Original Article

Improvement of *Petroselinum crispum* on Morphine Toxicity in Prefrontal Cortex in Rats

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Abstract

Background: *Petroselinum crispum* (*P. Crispum*) is an associate of the umbelliferae family with several therapeutic attributes. Morphine is known as a major risk factor in the development of functional disorder of several organs. **Objective:** This study was designed to evaluate the effects of *P. Crispum* extract against morphine-induced damage to the brain prefrontal cortex (PC) of rats. **Materials and Methods:** In this experimental study, 64 Wistar male rats were randomly assigned to 8 groups: Sham group, Morphine group, *P. Crispum* groups (50, 100, and 150 mg/kg), and Morphine + *P. Crispum* groups. Daily intraperitoneal treatment applied for 20 days. Ferric reducing/antioxidant power method was hired to determine the total antioxidant capacity (TAC). The number of dendritic spines was investigated by Golgi staining technique. Cresyl violet staining method was used to determine the number of neurons in the PC region. Furthermore, Griess technique was used to determine the level of serum nitrite oxide. **Results:** Morphine administration increased nitrite oxide levels and decreased TAC, density of neuronal dendritic spines and neurons compared to the sham group significantly ($P < 0.05$). In whole doses of the *P. Crispum* and Morphine + *P. Crispum* groups, the number of neurons and neuronal dendritic spines increased significantly while nitrite oxide level and TAC decreased compared to the morphine group ($P < 0.05$). **Conclusion:** It seems that the administration of *P. Crispum* extract protects the animals against oxidative stress and nitrite oxide, also improves some PC parameters including the number of neurons, and dendritic spines because of the morphine application.

Keywords: *Morphine, Petroselinum crispum, prefrontal cortex*

Introduction

Opioids generate free radicals which lead to apoptosis in some cells. Morphine is an opioid analgesic drug, and the main psychoactive chemical exists in opium.[1] Morphine is an addictive agent causes physiological dependence.[2] This chemical spreads rapidly into the brain within 10–20 s and binds to the nicotinic acetylcholine receptors (nAChRs).[3] Morphine rapidly transmits through the blood–brain barrier and stimulates the mesolimbic dopamine system. This substance can regulate the brain neurotransmitters, including catecholamine and serotonin A.[4] Dopaminergic structure shows a vigorous role in memory control and mainly reward behaviors.[5] The receptors of morphine acetylcholine are found in the neural pathways of accumbens nucleus and ventral tegmental. Stimulation of these receptors increases the dopamine release in accumbens nucleus, and prefrontal cortex (PC) that finally induces the feeling of joyfulness[6] yet the morphine can induce oxidative stress in the brain.[7] Pathologic changes associated with neuronal apoptosis have been reported due to the use of morphine.[8] Furthermore, morphine can induce increased oxidative stress and neuronal apoptosis, DNA destruction, production of reactive oxygen species.[9] This compound seems to activate some areas of the brain that play a crucial role in drug addiction and learning process. Among the brain areas that greatly affected by morphine, the mesocorticolimbic and brain's PC regions exist.[10] The PC region shows a key role in personal character and state of mind.[11] The role of PC region in regulatory performance, judgment, and behavior leads to choice of this region to get the survey.[12] Vegetables show a potent effect in reducing hunger in the eating habits of many people. They are rich in nutrients such as vitamins, minerals, proteins, and fibers.[13] *Petroselinum*

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crispum (Parsley) is a biennial plant of the Umbelliferae family that is widely used in nutrition and pharmacological interventions.[14] Frequent properties of *P. Crispum* extract are informed such as anti-hepatic fibrosis, antidiuretic, anti-microbial, anti-menorrhagia, and antioxidant.[15-19] Additional properties include the blood pressure regulation, treatment of eczema, and antiurolithiasis effects.[20] The *P. Crispum* leaves are used as a hot application against the inflammatory condition.[21] The chemical compounds available in *P. Crispum* extract contain various minerals such as iron, ascorbic acid, phenylpropanoids, carotenoids, flavonoids, different terpenoic compounds, coumarin, and tocopherol. Dietary supplements containing the leaf of *P. Crispum* extract significantly increase the antioxidant level.[22] According to the antioxidant effects of *P. Crispum* extract, it seems that this vegetable can protect the PC against morphine-induced oxidative damage. A review of the literature shows that no study has evaluated the effects of *P. Crispum* extract against morphine-induced oxidative stress in PC of male rats. Therefore, the present study was designed and conducted to investigate the effects of *P. Crispum* extract on morphine-induced toxicity in the brain's PC of male rats.

Materials and Methods

Animals

This experimental study was done from May 2018 to February 2019 in the anatomical department of medical school in Kermanshah University of Medical Sciences in Iran. 64 male *Wistar* rats) 220–250 g (were purchased from the Pasteur Institute and transferred to the animal house in medical school. During the study, the animals were kept under standard conditions (i.e., 12 h light/12 h dark and 22°C ± 2°C), the humidity of 50%–60%, in special cages and on a straw bed. Treated municipal water and standard food plates were freely available to the animals. All investigations conformed to the ethical and human principles of research and were approved by the Ethics Committee (ethics certificate No. 95350).[19]

Petroselinum crispum extract preparation

P. Crispum plant was obtained from a local store and its impurities were removed (time to pick and buy this plant in the spring in the west of Iran). After confirmation by a botanist, the plant was purging. The leaves and stems were desiccated in shadow for 5 days and subsequently grounded using a grinder. 100 g of this powder was added to 70% ethanol. The acquired solution was reserved in a warm water bath (36°C) under dark condition. Thereafter, the solution was progressively poured on Buchner funnel filter paper and cleaned by a vacuum pump. Then, the resulting solution transferred to a rotary device to obtain the extra solvent. The isolation process continued until the concentrated extract was obtained. The extract was dissolved in distilled water and administered intraperitoneally per a kilogram of animal's weight. It was sterilized after double filtration through a 0.2-m filter.[20]

Study groups and treatment of animals

A total of 64 male rats were randomly divided into 8 groups with 8 rats in each one. The first group was sham group, received normal saline through intraperitoneal injection equivalent to the amount of experimental groups. In the second morphine group, morphine administered via interaperitoneal injection (20 mg/kg once daily in the first 5 days and double per day in the following 5 days. On the eleventh to 20th days, a dose of up to 30 mg/kg doubles each day). The third to fifth groups including the *P. Crispum* groups, the animals respectively received 100, 150, and 200 mg/kg of *P. Crispum* intraperitoneally once daily, on 1–20 days. The sixth to eighth groups include morphine + *P. Crispum* groups, each animal on the days 1–20, received 100, 150, and 200 mg/kg of *P. Crispum* once daily plus morphine intraperitoneally.[2,19]

Transcardiac perfusion

The transcardiac approach was used for tissue fixation. In this process, 24 h after the last injection of the drug, animals were anesthetized with ketamine (87 mg/kg) and xylazine (13 mg/kg). The chest was opened in the midline, and the apex of the left ventricle was pierced after the completion of thoracotomy. Next, a glass cannula of 1 mm diameter was inserted and fixed in the ascending aorta. The pericardium and the right ventricle were cut. The left ventricle pathway was cut and the ascending aorta was connected to a plastic tube by the glass cannula and descending aorta was clamped right above the diaphragm. The cannula linked to the normal saline solution was implanted into the aorta through making an incision in the left ventricle. The descending aorta was fastened, and after washing the brain, the solution was removed through the incision made in the right atrium. For brain fixation, formalin 4% and buffer phosphate 6% were injected using brain cannula in a 15-minute period. After perfusion, the brains were dissected from the skull and stored in the same perfusion solution for 3 days.[23]

Golgi method

The Golgi method was used to observe neuron dendrites in the brain PC region. This method was applied using potassium dichromate followed by silver nitrate. After the brain fixation, tissue blocks were put inside 3% potassium dichromate solution in a dark environment for 48 h. The blocks were washed in 0.75% silver nitrate solution and were put inside the solution for 72 h. The tissues were washed in 1% silver nitrate solution. Then, tissue processing, dehydration, clearing, and embedding were performed. Microscopic sections (5 μm) were prepared and examined morphologically.[6]

Cresyl violet staining method

This staining method was used to determine the number of living cells in the PC. In brief, the slips were stable again (10 min) in 4% paraformaldehyde solution. The slide was immersed in 70% and 100% ethanol and xylene for 20 min. They were then immersed back through the ethanol descent concentrations. They were stained for 5 min in filtered Cresyl violet solution. Then, the slides were dehydrated again in ethanol. They were put in xylene for the next 10 min and then the cover slipped. After preparation of microscopic images, the number of cells was counted in a square millimeter (mm^2). In the slides stained by means of Cresyl violet technique, the round cells without peak nose were considered as living cells.[23]

Dendritic thorns

Dendritic thorns were counted via an optical microscopic and the use of Motic and Image tool IT (Moticam 2000; Madrid, Spain) Softwares. In the slides stained through the Golgi technique, the neurons entirely stained as the cell bodies located in the central part of the slices distant from the surrounding stained neurons were included. The dendritic tree of pyramidal neurons was demonstrated through a camera lucida at ×750 magnification, and the dendritic exclusion order from the cell body was used for counting the dendritic pieces.[6]

Griess technique

Nitrite oxide measured by Griess technique. Through this process, the mixture of zinc sulfate powder (6mg) and the serum samples (400 µl) vortexed for 1 min. The samples were centrifuged at 4°C for 10 min at 12,000 rpm, and the supernatant was used to measure the nitrite oxide. Briefly, 50 µl of sample was added to 100 µl of Griess reagent (Sigma; USA) and the reaction mixture was incubated for about 30 min at room temperature. The optical density of the sample was measured according to manufacturer protocol by an ELISA reader (Hyperion; USA) at a wavelength of 540 nm.[13]

Ferric-reducing/antioxidant power method

Ferric-reducing/antioxidant power method was used in order to measure the total antioxidant capacity (TAC) of the serum (Abcam, ab234626). Before the application of transcardiac method, the venipuncture from the animals' hearts (right ventricle) was done using a 5cc syringe. To clot formation, the blood sample was incubated 37° for 15 min. Then, the clot was centrifuged for 15 min at 3000 rpm to the serum separation. The separated serum was stored in the temperature of −70°C to the measurement of antioxidant capacity. In this technique, the ability of the plasma to reinstate the ferric ions was assessed. This process required a great quantity of Fe^{III}. A blue stain was formed when the compound of Fe^{III}-TPTZ in acidic pH returned to Fe^{II} and absorption at the maximum wavelength of 593 nm. The only factor defining the speed of the Fe^{II}-TPTZ and the blue color was the vitalizing power of the sample. TAC values were strategized by means of the standard curve with diverse concentrations of iron sulfate.[13]

Statistical analysis

The Kruskal–Wallis test was used to examine the data normality and the homogeneity of variance at a significance level of 0.05. The one-way analysis of variance and Tukey *post hoc* test were used for statistical analysis and determination of the differences between groups, respectively. SPSS 16 was used for data analysis. The obtained results were expressed as mean ± standard error, and $P < 0.05$ was considered as significant statistically.

Results

Number of neurons

The results of neuron counting in the brain PC region showed a significant decrease in the morphine group compared to the sham group ($P < 0.05$). The mean number of neurons in all *P. Crispum* groups was not significant compared to the sham group ($P > 0.05$). Also, the mean of pyramidal neurons increases significantly in *P. Crispum* and morphine + *P. Crispum* groups in all doses compared to the morphine group ($P < 0.05$) [Figure 1].

Dendritic spines

The mean number of neuronal dendritic spines in experimental groups showed a significant decrease between the sham and morphine groups ($P < 0.05$). Furthermore, the mean number of neuronal dendritic spines was not significant in all *P. Crispum* groups compared to the sham group ($P > 0.05$). Further, in the *P. Crispum* and morphine + *P. Crispum* groups, the mean number of neuronal dendritic spines increases significantly in all treated groups in comparison with the morphine group ($P < 0.05$) [Figure 2].

Nitrite oxide

The results of blood serum nitrite oxide analysis showed a significant increase in morphine group compared to the sham group ($P < 0.05$). The mean nitrite oxide level in the blood serum was not significant in all *P. Crispum* groups compared to the sham group ($P > 0.05$). Furthermore, the mean of nitrite oxide in blood serum declined significantly in *P. Crispum* and morphine + *P. Crispum* groups in all doses compared to the morphine group ($P < 0.05$) [Figure 3].

Total antioxidant capacity

The results displayed that the TAC serum level reduced significantly in the morphine group compared to the sham group ($P < 0.05$). The TAC level enhanced significantly in all *P. Crispum* and morphine + *P. Crispum* groups compared to the morphine group ($P < 0.05$) [Figure 4].

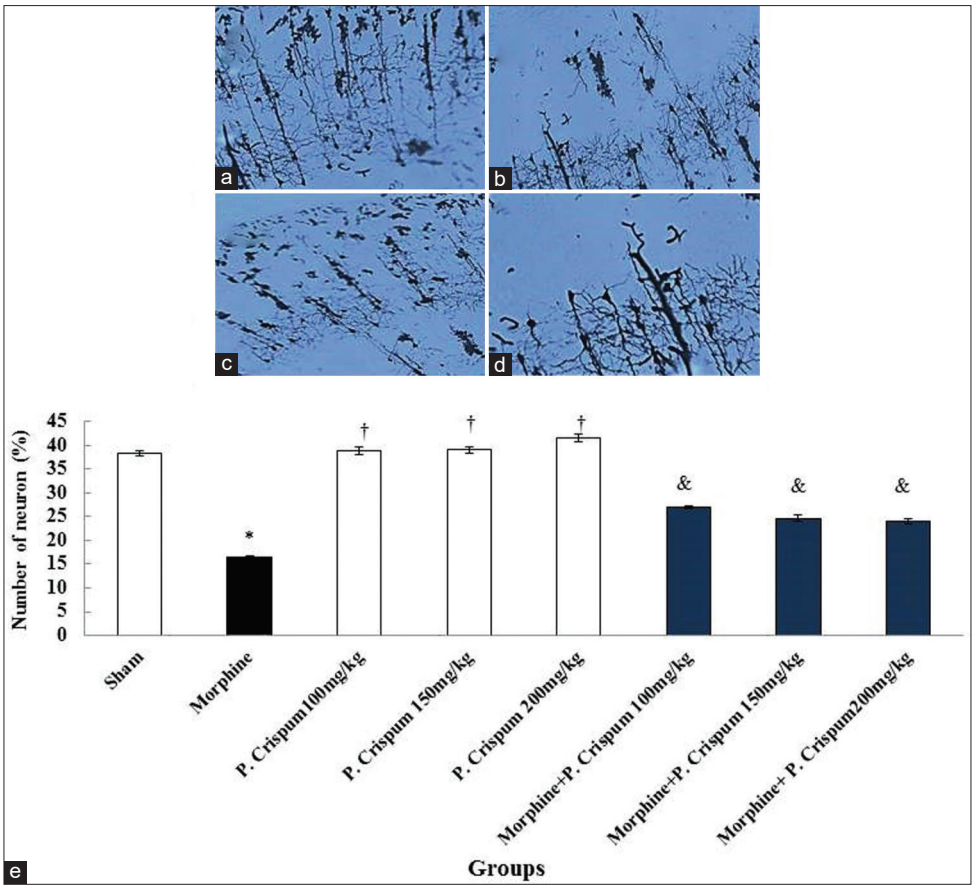

Figure 1: Effect of morphine and *Petroselinum crispum* administration on the number of neurons. (a-d) Five micrometer thick sections, Cresyl violet staining, at × 100 magnification. Prefrontal cortex section in the sham group (a), the normal number of neurons; morphine group (b), reduced neurons cells; *Petroselinum crispum* (200 mg/kg) group (c), a normal number of neurons. Morphine + *Petroselinum crispum* (200 mg/kg) a normal number of neurons. (e) *Significant difference with the sham group (*P* < 0.05). †Significant difference with the morphine group (*P* < 0.05). and Significant difference with the morphine group (*P* < 0.05)

Discussion

The PC of the brain displayed an important role in personality and state of awareness.[5] Morphine causes many implications and side effects by influencing the central and peripheral nervous systems.[24] The patients suffering from shocks in the region of PC mislay the abnormalities of the community consciousness; thus the life tends to hard.[25] The present study was aimed to investigate the effects of *P. Crispum* on morphine-induced disorders in the PC. Based on the results of this study, it can be generally argued that morphine has destructive effects on the PC. The results of the current study showed that the number of neurons and dendritic thorns decreased significantly in morphine group in comparison with the sham group. In all *P. Crispum* and Morphine + *P. Crispum* groups, there was a significant increase in the number of dendritic thorns compared to the morphine group. The results may indicate that the control of apoptosis and neurodegeneration occurs by administration of different doses of *P. Crispum* extract.[26] The results of Montel *et al*. were consistent with those of the present study that showed morphine could damage the cells in the brain cortex by increased protein accumulation in the membrane and reduced cell size.[27] It seems that morphine induces the oxidative stress and consequently, the production of free radicals such as superoxide and hydroxyl radicals, which can cause cell damage.[2] Generated free radicals following the oxidative stress may have the potential ability to damage the cellular compositions, including proteins, lipids, and DNA.[13] The lipid in the membrane of the neurons has a high content of oxidized unsaturated fatty acids. Therefore, it seems that Morphine can produce the reactive oxygen species via the activity of P-450 enzyme and cause the nuclear destruction in neurons.[28] Dendritic thorns play a major role in synaptic transmission. In this regard, many brain diseases are associated with changes in the morphology and density of dendritic thorns.[6] Morphine can reduce the length and the number of dendritic thorns in nucleus accumbens by affecting the neurotrophic factors in the striatum.[29] A study by Robinson and Kolb showed that morphine injections could reduce the length and the number of dendritic thorns; that is consistent with the results of our study.[30] It seems that morphine can destroy dendritic thorns by deactivation of β2-nAChRs in postsynaptic cells located in PC region.[31] Moreover, morphine can reduce the number of thorns by deactivating the α4 β2-nAChRs in the presynaptic membrane and

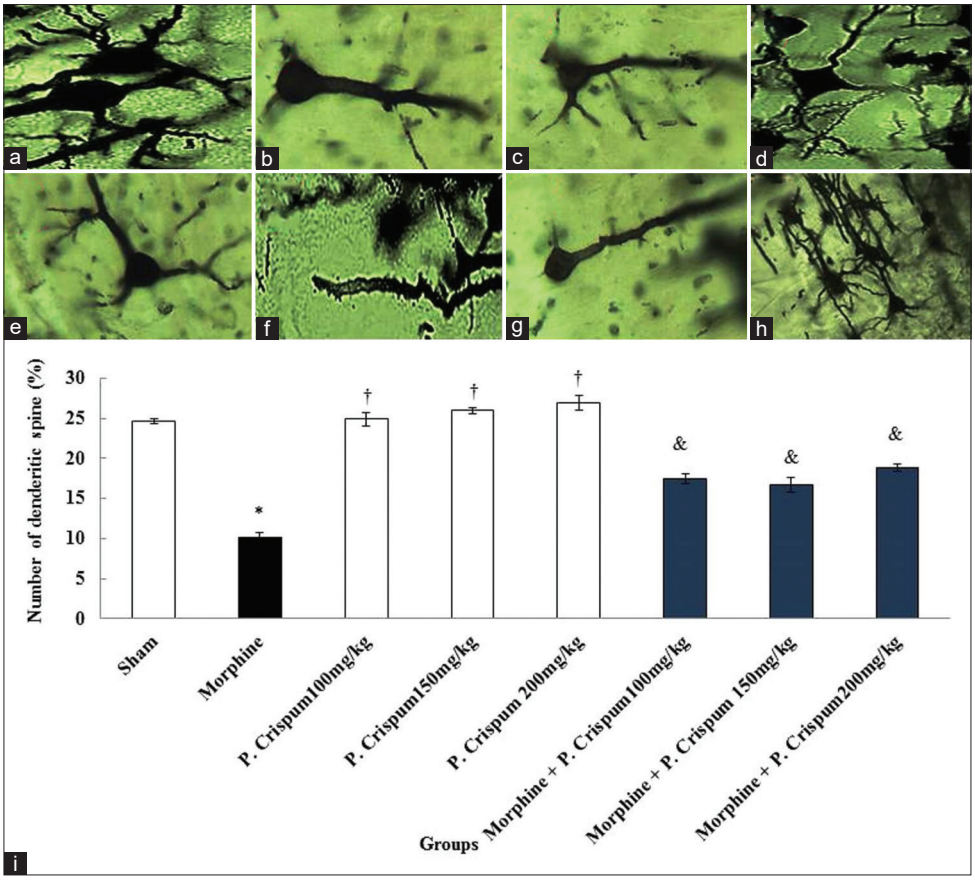

Figure 2: Comparison of morphine, *Petroselinum crispum*, and morphine + *Petroselinum crispum* groups concerning the number of dendritic processes in prefrontal cortex. (a-i) Five-micron thick sections, Golgi staining, magnification × 400. Prefrontal section in the sham group (a, neuronal dendritic, e, dendritic spines), normal structure. Morphine groups (b, neuronal dendritic, f, dendritic spines) reduced number of dendritic and spines processes. *Petroselinum crispum* (200 mg/kg) group (c, neuronal dendritic, g, dendritic spines), normal structure. Morphine + *Petroselinum crispum* (200 mg/kg) (d, neuronal dendritic, h, dendritic spines), normal structure. (i) *Significant difference with the sham group (*P* < 0.05). †Significant difference with the Morphine group (*P* < 0.05). and Significant difference with the morphine group (*P* < 0.05)

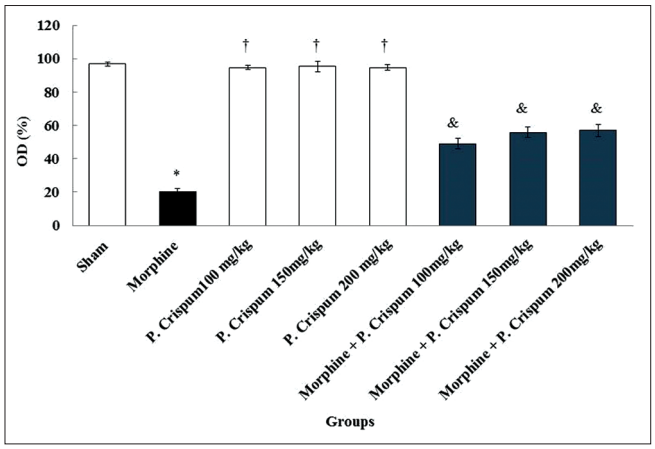

Figure 3: Effects of *Petroselinum crispum*, morphine, and morphine + *Petroselinum crispum* on the mean of nitrite oxide level. *Significant increase of nitrite oxide in the morphine group compared to the sham group (*P* < 0.05). †Significant decrease in all *Petroselinum crispum* groups compared to the morphine group (*P* < 0.05). and Significant decrease in all morphine + *Petroselinum crispum* groups compared to the morphine group (*P* < 0.05)

disruption in release of glutamate neurotransmitters.[32] *P. Crispum* extract is a purifier of reactive oxygen species and has the potential to destroy the oxidative stress.[19] The results of the study by Jassim confirmed the results of the present study that *P. Crispum* extract could prevent cell death and development oxidative stress due to sodium valproate administration.[18] *P. Crispum* extract seems to control the production of cyanide-induced superoxide.[14] This extract can enhance the effects of antioxidant enzymes such as catalase and superoxide dismutase and reduce the ROS production.[15] The results of this study showed that there was a significant increase in serum nitrite oxide and serum total antioxidant levels in the morphine group compared to the sham group. In all *P. Crispum* and morphine + *P. Crispum* groups, there was a significant decrease in serum nitrite oxide and serum total antioxidant levels in comparison with the morphine group. Nitrite oxide is a free radical and can regulate angiogenesis, apoptosis, cell cycle, invasion, and metastasis.[2] Morphine can stimulate nitrite oxide receptors in the brain and increase the glutamate release and N-methyl-D-aspartate activation. This activation may increase the formation of nitrite oxide in the PC.[33] The results of a study by Keser *et al*. showed that exposure of the morphine to the mouse brain increases the activity of nitrite oxide in the frontal cortex, consistent

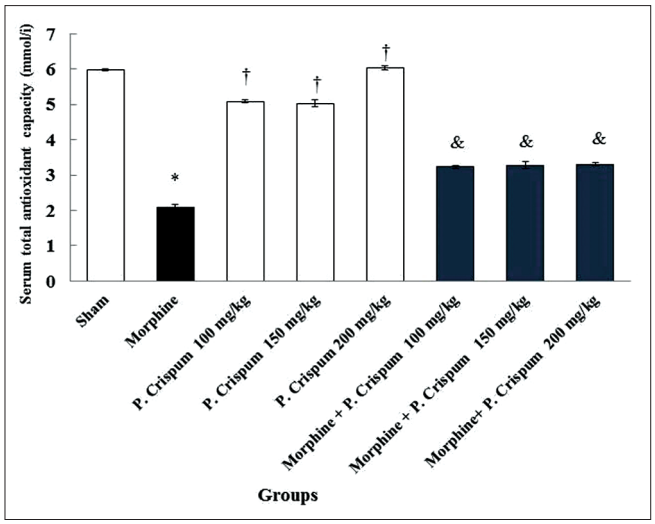

Figure 4: Comparison of total antioxidant capacity in morphine, *Petroselinum crispum*, and morphine + *Petroselinum crispum* groups. *Significant decrease in the morphine group compared to the sham group (*P* < 0.05). †Significant increase in all *Petroselinum crispum* groups compared to the morphine group (*P* < 0.05). and Significant increase in all morphine + *P Petroselinum crispum* groups compared to the morphine group (*P* < 0.05)

with the results of the present study.[34] Jalili *et al*. showed that nicotine administration led to morphometrically variations in neurons in the hippocampal region in rats, which included reduction of neuron cells count and dendritic spines in comparison with control group which is consistent with the results of the current study.[5] The reduction in the TAC level in this study shows the effects of PC oxidative stress induced by morphine. Morphine induces oxidative stress in neural tissue that is demonstrated as a growth in the levels of ROS and a reduction in the action of antioxidant enzymes like TAC.[35] In the present study, improved levels of TAC in rats treated with *P. Crispum* extract highlight the antioxidant effects of *P. Crispum*.[19] A total increased of antioxidant level due to the administration of morphine indicates the positive impact of *P. Crispum* extract on magnified antioxidant effects and also the inhibition of morphine-induced inflammation and destruction process of neurons in the brain. Further, it is assumed that *P. Crispum* extract inhibits the synthesis of nitrite oxide induction enzyme.[36] The results of the study by Jalili *et al*. are consistent with the results of the present study, which indicated that the *P. Crispum* extract administration could inhibit the nitrite oxide production.[20] The results of the present study showed that all doses of *P. Crispum* extract administration may have a positive effect on morphine-induced toxicity, as oxidative stress, in the neurons of the PC, and these effects are not associated with an increase in the dose of *Falcaria* extract.

Conclusion

It appears that *P. Crispum* extract provides protection against oxidative stress resulting from morphine in the PC. Such an ability of the *P. Crispum* may be due to its strong potential antioxidant attributes. *P. Crispum* extract administration moderates the antioxidant agents in the extract. As a result, it leads to PC recovery and prevention of morphine adverse effects on TAC, nitrite oxide, number of neurons, and dendritic spines as evidenced in the male rats.

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Conflicts of interest

There are no conflicts of interest.

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