Neuroprotective and anti-inflammatory effect of *Hedyotis corymbosa* extract on chronic stress-induced depression model of rat-A *in vivo* and *ex vivo* study

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Abstract

Background: Depression is a well-known mood disorder characterized by persistently low mood and loss of interest and a feeling of sadness. Plants and plant-derived agents have recently attracted the interest of researchers for their therapeutic effects against several illnesses, including mental disorders, and several herbal plants and herbal formulations are useful against experimental depression. In this study, the neuroprotective effects of *Hedyotis corymbosa* extract were investigated in rats induced with chronic mild stress.

Methods: Animals were designated into the following groups: control, 0, 150, and 300 mg of extracts. The dose was given for 30 consecutive days via the oral route. Sucrose preference analysis, forced swim, and open field tests were performed, and serum cortisol and monoamine levels in brain tissue were determined. Expression of brain-derived neurotrophic factor (BDNF) was also examined.

Results: Supplementation with extract increased the sucrose preference ratio, locomotor activity, and monoamines and decreased serum cortisol levels. The protein and mRNA expression of BDNF in the brain tissue was decreased by 62.58% and 73% in control rats. However, supplementation with extracts significantly increased BDNF mRNA expression (by 107% and 229.6% in groups 150 mg and 300 mg, respectively). Similarly, the protein expression of BDNF increased by 82.3% and 141.2% in groups 150 mg and 300 mg, respectively.

Conclusion: In summary, experimental results suggest that supplementation with extracts of *Hedyotis corymbosa* may be effective against depression.

Background

Depression is a state of aversion to activity and low mood. Depression affects behavior, thoughts, feelings, sense of well-being, and tendencies. It is a chronic, recurring, and severe life-threatening illness that affects people globally (Krishnan and Nestler, 2011; Li et al. 2018; Schuch et al. 2018). Depression can also be a side effect of medical treatments and physical exercise or a symptom of dysthymia (Pillemer et al. 2010). Monoamine oxidase A, tricyclic antidepressants, and specific serotonin and noradrenaline reuptake inhibitors are medically available drugs for depression treatment (Freitas et al. 2013). However, sleep disorder, weight gain, cardiac toxicity, sexual dysfunction, and hypokinesia are major adverse effects of these drugs (Ashok Kumar et al. 2014). Therefore, novel agents for the treatment of depression without any major side effects are needed.

Plants and plant-derived agents have recently attracted the interest of researchers based on their therapeutic effects against several illnesses, including mental disorders. Jawaid *et al*. (2011) reported the antidepressant activity of plants derived compounds against animal models. Within the genus, *Hedyotis* (family: Rubiaceae), found in the Pacific region and Asia. Researchers have reported that medicinal use of *H. corymbosa*, *H. diffusa*, and *H. biflora* (Inge et al. 2009). The plant
Rubiaceae *Hedyotis corymbosa* (L.) Lam. is reportedly effective against several illnesses, including

depression (Sivapraksam et al. 2014). Anil et al. (2018) have reported the antidepressant potential of *Hedyotis corymbosa* extract against olfactory bulbectomy rats. *H. corymbosa* contains saponins, phenols, carbohydrates, tannins, proteins, terpenoids, and steroids (Sivapraksam et al. 2014), and also reportedly exerts various biological activities, such as anticancer, antimalarial, antiulcer, analgesic, antimicrobial, and hepatoprotective effects (Sivapraksam et al. 2014). Brain-derived neurotrophic factor (BDNF) is neurotrophic factor which is regulated by neuronal activity (Lee and Kim 2010). Reduced brain BDNF level is associated with depression which is the major neurotrophic hypothesis of depression. Dumans (2002) have reported that the antidepressant treatments ameliorate depression through increased BDNF level. Thus, the present study evaluated the effect of extracts of *Hedyotis corymbosa* on chronic mild stress-induced depression.

Material And Methods

Animals

Male albino rats (weight: 180-210 g) were purchased from the Animal house of Peking University Shenzhen Hospital, China. The rats were maintained in rat polypropylene cages with standard atmospheric conditions of 12 h of light and dark periods. Temperature of 25 ± 0.5°C and relative humidity of 60 ± 5% was maintained.

Quantitative analysis of saponin from *H. corymbosa* extract

H. corymbosa plants extract was prepared according to Anil et al. (2018). Qualitative analysis of *H. corymbosa* extract was carried out using high-performance thin-layer chromatograph (HPTLC) as previously described (Jiang and Tu 2009). A methanol extract of *H. corymbosa* and gallic acid were dissolved in methanol for analysis. The sample solution was applied on prewashed and activated pre-coated silica gel aluminum HPTLC plate $60F_{254}$in the form of band of 6 mm. Then, HPTLC plate was developed for 8 cm with 20 ml mobile phase (toluene (4.7: ethyl acetate (3): Formic acid (0.3). Linear ascending developments were performed. The chamber saturation time for the solvent system was 20 min at 25 ± 2°C, and relative humidity of 62% ± 5%. Camag TLC scanner III was used for the densitometric scanning (Camag, Muttenz, Switzerland) at 278 nm.

Induction of chronic mild stress

Chronic mild stress was induced in rats according to a previously described procedure with slight modifications (Ducottet et al. 2003). Briefly, animals were trained to consume 1% sucrose solution before applying stress. Chronic mild stress protocols contains several unpredictable mild stressors such as one period of tilted cage (3 h), one period of shaking (15 minutes), one period of exposure to empty bottle (1 h), one period of continuous light (36 h), one period of paired caging (2 h), one period of wet cage (21 h) and one period of water and food deprivation. The procedure was repeated for 28 days.

Groups and treatments

Rats were designated into four groups: control, 0 mg, 150 mg, and 300 mg of extracts. The dose was given for 30 consecutive days via the oral route. Each group contained six rats.

Sucrose preference analysis

The sucrose preference analysis was carried out according to Willner et al. (1987). Briefly, the two bottles of sucrose solution (1%) were placed on the rat cage separately. Then, free access was provided to the rats to drink water from these cages for one day. Then, one bottle was continued with the same sucrose solution and another bottle was filled with water for the next 24 h. Bottles position was changed to avoid the influence of bottle position. Then, all the animals were deprived of water for another 23 h, and sucrose preference examination was performed for each rat. Then, the amount of water and sucrose solution consumed was recorded and calculated.

Behavioral test

Forced swim and open field tests were carried out as previously described (Jun et al. 2016). All the rats were placed in the center of open field [square chamber (80 cm), high walls (40 cm) and light (80 lux)] for 180 seconds in a silent room following rat weighed. Times of rearing and number of crossing squares were recorded.

Determination of serum cortisol

The serum cortisol level was estimated using an enzyme-linked immunosorbent assay (Anil et al. 2018). At the end of the treatment, rats were dissected and blood was collected and processed with the serum for the determination of cortisol level.

Determination of monoamine

The level of monoamines (i.e., 5-hydroxytryptamine [5-HT], noradrenaline, and 5-hydroxyindoleacetic acid [5-HIAA]) in brain tissue homogenate was determined as previously described (Schlumpf et al. 1974). Briefly, brain tissue was surgically removed and homogenized. The clear supernatant was collected, and monoamine levels were determined by using a radioimmunoassay kit (Abcam, UK).

Analysis of BDNF and Muscle related markers by Western Blot

Proteins were extracted from 100 mg of brain tissue homogenate samples using radioimmunoprecipitation assay (RIPA) buffer to determine protein expression levels of BDNF and myogenin in experimental groups. Protein concentrations were determined using a BIO-RAD protein assay kit (BIO-RAD). Extract samples containing 50 µg of protein were solubilized in *Laemmli buffer,* separated by 12% acrylamide gel, and then transferred to Hybond-P PVDF membranes (GE Healthcare Inc., Amersham, UK) for 60 min at 200 mA. These PVDF membranes were blocked with 5% skimmed milk powder in 0.5 M of Tris-buffered saline (pH 7.4) with 0.05% Tween 20 (TBST) at room temperature for 2 h. Western immunoblotting with BDNF primary antibodies (1:2500 dilution) was performed at 4°C

overnight. After washing three times with TBST, these membranes were incubated with HRP-conjugated secondary antibodies (1:5000 dilutions) at room temperature for 60 min and then washed three times with TBST (10 min each wash). Protein bands were visualized using a Chemiluminescent assay kit from Thermo Scientific for 1–5 min. Bands were imaged with an iBright™ CL1000 Imaging System (Invitrogen in Thermo Fisher Scientific) and quantified using Image J Software. The relative density of the band was normalized to that of β-actin as an internal control.

RT-PCR

Total RNA was extracted from the brain tissue homogenate using a TriZol reagent and the prepared RNA's purity was checked with a IDrop plate (Thermo Fisher Scientific, USA). The cDNA was synthesized with the iScriptTM cDNA Synthesis Kit from BIO-RAD, using 2 µg of the total RNA. Reverse transcription polymerase chain reaction assays were performed with the CFX96TM Real-Time PCR detection system (BIO-RAD). The cDNA was amplified for each gene and the reactions carried out according to the manufacturer's instructions (BIO-RAD). Real-time PCR was performed using a cDNA equivalent from each sample's total RNA in the amount of 10 ng with primers specific for BDNF and a housekeeping gene, GAPDH (Table 1). The statistical analysis of the real-time PCR results was calculated by using the DCt (cycle threshold) value (Ct_{gene} of interest-$Ct_{reporter\ gene}$). Relative gene expressions were obtained by DDCt methods (DCt_{sample}- $DCt_{calibrator}$). The conversion between DDCt and relative gene-expression levels was as follows: Fold induction = 2^{-DDCt}, where 2^{-DDCt} is the relative gene expression [16].

Immunohistochemistry

The brain hippocampal region was dissected and sectioned immediately fixed with 10% neutral buffered formalin (NBF), and processed in an auto processor (Excelsior ES, Thermo Scientific,Waltham, MA, USA). After embedding in paraffin, 5-µm sections were made and subjected to BDNF was performed according to a previously described method (Serra et al. 2017). Digital images were obtained using a Leica DM2500 microscope (Leica Microsystems, Wetzlar, Germany) at fixed 100x (200x) magnification.

Statistical analysis

Data are presented as means ± standard deviation (SD) from six determinations from each group. All values were compared and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test post hoc following ANOVAs. A *P*-value < 0.05 was considered statistically significant.

Results

The study evaluated the effect of extracts of *H. corymbosa* on chronic mild stress-induced depression. The quantitative analysis *H. corymbosa* extract revealed the presence of saponins, flavonoids, carbohydrates, proteins, steroids, tannins, and phenolic compounds (Figure 1, Table 1). Figure 2 shows

the sucrose preference ratio of control and treated rats. The sucrose preference ratio was substantially reduced by 50.5% in 0 mg rats compared to control rats. However, extracts increased the sucrose preference ratio to 74.4% and 93.6% in groups 150 mg and 300 mg, respectively (Figure 2, $P < 0.034$).

Behavioral parameters, such as rearing, crossing, and immobility time, were determined in control and treated rats. Rearing capacity was substantially reduced by 69.6% in 0 mg rats. However, extracts treatment increased rearing by 83.5% and 187% in groups 150 mg and 300 mg, respectively (Figure 3, $P < 0.041$). Crossing counts were substantially reduced by 77.3% in 0 mg rats. Extract supplementation increased crossing counts by 166% and 297.2% in groups 150 mg and 300 mg, respectively (Figure 4, $P < 0.025$). Immobility time was substantially increased by 125.9% in control rats. However, extracts treatment reduced immobility time by 32.8% and 47.5% in groups 150 mg and 300 mg, respectively (Figure 5, $P < 0.05$).

The serum cortisol level was substantially increased by 147.3% in 0 mg rats. However, supplementation with extracts reduced the cortisol level by 27.2% and 51.4% in groups 150 mg and 300 mg, respectively (Figure 6, $P < 0.044$). The level of monoamines (e.g., 5-HT, noradrenaline, and 5-HIAA) was substantially reduced in brain tissue homogenate. However, supplementation with extracts significantly increased these monoamine levels to near-normal levels (Table 3, $P < 0.032$). In brain tissue, the protein and mRNA expression of BDNF was substantially reduced by 62.58% and 73% in 0 mg rats respectively. However, supplementation with extracts significantly increased protein and mRNA expression BDNF by 107% and 229.6% in groups 150 mg and 300 mg, respectively (Figure 7, $P < 0.041$). Similarly, BDNF protein expression was increased by 82.3% and 141.2% in groups 150 mg and 300 mg, respectively (Figure 8, $P < 0.043$).

Discussion

In this study, we have investigated the biochemical, behavioral and molecular approaches to understand the effect of extracts on chronic mild stress induced depression model of rats. We observed that the extracts treatment exhibited protective effect against depression and prevented the hormone dysregulation. Depression induced rats showed reduced sucrose preference, rearing, crossing and increased immobility time, which agrees with findings of other researchers (Belovicova et al. 2017; Liu et al. 2013). These results serve as evidence for the successfulness in induction of depression. Saponin treatment in depression induced rats showed increased sucrose preference and locomotor activity, which indicates the antidepressant activity of extracts. Wang et al. (2013) have demonstrated the protective effect saponins on sucrose preference and behavioral parameters such as rearing, crossing and increased immobility time in chronic mild stress depression rats.

Neuroendocrine disorder is closely associated with stress induced depression. Several researchers have reported that the stress induced depression increased the cortisol level, which leads to accumulation of cortisol in the hippocampus region and neuronal damage in the hippocampus (Anacker et al. 2013; Dienes et al. 2013). Researchers have reported that an increased level of serum cortisol leads to severe

behavioral alterations, such as depression (Busquet et al. 2010). In this study, cortisol level was drastically increased in depression induced rats, and return to the near normal range following extracts treatment, which confirms the protective effect against depression and neuroendocrine disorders. Wang et al. (2013) have reported the inhibitory potential of saponins against increased serum cortisol in chronic mild stress depression rats.

The absolute or relative deficiency of monoamines such as 5-HT, noradrenaline, and 5-HIAA could play major role in depression pathogenesis (Lopez-Munoz and Alamo 2009). In this study, 5-HT, noradrenaline, and 5-HIAA levels were drastically reduced in depression induced rat model, and return to the near normal range following extracts treatment, which confirms the protective effect against depression. Liang et al. (2016) have reported the treatment of saponins increased the levels of monoamines in rat brain. Researchers have reported the deficiency of neurotrophic factor plays major role in pathogenesis of depression (Wu et al. 2017). Reduced level of BDNF has been reported in hippocampus under depression and stress condition (Elfving et al. 2010). BDNF is known to regulate the expression of monoamines, neurogenesis, apoptosis which are associated with chronic stress-induced depression (Gururajan et al. 2014).

Conclusion

In this study, BDNF expression was drastically reduced in depression induced rat model, and return to the near normal range following extracts treatment, which confirms the protective effect against depression. Taking all these data together, it is suggested that the extract is a good therapeutic agent against chronic stress induced depression model of rats.

Abbreviations

BDNF:Brain-derived neurotrophic factor; HPLC: high-performance thin-layer chromatograph;5-HIAA:5-hydroxytryptamine [5-HT], noradrenaline, and 5-hydroxyindoleacetic acid;RIPA: radioimmunoprecipitation assay; TBST: Tween 20; NBF: neutral buffered formalin.

Declarations

Compliance with ethical standards

Ethics approval and consent to participate

All animal experiments were approved by the ethical committee of Department of Ultrasound, Peking University Shenzhen Hospital, Shenzhen, China.

Consent for publication: Not applicable

Availability of data and materials: Corresponding author could provide the all experimental data on valid request

Competing interests: Authors declare that they have no conflict of interest

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Authors' contributions: TZ, JZ, YS, JP and LL conducted experiments and collected data. HX, YH, KC, XZ and YC carried out data interpretation, review of literature and manuscript drafting. All authors read and approved the final manuscript.

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Tables

Table 1: Qualitative phytochemical analysis of *Hedyotis* corymbosa leaf methanol extracts

S. No	Compounds
1	Saponins
2	Carbohydrates
3	Flavonoids
4	Protein
5	Steroids
6	Tannins and Phenolic compounds

Table 2: List of primers used in RT-PCR reaction for the amplification of brain-derived neurotrophic factor

S.NO	Markers	Forward primer	Reverse primer
1	GAPDH	5′-TCCCTCAAGATTGTCAGCAA-3′	5′-AGATCCACAACGGATACATT-3′
2	BDNF	5′-TGCAGGGGCATAGACAAAAGG-3′	5′-CTTATGAATCGCCAGCCAATTCTC-3′

Table 3: Effect of Hedyotis corymbosa extract on monoamine levels in on chronic mild stress-induced rats

Monoamines	Control	0 mg	150 mg	300 mg
5-HT (ng/g)	291.5 ± 13.6	54.2 ± 3.1*	147.4 ± 8.5#	266.6 ± 11.5#
Noradrenaline (ng/g)	137.7 ± 7.5	34.3 ± 2.5*	77.9 ± 4.2#	121.5 ± 8.2#
5-HIAA (ng/g)	274.3 ± 12.8	63.5 ± 3.3*	144.3 ± 8.4#	245.1 ± 10.8#

*P<0.05 & #P<0.05

Figures

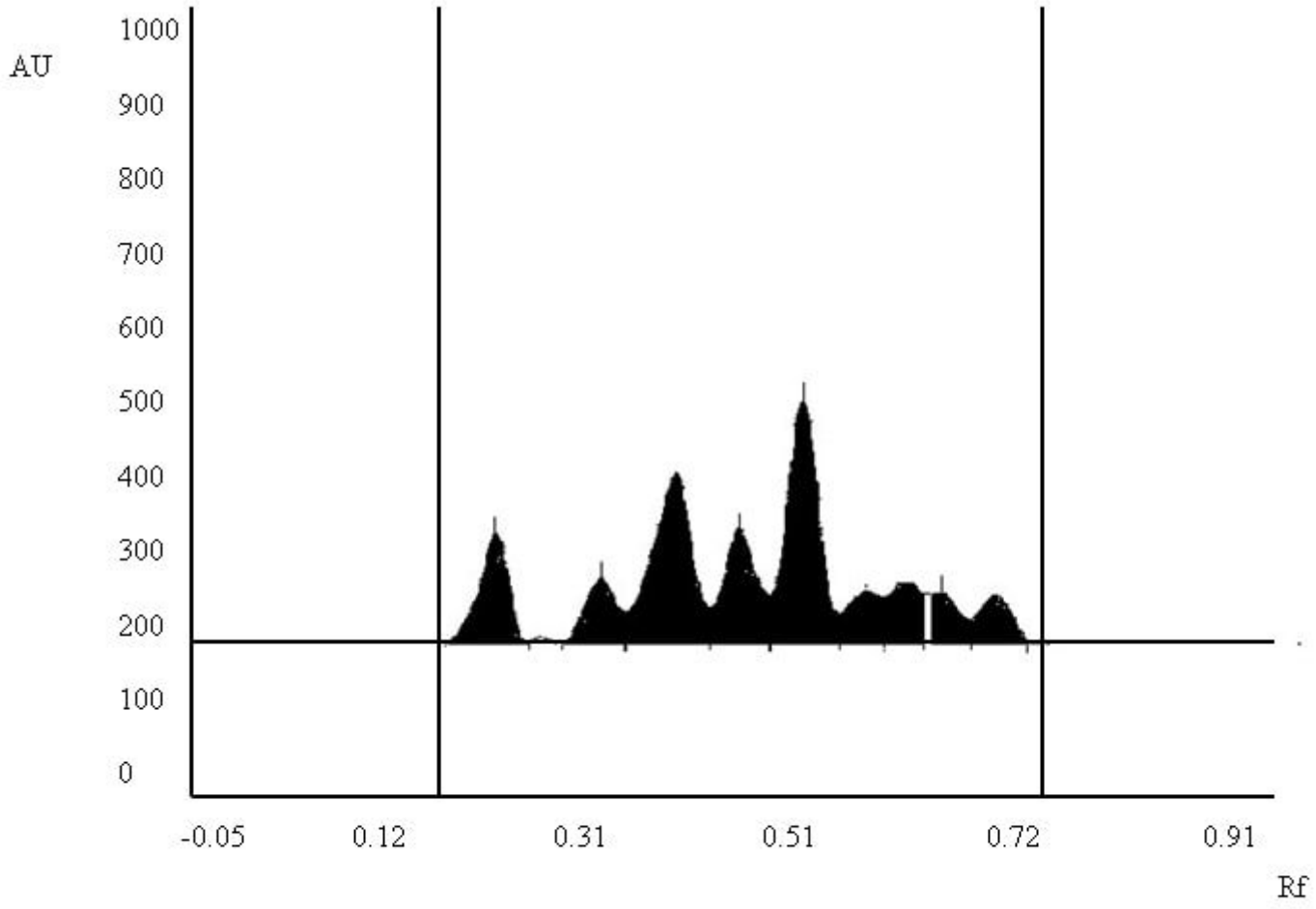


Figure 1

Figure 1

High-performance thin layer chromatograph densitogram of methanolic extract of Hedyotis corymbosa standardized scanned at 278 nm.

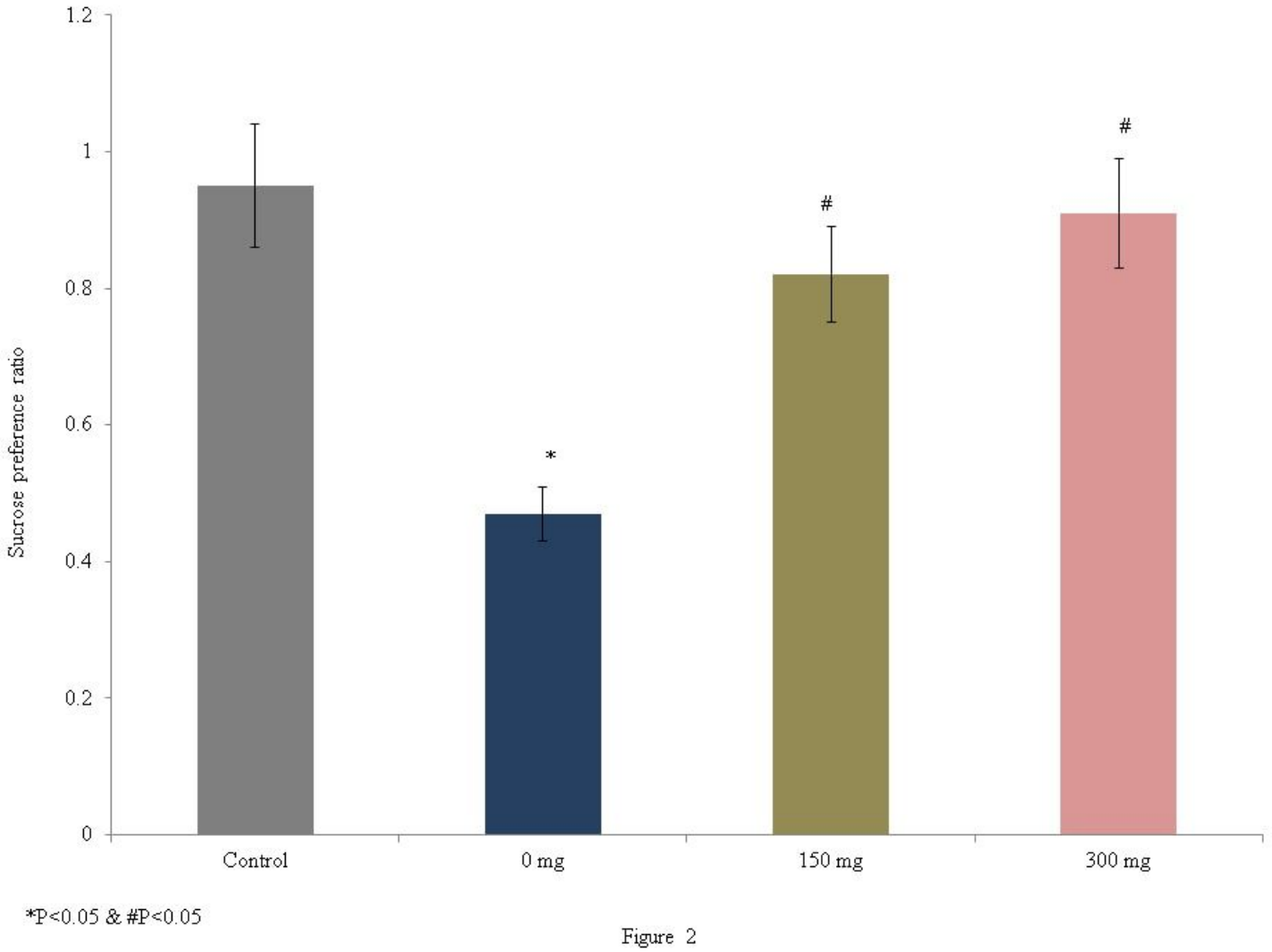


Figure 2

Protective effect of extracts on sucrose preference test in an experimental model of chronic mild stress-induced depression.

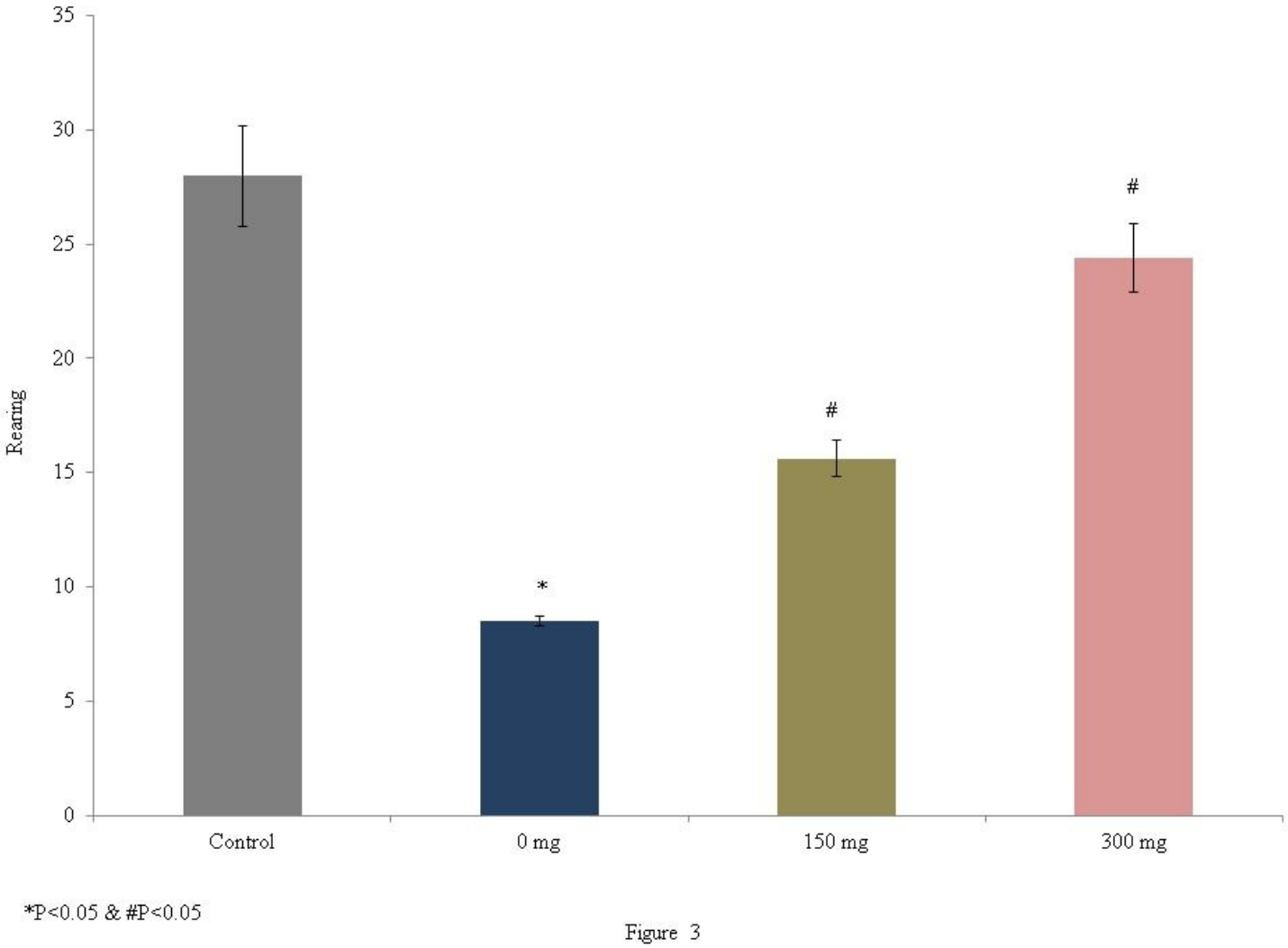


Figure 3

Protective effect of extracts on the rearing behavioral test in an experimental model of chronic mild stress-induced depression.

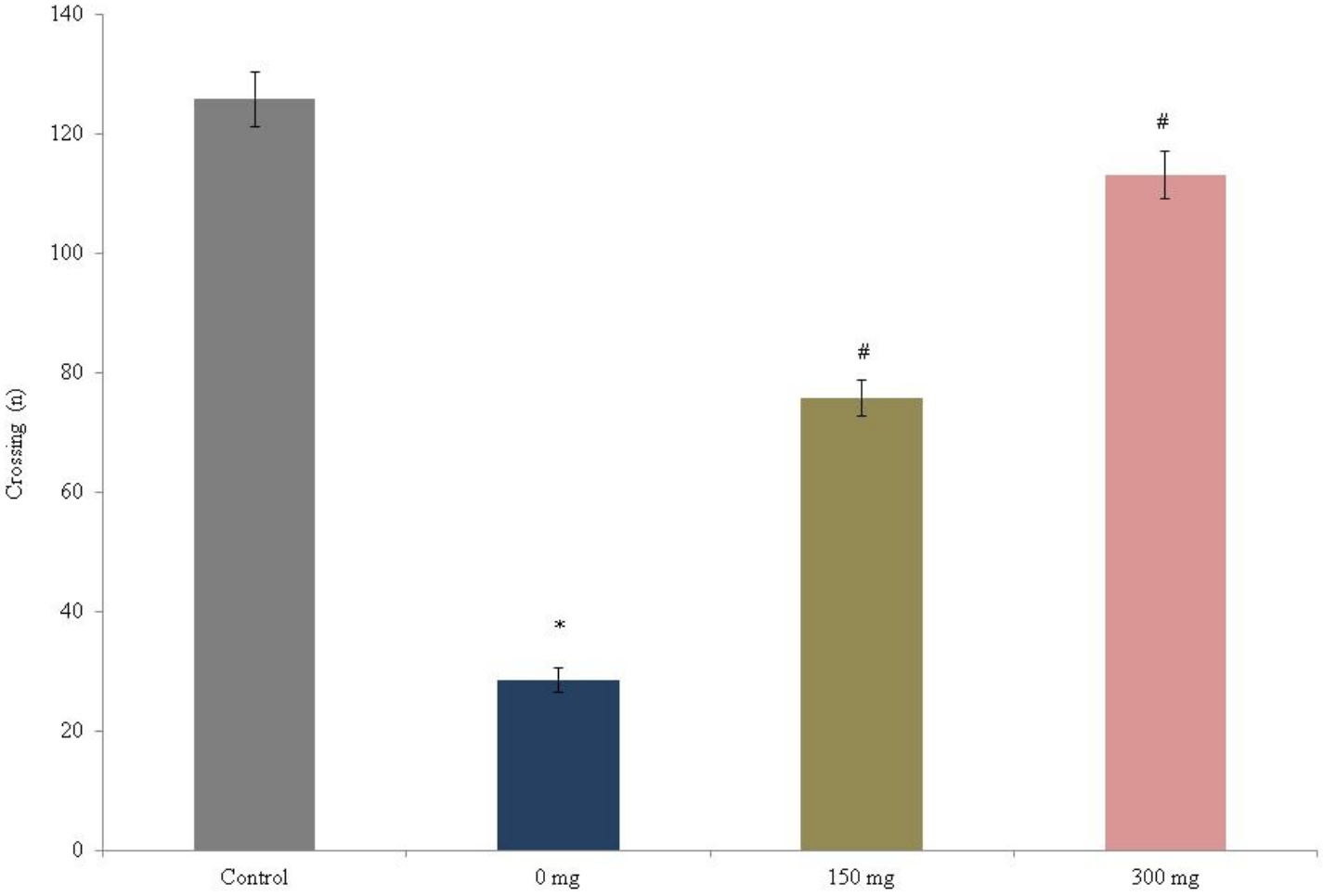


Figure 4

Protective effect of extracts on the crossing behavioral test in an experimental model of chronic mild stress-induced depression.

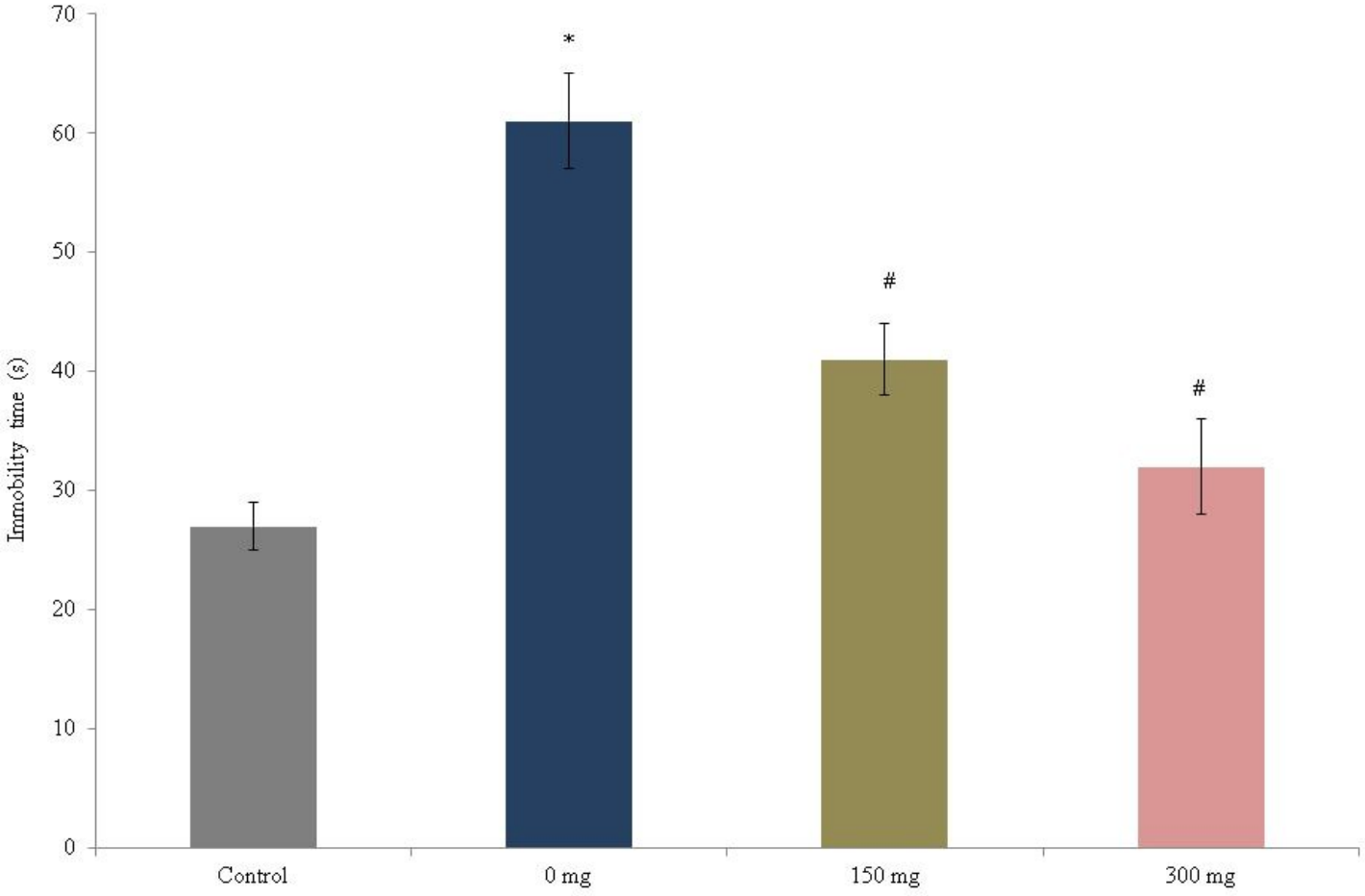


Figure 5

Protective effect of extracts on immobility time in an experimental model of chronic mild stress-induced depression.

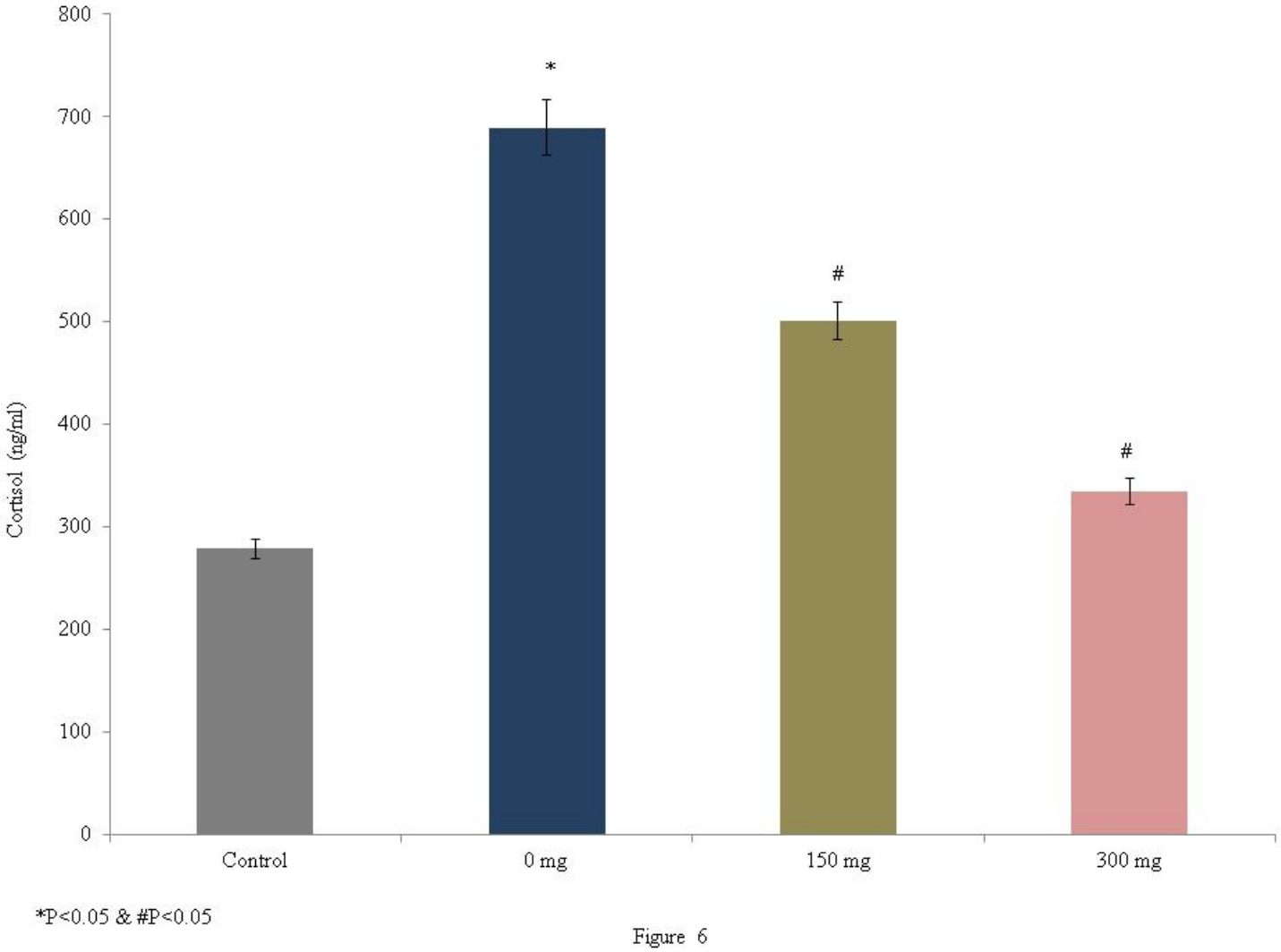


Figure 6

Protective effect of extracts on serum cortisol in an experimental model of chronic mild stress-induced depression.

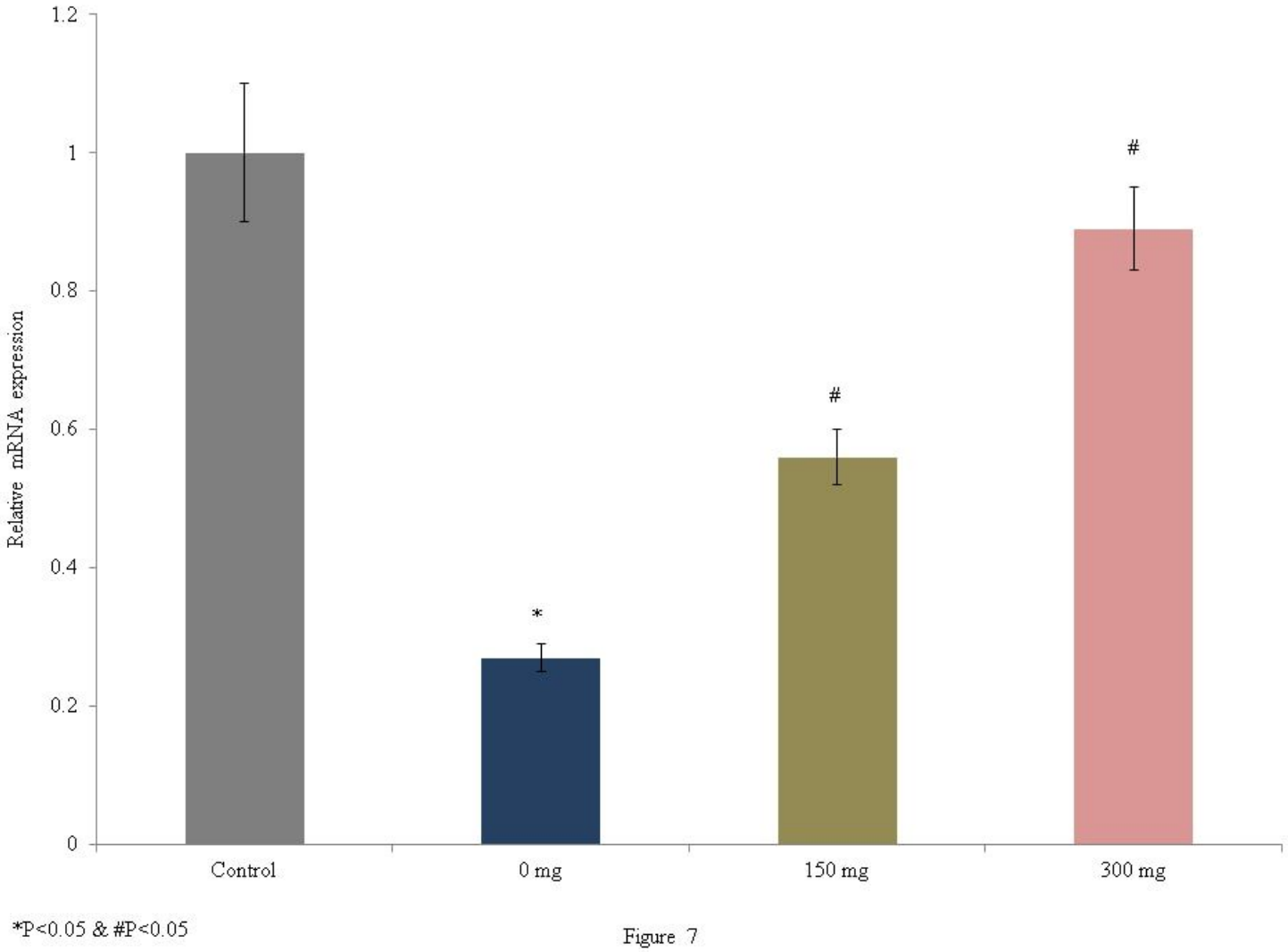


Figure 7

Protective effect of extracts on the mRNA expression of BDNF in an experimental model of chronic mild stress-induced depression.

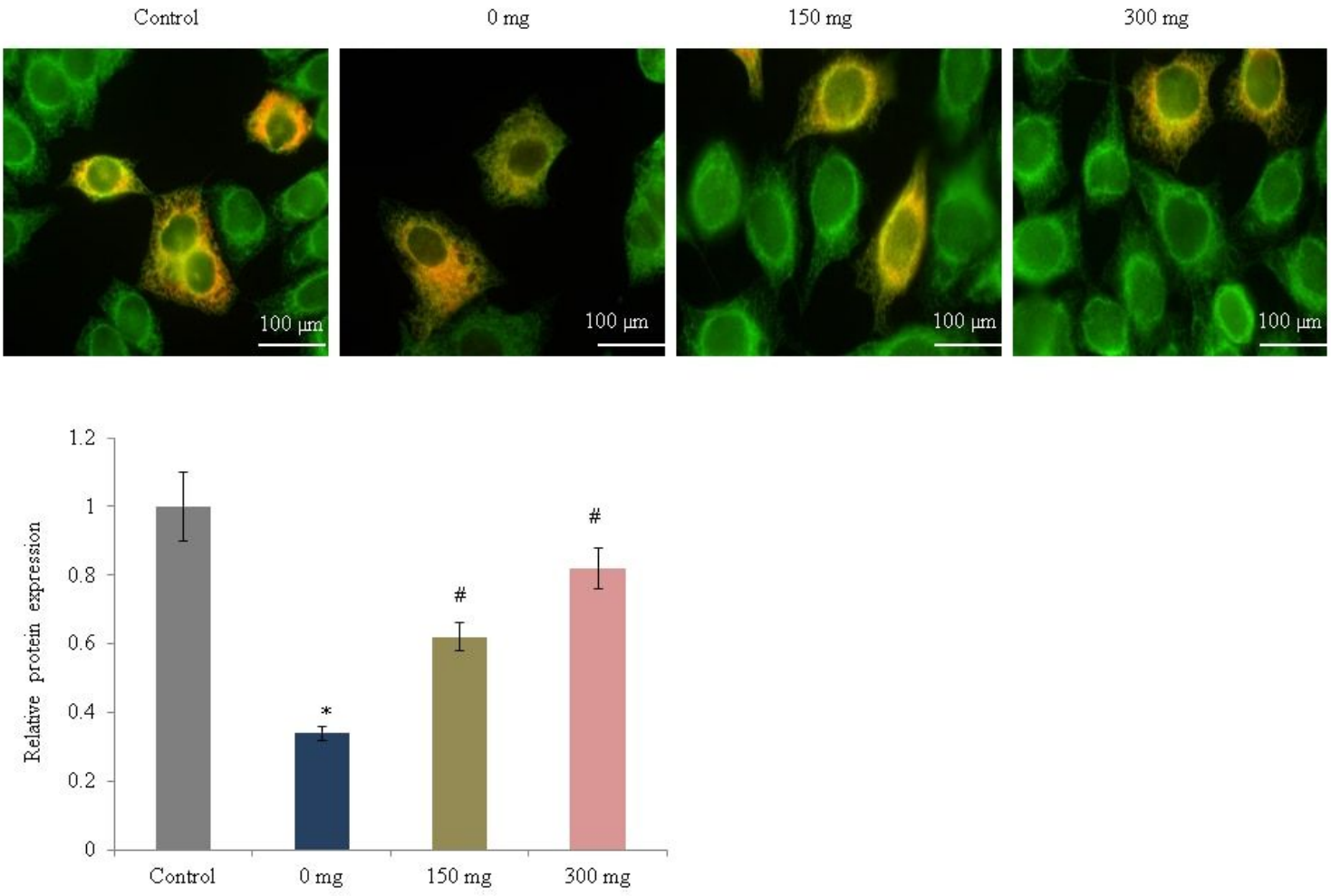
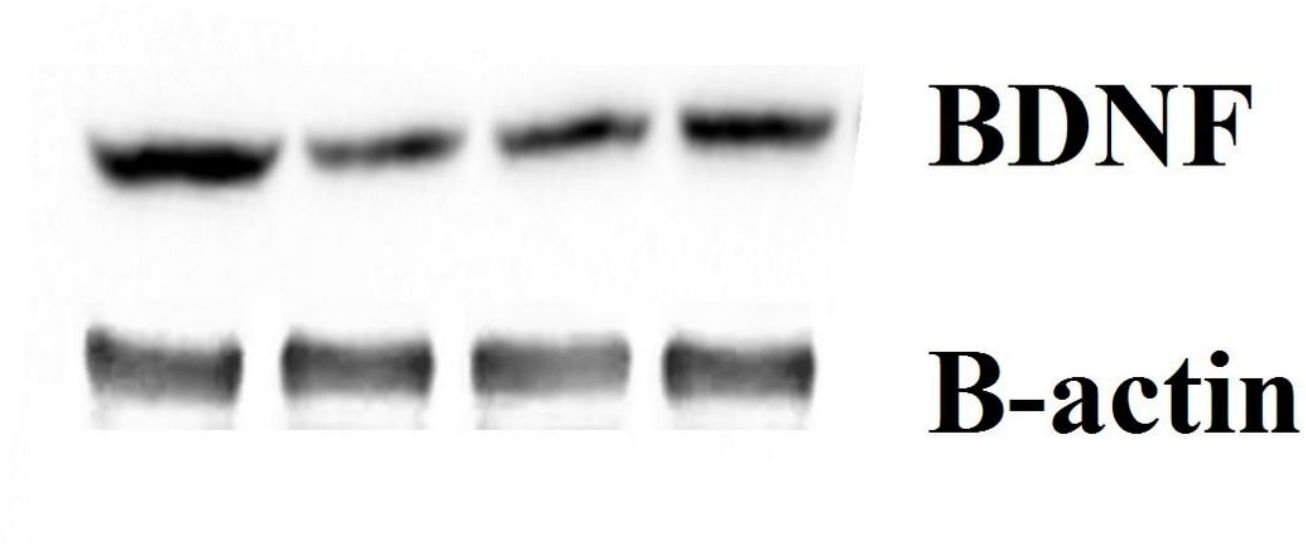


Figure 8

Protective effect of extracts on the mRNA expression of BDNF in an experimental model of chronic mild stress-induced depression.



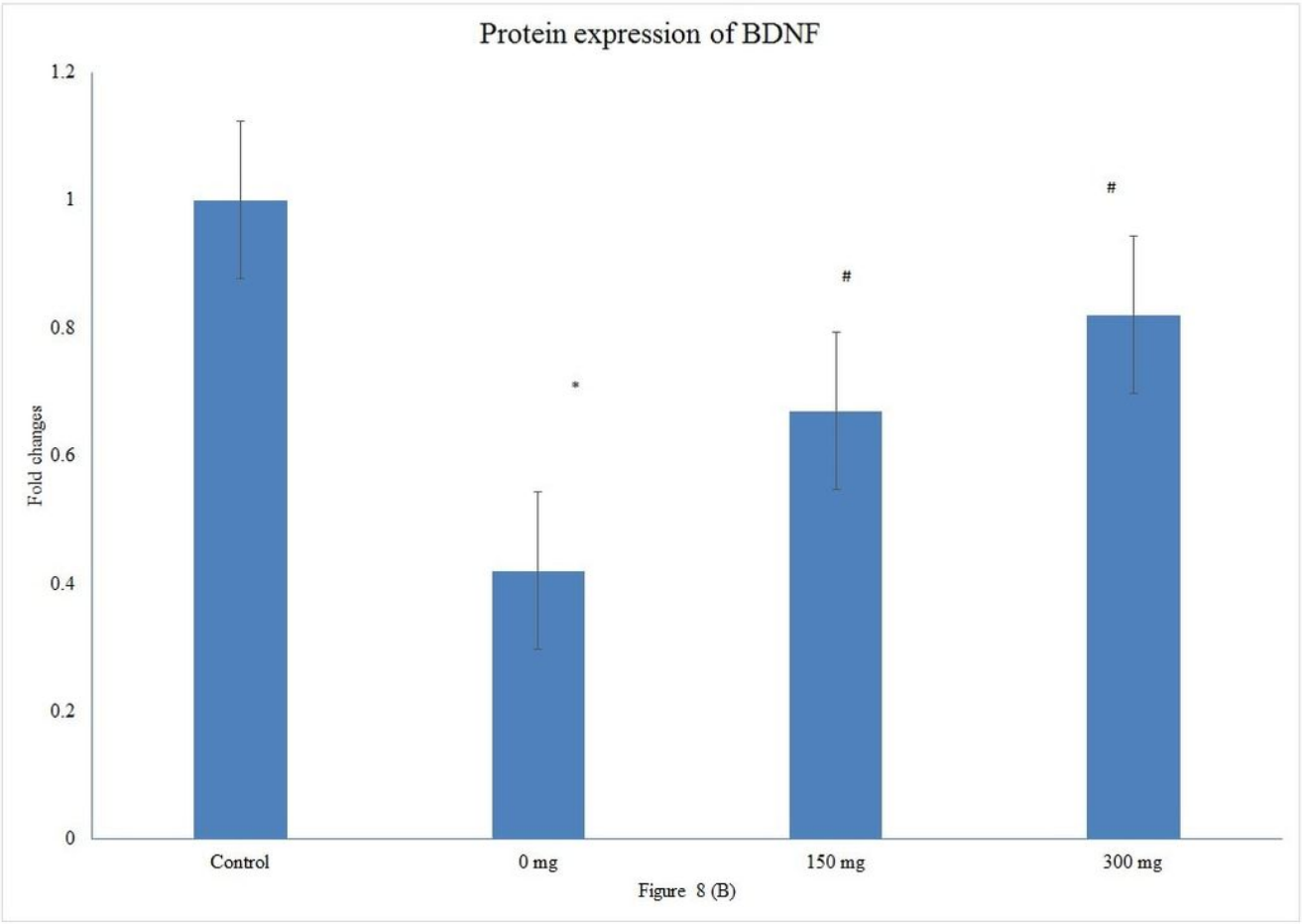


Figure 9

Protective effect of extracts on the protein expression of BDNF in an experimental model of chronic mild stress-induced depression.