 2 ml of the above mixture, 0.05 ml of hydroxylamine and 0.05 ml of the supernatant were added and auto-oxidation of hydroxylamine was measured for 2 min at 30 s intervals by measuring absorbance at 560 nm using a PerkinElmer Lambda 20 spectrophotometer (Norwalk, CT, USA).

2.5.5. Catalase

Catalase activity was determined by the method of Luck [30], wherein the breakdown of hydrogen peroxide (H_2O_2) is measured at 240 nm. Briefly, the assay mixture consisted of 3 ml of H_2O_2, phosphate buffer and 0.05 ml of supernatant of tissue homogenate (10%), and the change in absorbance was recorded at 240 nm using a PerkinElmer lambda 20 spectrophotometer (Norwalk, CT, USA). The results were expressed as micromoles of H_2O_2 decomposed per milligram of protein/min.

2.5.6. Protein

The protein content was estimated by biuret method [31] using bovine serum albumin as a standard.

2.6. Serum corticosterone estimations

2.6.1. Preparation of serum

Blood was collected (1.0 ml) between 8.00 and 9.00 AM through retro orbital bleeding in the test tube and allowed to clot at room temperature. The tubes were then centrifuged at 2000 rpm for 10 min. The straw colored serum was separated and stored frozen at −20 °C.

2.6.2. Corticosterone assessment

For extraction of corticosterone the method of Silber et al. [32] was modified as described. 0.1–0.2 ml of serum was treated with 0.2 ml of freshly prepared chloroform: methanol mixture (2:1, v/v), followed by 3 ml of chloroform instead of dichloromethane used in the procedure of Silber and its group [32]. The step of treatment of petroleum ether was omitted. The samples were vortexed for 30 s and centrifuged at 2000 rpm for 10 min. The chloroform layer was carefully removed with the help of a syringe with a long 16 gauge needle attached to it and was transferred to a fresh tube. The chloroform extract was then treated with 0.1 N NaOH by vortexing rapidly and NaOH layer was rapidly removed. The sample was then treated with 3 ml of 30 N H_2SO_4 by vortexing vigorously. After phase separation, chloroform layer on top was removed using a syringe as described above and discarded. The tubes containing H_2SO_4 were kept in dark for 30–60 min and thereafter fluorescence measurements were carried out in a fluorescence spectrophotometer (make Hitachi, model F-2500) with excitation and emission wavelength set at 472 and 523.2 nm respectively. The standard curve depicting the fluorescence yield versus corticosterone concentration was used for result analysis.

2.7. Molecular estimations

2.7.1. BDNF and TNF-α ELISA

The quantifications of BDNF and TNF-α were done with the help and instructions provided by Chemikine and R&D Systems immunoassay kits respectively. All samples were assayed in duplicate and absorbance was read on an ELISA plate reader (iMark™ microplate absorbance reader, BIO-RAD) and the concentration of each sample was calculated by plotting the absorbance values on standard curve with known concentrations generated by the assay.

2.7.2. Caspase-3 colorimetric assay

Caspase-3, also known as CPP-32 is an intracellular cysteine protease that exists as a pro-enzyme, becoming activated during the cascade of events associated with apoptosis. The tissue lysates/homogenates can then be tested for protease activity by the addition of a caspase specific peptide that is conjugated to the color reporter molecule p-nitroaniline (pNA). The cleavage of the peptide by the caspase releases the chromophore pNA, which can be quantitated spectrophotometrically at a wavelength of 405 nm. The level of caspase enzymatic activity in the cell lysate/homogenate is directly proportional to the color reaction. The enzymatic reaction for caspase activity was carried out using R&D systems caspase-3 colorimetric kit.

2.8. Statistical analysis

Data are expressed as mean ± S.E.M. The data was analyzed by one-way ANOVA followed by Tukey's test. $p < 0.05$ was considered as statistically significant. All statistical procedures were carried out using SigmaStat GraphPad Prism (GraphPad Software, San Diego, CA).

3. Results

3.1. Effects of P. quinquefolium (PQ) and its modulation by L-NAME or L-arginine on sucrose preference test

OBX animals showed a significant reduction in sucrose consumption as compared to sham group. Treatment with PQ (100, 200 mg/kg) significantly and dose dependently attenuated the reduction in sucrose consumption as compared to OBX control. PQ (50 mg/kg) did not show any significant effect on sucrose consumption as compared to control. Further, L-NAME (10 mg/kg) pretreatment with subeffective dose of PQ (100 mg/kg) potentiated its sucrose consumption which was significant when compared to their effects alone. However, pretreatment of L-arginine (100 mg/kg) with PQ (100 mg/kg) significantly reversed the protective effect of PQ (100 mg/kg). Besides, the effects of L-NAME (10 mg/kg) and L-arginine (100 mg/kg) treatments alone did not show any significant effect as compared to control [$F(9, 71) = 12.34$ ($p < 0.05$)] (Fig. 2).

3.2. Effects of P. quinquefolium (PQ) and its modulation by L-NAME or L-arginine in open field performance task

Ablation of olfactory bulbs caused a characteristic hyperactivity in the open field task as seen by increased ambulation, rearing, defecation (number of fecal pellets) and reduced grooming/licking episodes which were significant when compared to sham group. PQ (100, 200 mg/kg) treatment significantly reduced ambulation, rearing, defecation and improved grooming/licking episodes when compared to OBX control. PQ (50 mg/kg) did not show any significant effect on open field behavior as compared to control. Further, pretreatment of L-NAME (10 mg/kg) with subeffective dose of PQ (100 mg/kg) significantly potentiated their protective effects in open field task as compared to their effects alone. However, pretreatment of L-arginine (100 mg/kg) with PQ (100 mg/kg) significantly reversed the protective effect of PQ (100 mg/kg). Besides, the effects of L-NAME (10 mg/kg) and L-arginine (100 mg/kg) treatments alone did not show any significant effect on ambulation [$F(9, 71) = 56.35$ ($p < 0.05$)], rearing [$F(9, 71) = 121.42$ ($p < 0.05$)], grooming [$F(9, 71) = 45.58$ ($p < 0.01$)] and defecation [$F(9, 71) = 13.41$ ($p < 0.05$)] parameters as compared to control group (Table 1).

3.3. Effects of P. quinquefolium (PQ) and its modulation by L-NAME or L-arginine on immobility period

Olfactory bulbectomy caused a significant increase in the immobility period as compared to sham animals. Treatment with PQ (100, 200 mg/kg) significantly shortened the immobility time when compared to OBX control. PQ (50 mg/kg) did not show any significant effect on immobility period as compared to control. Further, pretreatment of L-NAME (10 mg/kg) with subeffective dose of PQ (100 mg/kg) potentiated their protective effects (shortened immobility period) which was significant as compared to their effect alone. However, L-arginine (100 mg/kg) pretreatment with PQ (100 mg/kg) significantly reversed the protective effect of PQ (100 mg/kg). Besides, the per se effects of L-NAME (10 mg/kg) and L-arginine (100 mg/kg)

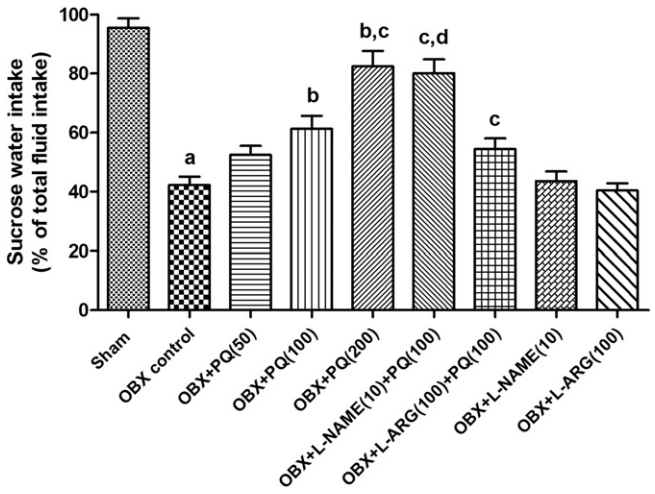

Fig. 2. Effect of *Panax quinquefolium* and its modulation by L-NAME and L-arginine on sucrose consumption test. Values are expressed as mean ± SEM. For statistical significance, $^{a}P < 0.05$ as compared to sham group; $^{b}P < 0.05$ as compared to OBX control; $^{c}P < 0.05$ as compared to OBX + PQ (100); $^{d}P < 0.05$ as compared to OBX + L-NAME (one-way ANOVA followed by Tukey's test). OBX, olfactory bulbectomy; PQ, *P. quinquefolium*; L-ARG, L-arginine.

Table 1
Effect of *Panax quinquefolium* and its modulation by L-NAME and L-arginine on open field behavior. Values are expressed as mean ± SEM. For statistical significance, [a]$P < 0.05$ as compared to sham group; [b]$P < 0.05$ as compared to OBX control; [c]$P < 0.05$ as compared to OBX + PQ (100); [d]$P < 0.05$ as compared to OBX + L-NAME (10) (one-way ANOVA followed by Tukey's test). OBX, olfactory bulbectomy; PQ, *Panax quinquefolium*; L-ARG, L-arginine.

Treatment (mg/kg)	Open field behavior			
	Ambulation	Rearing	Grooming/licking episodes	Number of fecal pellets
Sham	88.2 ± 3.12	20.1 ± 1.84	7.5 ± 0.86	0.8 ± 0.22
OBX control	167.2 ± 5.21[a]	61.1 ± 2.62[a]	1.2 ± 0.38[a]	5.3 ± 0.44[a]
OBX + PQ (50)	152.0 ± 4.84	57.2 ± 2.10	2.0 ± 0.33	4.2 ± 0.85
OBX + PQ (100)	125.5 ± 2.45[b]	43.1 ± 2.11[b]	3.2 ± 0.56[b]	3.8 ± 0.55[b]
OBX + PQ (200)	100.8 ± 3.62[b,c]	22.8 ± 1.52[b,c]	5.4 ± 0.77[b,c]	2.2 ± 0.35[b,c]
OBX + L-NAME (10) + PQ (100)	102.6 ± 4.57[c,d]	23.2 ± 1.40[c,d]	5.2 ± 0.84[c,d]	2.3 ± 0.51[c,d]
OBX + L-ARG (100) + PQ (100)	153.4 ± 4.12[c]	55.4 ± 2.65	2.1 ± 0.30[c]	5.5 ± 0.80[c]
OBX + L-NAME (10)	158.2 ± 3.58	60.8 ± 2.60	1.6 ± 0.34	5.0 ± 0.85
OBX + L-ARG (100)	170.5 ± 4.41	64.2 ± 2.32	1.0 ± 0.14	5.2 ± 0.34

treatments did not show any significant effect on immobility time when compared to control [$F(9, 71) = 32.11$ ($p < 0.01$)] (Fig. 3).

3.4. Effect of P. quinquefolium (PQ) and its modulation by L-NAME or L-arginine on lipid peroxidation (MDA), reduced glutathione (GSH), nitrite concentration, superoxide dismutase (SOD) and catalase enzyme levels

OBX rats showed a significant increase in oxidative damage as evidenced by a rise in MDA and nitrite levels and depletion of reduced GSH, SOD and catalase levels as compared to sham group. Chronic treatment with PQ (100, 200 mg/kg) significantly attenuated the oxidative damage (reduced MDA, nitrite levels, restoration of reduced GSH, SOD and catalase levels) as compared to OBX control. PQ (50 mg/kg) did not show any significant effect on oxidative stress markers as compared to control. Further, pretreatment of L-NAME (10 mg/kg) with subeffective dose of PQ (100 mg/kg) potentiated their antioxidant like effect which was significant as compared to their effect alone. However, L-arginine (100 mg/kg) pretreatment with PQ (100 mg/kg) significantly reversed the protective effect of PQ (100 mg/kg). In addition, L-NAME (10 mg/kg) and L-arginine (100 mg/kg) treatments alone did not produce any significant effect on LPO [$F(9, 71) = 34.23$ ($p < 0.05$)], GSH [$F(9, 71) = 14.45$ ($p < 0.01$)], nitrite [$F(9, 71) = 56.23$ ($p < 0.05$)], SOD [$F(9, 71) = 23.82$ ($p < 0.01$)] and catalase [$F(9, 71) = 13.41$ ($p < 0.05$)] levels as compared to control (Table 2).

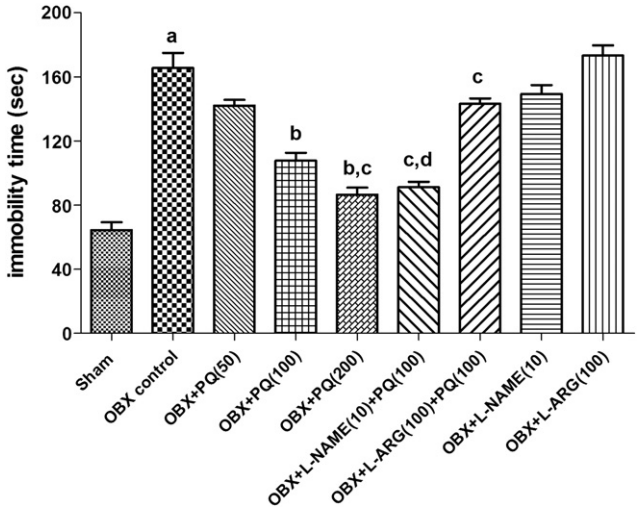

Fig. 3. Effect of *Panax quinquefolium* and its modulation by L-NAME and L-arginine on immobility time in FST. Values are expressed as mean ± SEM. For statistical significance, [a]$P < 0.05$ as compared to sham group; [b]$P < 0.05$ as compared to OBX control; [c]$P < 0.05$ as compared to OBX + PQ (100); [d]$P < 0.05$ as compared to OBX + L-NAME (one-way ANOVA followed by Tukey's test). OBX, olfactory bulbectomy; PQ, *P. quinquefolium*; L-ARG, L-arginine.

3.5. Effect of P. quinquefolium (PQ) and its modulation by L-NAME or L-arginine on serum corticosterone (CORT) levels

OBX group of animals showed a significant increase in serum CORT levels as compared to sham group. PQ (100, 200 mg/kg) significantly attenuated the increased serum CORT levels as compared to OBX control. PQ (50 mg/kg) did not show any significant effect on CORT levels as compared to control. In addition, pretreatment of L-NAME (10 mg/kg) with subeffective dose of PQ (100 mg/kg) significantly attenuated the increased serum CORT level as compared to their effects alone. Further, L-arginine (100 mg/kg) pretreatment with PQ (100 mg/kg) significantly reversed the protective effect of PQ (100 mg/kg). However, treatment with L-NAME (10 mg/kg) and L-arginine (100 mg/kg) alone did not cause any significant difference on serum CORT levels as compared to control group [$F(9, 71) = 211.32$ ($p < 0.01$)] (Fig. 4).

3.6. Effect of P. quinquefolium (PQ) and its modulation by L-NAME or L-arginine on brain tissue necrosis factor (TNF-α) and caspase 3 level

There was a significant increase in TNF-α and caspase 3 levels in OBX animals when compared to sham group. Treatment with PQ (100, 200 mg/kg) significantly attenuated the increased levels of TNF-α and caspase 3 as compared to OBX control. PQ (50 mg/kg) did not show any significant effect on TNF-α and caspase 3 levels as compared to control. Further, pretreatment of L-NAME (10 mg/kg) with subeffective dose of PQ (100 mg/kg) potentiated its protective effect (lowered TNF-α and caspase 3 levels) which was significant as compared to their effects alone. However, pretreatment of L-arginine (100 mg/kg) with PQ (100 mg/kg) significantly reversed the protective effect of PQ (100 mg/kg). In addition, treatment with L-NAME (10 mg/kg) and L-arginine (100 mg/kg) per se did not produce any significant effect on TNF-α [$F(9, 71) = 19.24$ ($p < 0.05$)] (Fig. 5) and caspase 3 levels [$F(9, 71) = 42.75$ ($p < 0.05$)] as compared to control (Fig. 6).

3.7. Effects of P. quinquefolium (PQ) and its modulation by L-NAME or L-arginine on brain derived neurotrophic factor (BDNF) levels

OBX animals showed a significant reduction in BDNF levels as compared to sham group. PQ (100, 200 mg/kg) treatment significantly restored the BDNF level as compared to OBX control. PQ (50 mg/kg) did not show any significant effect on BDNF levels as compared to control Further, pretreatment of L-NAME (10 mg/kg) with subeffective dose of PQ (100 mg/kg) significantly potentiated its protective effect (elevated BDNF level) which was significant as compared to their effects alone. However, pretreatment of L-arginine (100 mg/kg) with PQ (100 mg/kg) significantly reversed the protective effect of PQ (100 mg/kg). In addition, treatment with L-NAME (10 mg/kg) and L-arginine (100 mg/kg) per se did not produce any significant effect on BDNF levels [$F(9, 71) = 37.13$ ($p < 0.01$)] as compared to control (Fig. 7).

Table 2
Effect of *Panax quinquefolium* and its modulation by L-NAME and L-arginine on oxidative stress parameters. Values are expressed as mean ± SEM. For statistical significance, [a]$P < 0.05$ as compared to sham group; [b]$P < 0.05$ as compared to OBX control; [c]$P < 0.05$ as compared to OBX + PQ (100); [d]$P < 0.05$ as compared to OBX + L-NAME (10) (one-way ANOVA followed by Tukey's test). OBX, olfactory bulbectomy; PQ, *Panax quinquefolium*; L-ARG, L-arginine.

Treatment (mg/kg)	LPO (nmol of MDA/mg pr)	GSH (μmol of GSH/mg pr)	Nitrite (μg/ml)	Catalase (μmol of H_2O_2 hydrolysed/min/mg pr)	SOD (μ/mg pr)
Sham					
Cerebral cortex	0.152 ± 0.03	0.075 ± 0.005	324.4 ± 9.2	0.77 ± 0.06	1.68 ± 0.55
Hippocampus	0.107 ± 0.06	0.052 ± 0.002	242.2 ± 5.5	0.54 ± 0.05	1.38 ± 0.42
OBX control					
Cerebral cortex	0.560 ± 0.08[a]	0.016 ± 0.003[a]	783.7 ± 14.6[a]	0.19 ± 0.03[a]	0.52 ± 0.02[a]
Hippocampus	0.370 ± 0.04[a]	0.011 ± 0.001[a]	510.2 ± 10.2[a]	0.14 ± 0.04[a]	0.32 ± 0.02[a]
OBX + PQ (50)					
Cerebral cortex	0.529 ± 0.09	0.021 ± 0.009	662.4 ± 9.2[b]	0.28 ± 0.04	0.61 ± 0.05
Hippocampus	0.342 ± 0.06	0.018 ± 0.002	461.0 ± 8.8	0.21 ± 0.04	0.41 ± 0.04
OBX + PQ (100)					
Cerebral cortex	0.406 ± 0.04[b]	0.038 ± 0.004[b]	587.2 ± 6.4[b]	0.41 ± 0.02[b]	0.82 ± 0.05[b]
Hippocampus	0.285 ± 0.06[b]	0.026 ± 0.001	362.8 ± 5.8[b]	0.31 ± 0.01[b]	0.78 ± 0.08[b]
OBX + PQ (200)					
Cerebral cortex	0.258 ± 0.02[b,c]	0.057 ± 0.002[b,c]	454.2 ± 8.7[b,c]	0.63 ± 0.06[b,c]	1.28 ± 0.04[b,c]
Hippocampus	0.192 ± 0.01[b,c]	0.044 ± 0.004[b,c]	312.4 ± 6.7[b]	0.47 ± 0.05[b,c]	1.01 ± 0.04[b,c]
OBX + L-NAME (10) + PQ (100)					
Cerebral cortex	0.272 ± 0.08[c,d]	0.055 ± 0.003[c,d]	468.0 ± 7.9[c,d]	0.59 ± 0.04[c,d]	1.26 ± 0.08[c,d]
Hippocampus	0.196 ± 0.04[c,d]	0.043 ± 0.002[c,d]	325.8 ± 6.4[b]	0.45 ± 0.01[c,d]	0.98 ± 0.03[c,d]
OBX + L-ARG (100) + PQ (100)					
Cerebral cortex	0.511 ± 0.03[c]	0.020 ± 0.002[c]	647.2 ± 9.8[c]	0.33 ± 0.03	0.64 ± 0.06[c]
Hippocampus	0.330 ± 0.05[c]	0.019 ± 0.001[c]	472.1 ± 9.5[c]	0.22 ± 0.04[c]	0.43 ± 0.05[c]
OBX + L-NAME (10)					
Cerebral cortex	0.548 ± 0.08	0.018 ± 0.007	762.2 ± 11.2	0.22 ± 0.05	0.56 ± 0.05
Hippocampus	0.362 ± 0.01	0.013 ± 0.002	494.1 ± 8.2	0.16 ± 0.09	0.31 ± 0.04
OBX + L-ARG (100)					
Cerebral cortex	0.576 ± 0.04	0.013 ± 0.004	785.7 ± 12.4	0.14 ± 0.03	0.50 ± 0.06
Hippocampus	0.386 ± 0.06	0.009 ± 0.001	521.2 ± 11.8	0.11 ± 0.01	0.28 ± 0.08

4. Discussion

Olfactory bulbectomy (OBX) is a well studied and widely used experimental model of depression, results in several neurobiological and behavioral deficits that resemble key features of human depression [33] and are reversed by several pharmacotherapeutic interventions [2].

The present study was designed to evaluate the protective effects of *P. quinquefolium* (PQ) against olfactory bulbectomy induced-depression like behavior and to study the possible involvement of nitric oxide pathway in this regard. In our study, we observed a characteristic hyperactivity in the open field paradigm and an increased immobility time in the forced swim test in OBX rats; thereby showing the behavioral symptoms associated with the model of depression. These results are consistent with the previous reports [2]. These behavioral deficits induced by olfactory bulbectomy were significantly reduced in a dose-dependent manner on daily treatment with PQ. Our findings are supported by results from various laboratories [34,35]. Recently, Xu et al. [34] demonstrated antidepressant-like effect of an intestinal metabolite of ginseng, 20(S)-protopanaxadiol in rat olfactory bulbectomy depression model. In another study by Kim et al. [35], treatment with ginsenoside Re decreased immobilization stress induced immobility period in forced swim test. Olfactory bulbectomy is also associated with deficits in daily intake of sucrose solution [36]. Such changes in behavior are proposed to reflect anhedonic features (loss of interest or pleasure) of the OBX syndrome, which is also a characteristic feature of endogenous depression [4]. In the present investigation OBX rats showed significant reduction in sucrose consumption when compared to sham group, indicating a state of anhedonia. Further, PQ significantly restored the decrease in sucrose preference; thereby showing its antidepressant like effects. These results are consistent with the previous study on this model [37]. Similar to many antidepressants, PQ showed excellent effect on reversing the behavior deficits associated with olfactory bulbectomy rat model.

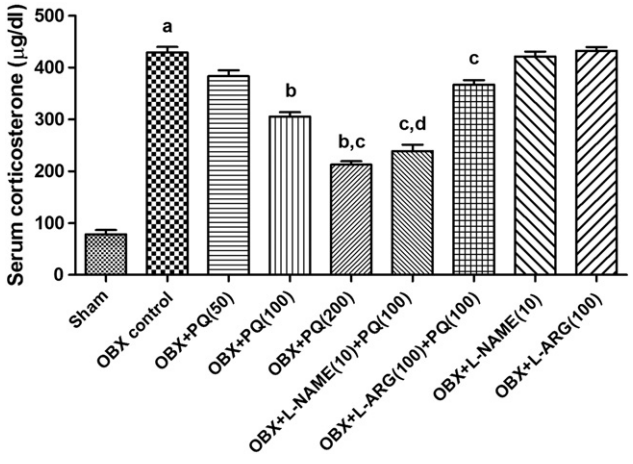

Fig. 4. Effect of *Panax quinquefolium* and its modulation by L-NAME and L-arginine on serum corticosterone levels. Values are expressed as mean ± SEM. For statistical significance, [a]$P < 0.05$ as compared to sham group; [b]$P < 0.05$ as compared to OBX control; [c]$P < 0.05$ as compared to OBX + PQ (100); [d]$P < 0.05$ as compared to OBX + L-NAME (one-way ANOVA followed by Tukey's test). OBX, olfactory bulbectomy; PQ, *P. quinquefolium*; L-ARG, L-arginine.

Ablation of olfactory bulbs in rat is known to activate hypothalamic–pituitary–adrenal (HPA) axis and increases the levels of blood corticosterone [38]. In our study, removal of olfactory bulbs resulted in a significant rise in the serum corticosterone levels indicating hyperactivity of the HPA axis [39]. It is well reported that increase in the corticosterone levels may lead to the behavioral alterations including depression-like behavior [40]. Recently, studies from Kumar et al. [41] showed that attenuation of corticosterone release through blockage of calcium

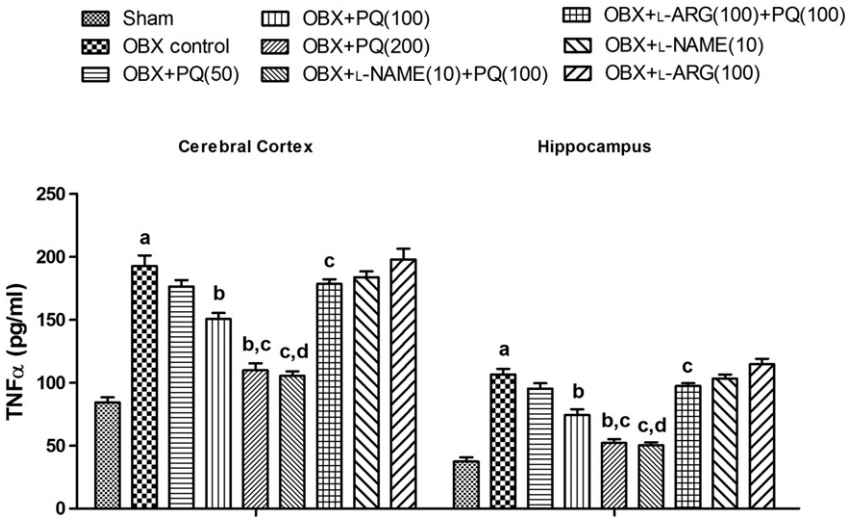

Fig. 5. Effect of *Panax quinquefolium* and its modulation by L-NAME and L-arginine on TNF-α activity. Values are expressed as mean ± SEM. For statistical significance, $^{a}P < 0.05$ as compared to sham group; $^{b}P < 0.05$ as compared to OBX control; $^{c}P < 0.05$ as compared to OBX + PQ (100); $^{d}P < 0.05$ as compared to OBX + L-NAME (one-way ANOVA followed by Tukey's test). OBX, olfactory bulbectomy; PQ, *P. quinquefolium*; L-ARG, L-arginine.

channels was beneficial in the restoration of behavioral alterations against immobilization stress. In the current study, treatment with PQ significantly restored the levels of serum corticosterone and the data is supported by findings from Xu et al. [34].

Oxidative stress is an emerging focus of research and is known to play an important role in the pathogenesis of depression [42]. Olfactory bulbectomy is reported to be associated with production of oxygen reactive species and saturation of antioxidant enzymes [43]. Clinical reports have also suggested a decrease in lipid peroxidation and levels of antioxidant enzymes in patients suffering from depression which later returned to normal on antidepressant treatment [44]. Similarly, in our study we found a significant increase in the levels of lipid peroxide and a marked reduction in the activity of reduced glutathione, catalase and superoxide dismutase in both cerebral cortex and hippocampal region of OBX rats. Our results are corroborated by findings of Tasset et al. [43] who found an increased oxidative and cell damage following olfactory bulbectomy in rats. Besides the enhanced level of oxygen reactive species, nitrite levels were also markedly increased in both the brain areas of OBX rats. Peroxynitrite is a harmful oxidant which results due to reaction between superoxide and nitric oxide, and causes oxidative modification of proteins leading to neuronal cell death [45]. This is further confirmed from clinical studies on depressed patients which showed elevated plasma nitrate levels [46]. Chronic treatment with PQ significantly reversed OBX induced alterations in the levels of lipid peroxide and antioxidants enzymes along with attenuation of enhanced nitrite levels in both the brain regions of OBX rats. These observations are supported by findings from Korivi et al. [47] who found that ginsenoside-Rg1 can provide significant protection against exhaustive exercise by mitigation of several oxidative and nitrosative stress markers.

Apart from increased oxidative and nitrosative stress, we also found a significant enhancement in the markers of inflammation (TNF-α) and apoptosis (caspase-3) in both the cerebral cortex and hippocampal regions of OBX rats, suggesting neuro-inflammation induced apoptosis.

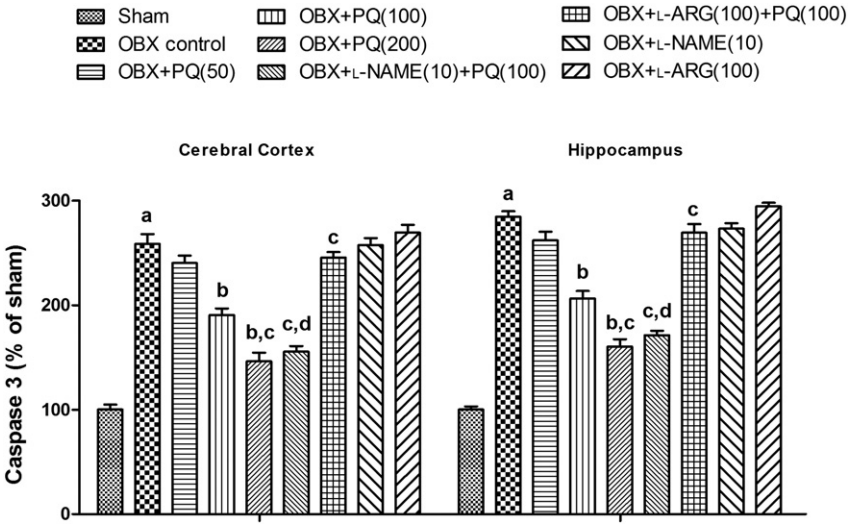

Fig. 6. Effect of *Panax quinquefolium* and its modulation by L-NAME and L-arginine on caspase-3 activity. Values are expressed as mean ± SEM. For statistical significance, $^{a}P < 0.05$ as compared to sham group; $^{b}P < 0.05$ as compared to OBX control; $^{c}P < 0.05$ as compared to OBX + PQ (100); $^{d}P < 0.05$ as compared to OBX + L-NAME (one-way ANOVA followed by Tukey's test). OBX, olfactory bulbectomy; PQ, *P. quinquefolium*; L-ARG, L-arginine.

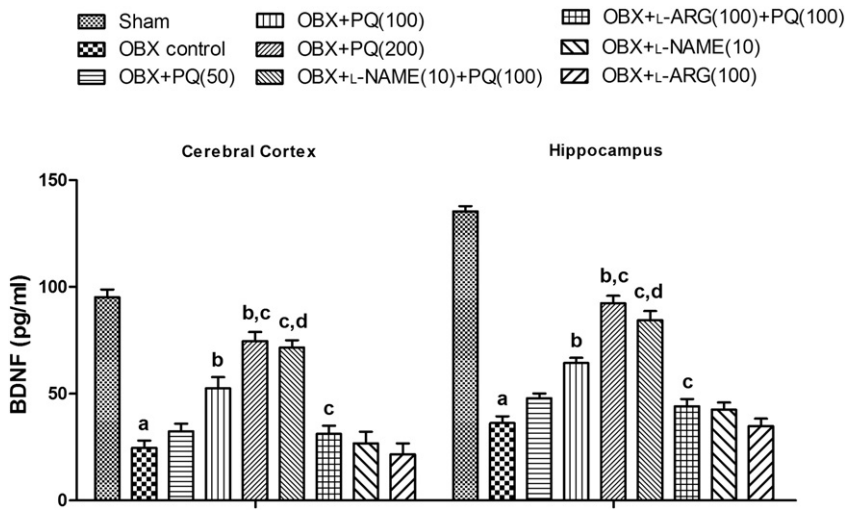

Fig. 7. Effect of *Panax quinquefolium* and its modulation by L-NAME and L-arginine on BDNF levels. Values are expressed as mean ± SEM. For statistical significance, $^{a}P < 0.05$ as compared to sham group; $^{b}P < 0.05$ as compared to OBX control; $^{c}P < 0.05$ as compared to OBX + PQ (100); $^{d}P < 0.05$ as compared to OBX + L-NAME (one-way ANOVA followed by Tukey's test). OBX, olfactory bulbectomy; PQ, *P. quinquefolium*; L-ARG, L-arginine.

Earlier, reports suggested that olfactory bulbectomy is linked with the generation of pro-inflammatory cytokines like TNF-α and IL-6 [48]. Studies from Hall and Macrides [49] found neuronal cell death (apoptosis) in different brain regions following olfactory bulbectomy. Our observations are also supported by findings from Song et al. [50], who found a decrease in synthesis of nerve growth factor due to neuroinflammatory response which caused neuronal death and lead to behavioral deficits in rat model of olfactory bulbectomy. Studies from Nesterova et al. [51] reported a significant degeneration in neurons of the temporal cortex and hippocampus following olfactory bulbectomy. In the current study, PQ treatment attenuated both TNF-α and caspase-3 levels in a dose-dependent manner which attributes to its potent anti-inflammatory properties. Our findings are supported by observations from Lee et al. [52] who found a significant inhibition of neuroinflammatory markers like TNF-α, IL-6, and IL-1β on treatment with ginsenoside Rb1 in mice. Recently, ginsenoside Rb1 has shown to exhibit neuroprotective effects on PC12 cells by preventing caspase-3 dependent apoptosis [53].

Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family and is involved in cell proliferation, differentiation and survival in the central nervous system [54]. The neurotrophin hypothesis of depression predicts that a reduction in BDNF is involved in the pathogenesis of depression [55]. In the present study, we observed a significant decrease in BDNF levels in OBX rat, thereby showing a reduced neurogenesis; a pathogenic mechanism in depression. Koo et al. [6] have reported a decreased brain neurogenesis following olfactory bulbectomy. However, treatment with PQ potentiated the neurogenesis process and increased BDNF levels in different regions of OBX rats. These results are consistent with the findings from Jiang et al. [56] who found activation of the BDNF signaling pathway and up-regulation of hippocampal neurogenesis on treatment with Ginsenoside Rg1. All these observations suggest that PQ could be useful in the management of behavioral, biochemical and molecular alterations associated with OBX-induced depression; however its mode of action is not yet clear. To further explore the specific mechanism of PQ, we combined its sub-effective dose with L-arginine (nitric oxide precursor) and L-NAME (nitric oxide synthase inhibitor) to elucidate whether modulation of NO mechanism is involved in the protective effects of PQ against depression like behavior associated with olfactory bulbectomy.

Nitric oxide (NO), synthesized from L-arginine by nitric oxide synthase (NOS) has been suggested to play an important role in the pathogenesis of major depression [57]. NO is as an endogenous activator of guanylyl cyclase and increases the levels of cyclic guanosine monophosphate (cGMP) [58]. Modulation of cGMP levels by nitric oxide is known to produce depression like state [59]. Reports from Harkin et al. [19] suggest that pretreatment with L-arginine attenuated the antidepressant effects of imipramine while pretreatment with N^{G}-nitro-L-arginine (an NOS inhibitor) augmented the behavioral effect of imipramine in the forced swim test. These results show that inhibition of nitric oxide synthase (NOS) is useful in promoting the efficacy of several antidepressants [19]. The levels of cGMP are controlled not only by guanylyl cyclase, but also by phosphodiesterase 5 (PDE5), which catalyzes the hydrolysis of the cGMP and cAMP to yield GMP and AMP, respectively [60]. Thus the time and extent of cGMP bioavailability are confirmed by the activity of PDE5 which augments the biological effects mediated by NO. Studies from Cashen et al. [61] suggested that nNOS isoform is crucial for the action of PDE5 inhibitors since treatment with sildenafil failed to correct the erectile function in mice lacking the nNOS. Thus, NOS inhibitors are defined as a novel class of therapeutics for the treatment of major depressive disorders by reducing cGMP and NO levels [62]. Similarly, in our current study we witnessed that pretreatment of L-arginine (a substrate of NOS) with sub-effective dose of PQ reversed the protective effect of PQ. Further, L-NAME (a non selective inhibitor of NOS) pretreatment potentiated the protective effect of PQ, suggesting the involvement of nitric oxide mechanism in its protective effects. However, the entire mechanism of nitric oxide cascade in the beneficial effect of PQ is still far from elucidation. From the current study it seems that modified nitrergic tone may have played a functional role in the protective effect of PQ and that in future nitric oxide modulators could prove to be a potent combination mechanism to enhance the therapeutic efficacy of herbal drugs like PQ.

5. Conclusion

In conclusion, the findings of the present study raised the possibility that: (i) Olfactory bulbectomy in rats caused behavioral, biochemical and molecular alterations along with oxidative–nitrosative damage which lead to depression like behavior; (ii) PQ has shown the protective effects against OBX induced alterations; and (iii) protective effect of PQ could be mediated through modulation of nitric oxide pathway. Together we have tried to prove that co-administration of PQ with NOS

inhibitors may provide a useful natural adjuvant to establish its clinical effectiveness in patients suffering from depression.

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