Original Article

Serum Malondialdehyde Levels Decreased Along with the Alleviating Effects of Marrubium Parviflorum on Morphine Withdrawal Syndrome in Rats

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ABSTRACT

As a major concern for the clinicians, better treatment of the patients hospitalized to stop opioid abuse has always been a target for the researchers working in this field. On the other hand, the therapeutic potential of medicinal plants has become of great interest to both researchers and consumers in recent years. Among the plants, we can mention those belonging to the genus Marrubium, which have been reported to exert many therapeutic effects. The aim of this research was to investigate the effect of Marrubium parviflorum on morphine withdrawal syndrome and its possible relationship with malondialdehyde (MDA), the indicator of lipid peroxidation that is elevated during the syndrome. To perform this study, 48 rats were divided into 6 groups as follows: 1) Saline-Saline 2) Saline-Morphine; 3, 4, 5) Different doses of the Extract-Morphine (10, 20 and 40 $mg.kg^{-1}$); 6) and the most effective dose of the extract-Saline. To evaluate the withdrawal syndrome, the increasing doses of morphine were injected subcutaneously for 9 days followed by a single dose of naloxone (4 $mg.kg^{-1}$, i.p.). Withdrawal symptoms were then assessedand the total withdrawal score (TWS) was calculated. On the other hand, to confirm the efficacy biochemically and to investigate the possible relationship between the observed effects and lipid peroxidation, blood samples were taken for malondialdehyde (MDA) measurement. According to the data, administration of the extract (in two higher doses) significantly alleviated the syndrome-related behavioral signs as well as MDA levels significantly. Overally, based on the results, aerial parts of Marrubium parviflorum seem to be beneficial for better coping with morphine withdrawal syndrome through complex pathways such as suppressing lipid peroxidation, further preclinical and clinical studies are required.

Keywords: Morphine, Dependence, Marrubium parviflorum, Lipid peroxidation, Malondialdehyde.

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1. Introduction

Morphine, which belongs to a group of analgesics called opioids, may be prescribed for severe pain. (1,2) However, the doses administered must be strictly monitored using approved and available screening and analytical methods such as High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC) (3). Regarding the mechanisms of action, morphine has been shown to act through the pharmacological pathways that are mostly mediated via activating the receptors particularly Mu, Delta and Kappa. Following the interactions, the interneurons would be hyperpolarized and the release of the relevant neurotransmitters would be depressed. Despite its efficacy, many challenges such as tolerance and dependence would be associated with morphine administration. (1, 2, 4) It is obvious that these challenges would lead to limitations in prescribing the drug (5). However, many pharmacological attempts have been made in recent years to alleviate these challenges (6-8). The pharmacological approach to natural compounds has always been accepted in societies, so that many people would meet their needs with these medicines (9, 10). On the other hand, several natural products have recently been studied for their potential health benefits (11-13). The plant family, Lamiaceae, contains many useful herbs with important therapeutic properties. In this regard, there are many records supporting the pharmacological properties of the genus, *Marrubium*, which belongs to the aforementioned family. Phytochemically, many important compounds have been found in the structure of these plants, among which we can point out polyphenols, alkaloids, diterpenes and flavonoids, each of which exerts a particular pharmacological result (14). *Marrubium parviflorum* (*M.parviflorum*), belonging to the aforementioned genus, is a perennial, herbaceous plant known for for some therapeutic effects. Historically, it has been used as a treatment for various medical conditions such as fever and colds (15, 16). Phytochemical analyses have reported significant antioxidant capacity of this plant as well as the metal chelating and DNA protective abilities. These properties have been proposed to be due to the presence of compounds such as phenols, flavonols and flavonoids (17). On the other hand, numerous studies have introduced oxidative stress (18-20), heavy metals interactions (21) and DNA damage (22) as the important mediating factors for morphine dependence. As mentioned above, these events have been suggested to be alleviated by M. parviflorum. Therefore, in this study we decided to evaluate the ability of this plantto attenuate morphine withdrawal syndrome and also the involvement of malondialdehyde as a mediating factor.

2. Materials and Methods

2.1. Plant Preparations

The plant was collected in the middle of the flowering season in the city of Marand, East Azerbaijan province. The aerial parts of the plant were cleaned from dirt and dust then dried and ground in the shade, away from direct sunlight, at laboratory temperature. Extraction from the aerial part of the plant was done by Soxhlet method using petroleum ether, then chloroform, followed by methanol. Flavonoids and phenylethanoids accumulated in methanol due to its high polarity. The resulting extract was then dried in an evaporator at low temperature and pressure. Finally normal saline was used as a solvent for the dry extract before injection.

2.2. In Vitro Analyses

2.2.1. Evaluating total flavonoid content

The total flavonoid content of the extract was evaluated by aluminum chloride colorimetric method. In this method, 0.5 ml of the methanol extract (1000 µg/ml) was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of one molar potassium acetate solution, and 2.8 ml of distilled water. After incubation for30 minutes at laboratory temperature, the absorbance of the solution was measured at 415 nm using a spectrophotometer. The standard curve of quercetin was plotted using concentrations of 25-250 µg/ml. Finally, the total flavonoid content of the methanol extract was calculated using the standard curve based on grams of quercetin per 100 grams of the dry plant powder(23).

2.2.2. Evaluating Total Phenolic Content

To evaluate the total phenolic content of the extract, the absorbance of a solution containing 0.5 ml of the methanol extract (1000 µg/ml), 5 ml of Folin-Ciocalteu reagent (10% v/v in distilled water) and 4 ml of one molar sodium carbonate was measured at 765 nm using a spectrophotometer A standard curve for . Gallic acid was also plotted using concentrations of 25-300 µg/ml. Finally, the total phenolic content of the methanol extract was calculated from the standard curve and the values obtained for the sample. (24).

2.2.3. In Vitro Sssessment of Antioxidant Activity

To evaluate the antioxidant activity *in vitro*, the scavenging capacity of the extract was measured against 2,2-diphenyl-1-picrylhydrazyl (DPPH), which is composed of stable free radical molecules (25). After preparing the stock solutions of the extract and the sequential twofold dilutions, they were integrated with similar volumes of DPPH (26-28) and incubated for 30 minutes. The absorbance was read at 517 nm and R% was calculated as follows:

R (%) = [(Blank's absorbance – Sample's absorbance) / Blank's absorbance] × 100

2.3. In Vivo Analyses

2.3.1. Animals

In this study, 48 adult male Wistar rats in the weighing range of 225-275 g were randomly divided into 6 groups randomly. The rats were maitained at a constant temperature of 23±2°C and relative humidity of 50±10% in standard polypropylene cages (4 rats per cage) under a 12-hour light-dark cycle with free access to food and water. All groups were habituated to the laboratory environment, the experimenter, and the cylindrical chamber (used for recording symptoms) for 30 minutes 3 days prior to the

experimental and behavioral studies. Rats were weighed daily using a digital scale and the amount of injections was adjusted according to their weight. Each animal was used only once in all experiments. All the procedures were in accordance with the international guidelines. The study protocol was designed and approved by the Ethics Committee for the Use of Animals in Research for Tabriz University of Medical Sciences. (Code: IR.TBZMED.VCR.REC.1398.043).

2.3.2.Experimental Design

Eight experimental groups were treated with saline and saline; saline and morphine; the extract (10, 20, 40 $mg.kg^{-1}$) and morphine; and the most effective dose of the extract and saline, respectively. Each group was treated for 8 consecutive days. The doses for morphine were considered incremental (from 5 to 25 $mg.kg^{-1}$) during the treatment period. Each animal was treated twice daily (every 12 hours). The first injection was administered intraperitoneally 30 minutes before the second injection, which was injected subcutaneously. On the ninth morning, only the second injections were applied followed by a single dose of naloxone (4 $mg.kg^{-1}$, i.p.). Morphine withdrawal symptoms were then recorded for 60 minutes and the animals were prepared for sampling in the final step.

2.3.3. Assessment of the Withdrawal Syndrome

During the 60 minutes of assessment, the behaviors and movements of animals were closely monitored and Total Withdrawal Score (TWS) was calculated for each animal (29), in such a way that, the behaviors associated with morphine withdrawal were scored, with the details presented in our previous research (30), as follows: 20 for standing on feet, 10 for teeth grinding, face wiping and body grooming, 5 for genital grooming, head and wet dog shaking , abdominal writhing as well as paw tremor and 4 for jumping.

2.3.4. Locomotor Activity Evaluation

A locomotor test was also performed to evaluate the effect of the extract on the mobility of the animals. Thus, the locomotor activity of the groups receiving morphine + saline and the group receiving the most effective dose of the extract with morphine was evaluated using the openfield methodon the ninth day before the injection. For this purpose, a 100 x 100 cm box (the floor was divided into 20 x 20 cm squares) and protected by 40 cm high edges was used. Each mouse was placed in the center of the area and the times it crossed the lines were recorded within 30 minutes (31).

2.3.5. Measurement of serum malondialdehyde levels

Finally, the rats were anesthetized one by one using ketamine and xylazine and 5 ml of blood was collected from the hearts. The samples were kept in the test tubes (without anticoagulant) at room temperature until clotting, then were centrifuged at 3500 rpm for ten minutes. The serum samples were separated, poured into two microtubes (1000 and 700 microliters), and kept frozen for 24 hours. Malondialdehyde (MDA) levels were measured using a spectrophotometer based on the reaction with thiobarbituric acid. (32).

2.4. Statistical Analysis

The latest available version of Sigma Plot software was used to analyze the results. The results were expressed as S.E.M ± mean, and student's t-test and one-way ANOVA (followed by Tukey's post-test) were used to compare the results between two groups and more than two groups, respectively. Statistically, $p<0.05$ was considered significant.

3. Results

3.1. In Vitro Analyses

3.1.1. Total Phenolic and Flavonoid Contents

The total phenolic and flavonoid contents of the extract which were analyzed by colorimetric methods, were determined and reported as presented (Table 1).

3.1.2. In Vitro Antioxidant Activity

To assess the antioxidant activity of the methanol extract, the absorbance was measured 3 times, the values were recorded, the antioxidant activity of the methanol extract was determined and the RC50 was calculated as shown (Table 2).

3.2. In Vivo Analyses

3.2.1. Effects on the withdrawal syndrome

As presented (Figures 1 to 8), the extract was able to significantly alleviate the morphine withdrawal syndrome, so that the quantity of the withdrawal-associated behaviors (standing on feet, teeth grinding, face wiping, body and genital grooming, head and wet dog shaking , abdominal writhing, paw tremor and jumping) and subsequently TWS were statistically lower for the extract-treated rats compared to morphine + saline group. According to Figure 1, the number of jumps which had dramatically increased in morphine-treated group, was significantly lower in the extract-treated groups (in the two higher doses). Figure 2 shows the comparison between the groups in terms of another behavioral indicator of withdrawal syndrome, wet dog shakes. As can be seen, the increase in the M+S group was alleviated in the groups treated with the extract (40 $mg.kg^{-1}$). As another indicator of morphine withdrawal syndrome, the rise in the number of abdominal writhing shows the occurrence of the syndrome in the M+S group. Figure 3 presents the ability of the extract to reduce the frequency of the studied behavior. The number of head shakes, the other behavioral indicator of withdrawal syndrome was also increased in morphine- treated group. It can be observed that the extract was able to reduce the number of head shakes only at the dose of 40 $mg.kg^{-1}$. (Figure 4). A Significant reduction in the frequency of genital grooming was also observed in the rats treated with the extract. According to the data presented in Figure 5, the reductions were significant at two higher doses. As presented, the other morphine withdrawal-related behavior which was alleviated in the groups treated with the extract (two higher doses) was teeth chattering (Figure 6).

Table 1. Total phenolic and flavonoid contents of M. parviflorum methanol extract analyzed by colorimetric methods.

The analyzed material	Total phenol content of the dried powder (g/100g)	Total flavonoid content of the dried powder (g/100g)
M. parviflorum methanol extract	1.74	2.63

Table 2. The antioxidant activity of M. parviflorum methanol extract compared with the standard solution of quercetin.

The calculated factor	The methanol extract	Quercetin
RC_{50} (µg/ml)	22.8	6.73

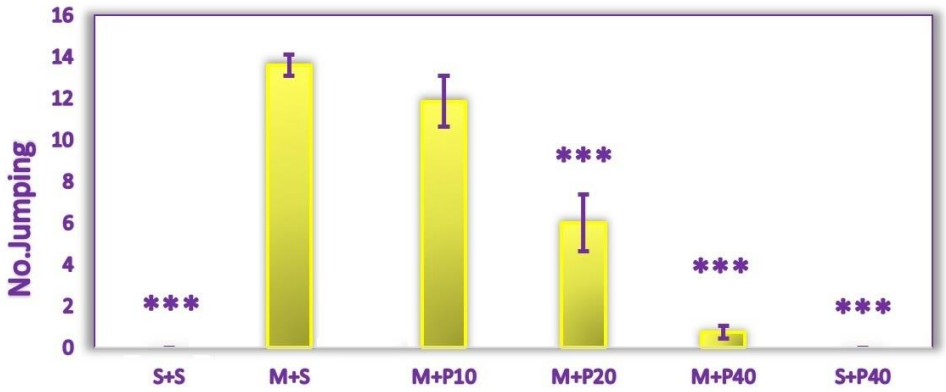

Figure 1. Effects of M. parviflorum on the number of jumps. n = 8 in each group. ***p<0.001 vs. M+S group (analyzed by ANOVA). S+S: The group treated by Saline and Saline, M+S: The group treated by Morphine and Saline, M+P10: The group treated by Morphine and The extract 10 mg.kg^{-1}, M+P20: The group treated by Morphine and The extract 20 mg.kg^{-1}, M+P40: The group treated by Morphine and the extract 40 mg.kg^{-1}, S+P40: The group treated by Saline and the extract 40 mg.kg^{-1}.

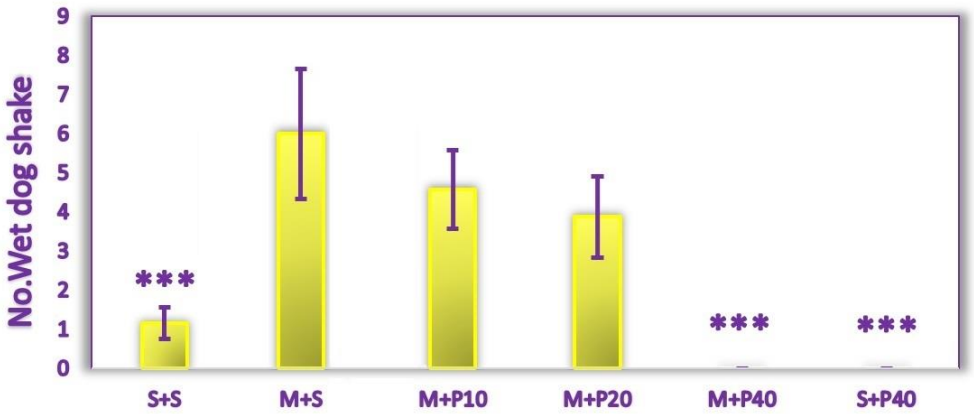

Figure 2. Effects of M. parviflorum on the number of wet dog shakes. n = 8 in each group. ***p<0.001 vs. M+S group (analyzed by ANOVA). S+S: The group treated by Saline and Saline, M+S: The group treated by Morphine and Saline, M+P10: The group treated by Morphine and the extract 10 mg.kg^{-1}, M+P20: The group treated by Morphine and the extract 20 mg.kg^{-1}, M+P40: The group treated by Morphine and the extract 40 mg.kg^{-1}, S+P40: The group treated by Saline and The extract 40 mg.kg^{-1}.

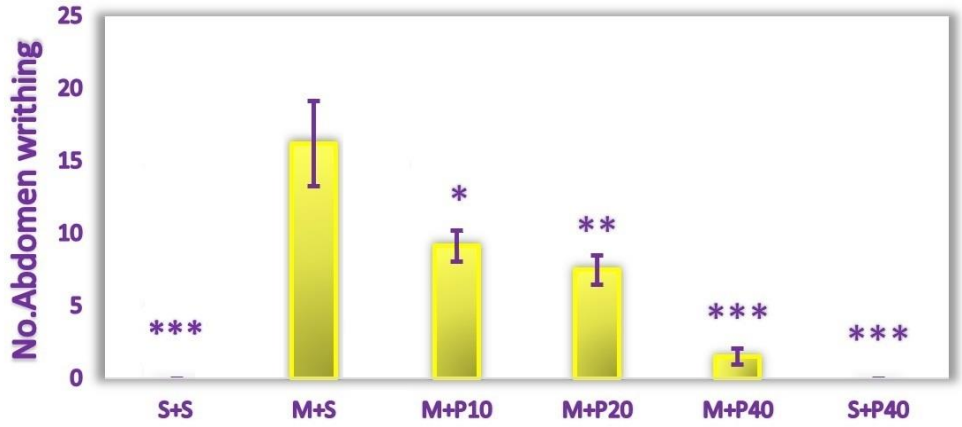

Figure 3. Effects of M. parviflorum on the number of abdominal writhing. n = 8 in each group. *p<0.05, **p<0.01, ***p<0.001 compared with M+S group (analyzed by ANOVA). S+S: The group treated by Saline and Saline, M+S: The group treated by Morphine and Saline, M+P10: The group treated by Morphine and The extract 10 mg.kg^{-1}, M+P20: The group treated by Morphine and The extract 20 mg.kg^{-1}, M+P40: The group treated by Morphine and The extract 40 mg.kg^{-1}, S+P40: The group treated by Saline and The extract 40 mg.kg^{-1}.

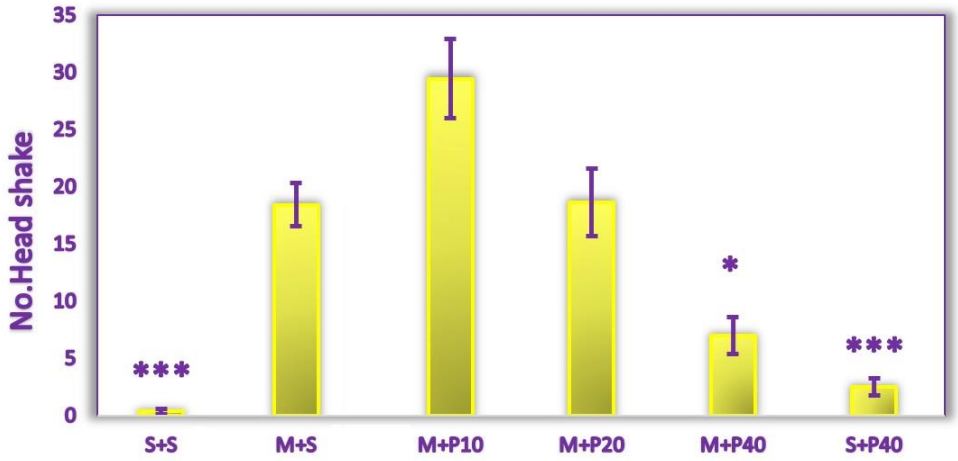

Figure 4. Effects of M. parviflorum on the number of head shakes. n = 8 in each group. *p<0.05, ***p<0.001 compared to M+S group (analyzed by ANOVA). S+S: The group treated by Saline and Saline, M+S: The group treated by Morphine and Saline, M+P10: The group treated by Morphine and The extract 10 mg.kg^{-1}, M+P20: The group treated by Morphine and The extract 20 mg.kg^{-1}, M+P40: The group treated by Morphine and the extract 40 mg.kg^{-1}, S+P40: The group treated by Saline and The extract 40 mg.kg^{-1}.

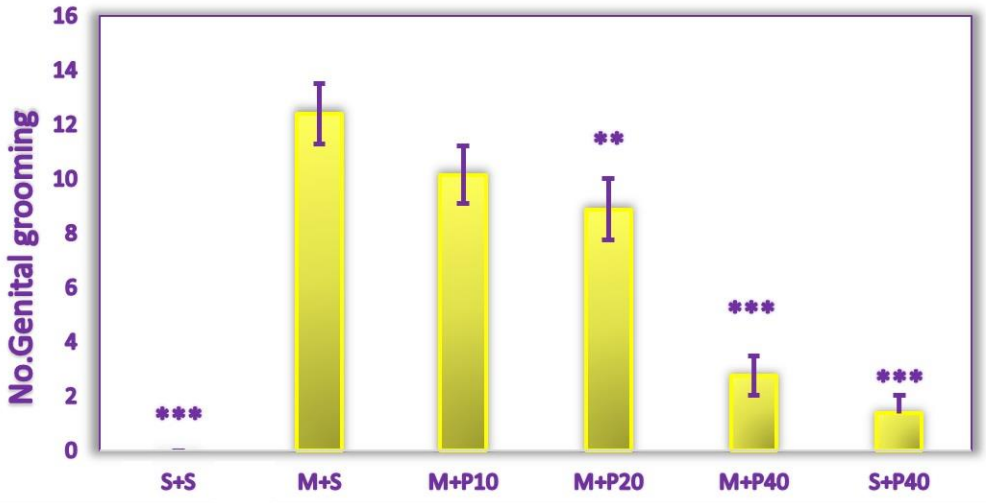

Figure 5. Effects of M. parviflorum on the number of genital grooming. n = 8 in each group. **p<0.01, ***p<0.001 vs. M+S group (analyzed by ANOVA). S+S: The group treated by Saline and Saline, M+S: The group treated by Morphine and Saline, M+P10: The group treated by Morphine and The extract 10 mg.kg^{-1}, M+P20: The group treated by Morphine and The extract 20 mg.kg^{-1}, M+P40: The group treated by Morphine and The extract 40 mg.kg^{-1}, S+P40: The group treated by Saline and The extract 40 mg.kg^{-1}.

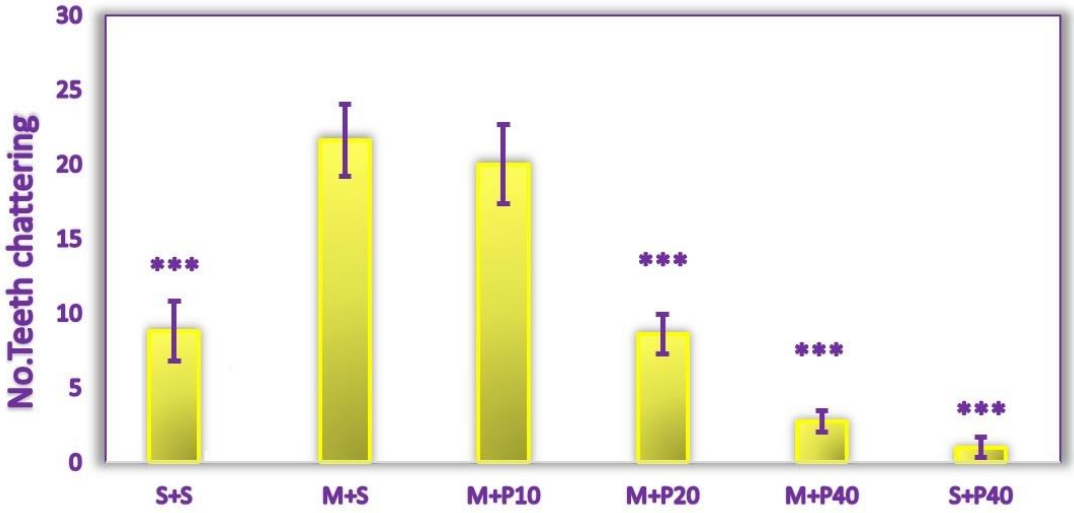

Figure 6. Effects of M. parviflorum on number of teeth chattering. n = 8 in each group. ***p<0.001 compared to M+S group (analyzed by ANOVA). S+S: The group treated by Saline and Saline, M+S: The group treated by Morphine and Saline, M+P10: The group treated by Morphine and The extract 10 mg.kg^{-1}, M+P20: The group treated by Morphine and The extract 20 mg.kg^{-1}, M+P40: The group treated by Morphine and The extract 40 mg.kg^{-1}, S+P40: The group treated by Saline and The extract 40 mg.kg^{-1}.

Two higher doses of the extract were also able to reduce the number of times the patient stood on their feet, the other behavioral consequence of morphine withdrawal. (Figure 7). Finally, according to Figure 8, the TWS was calculated for each group (based on the method described above), was statistically lower (compared to the M+S group) in the groups treated with the two higher doses of the extract.

3.2.2. Effects on the locomotor activity

According to the data, the locomotor activity of the rats treated with M. parviflorum methanol extract was significantly higher compared to those in morphine + saline group (Figure 9).

3.2.3. Effects on MDA levels

As can be seen (Figure 10), statistically lower levels of MDA were clearly observed in the groups treated with the extract.

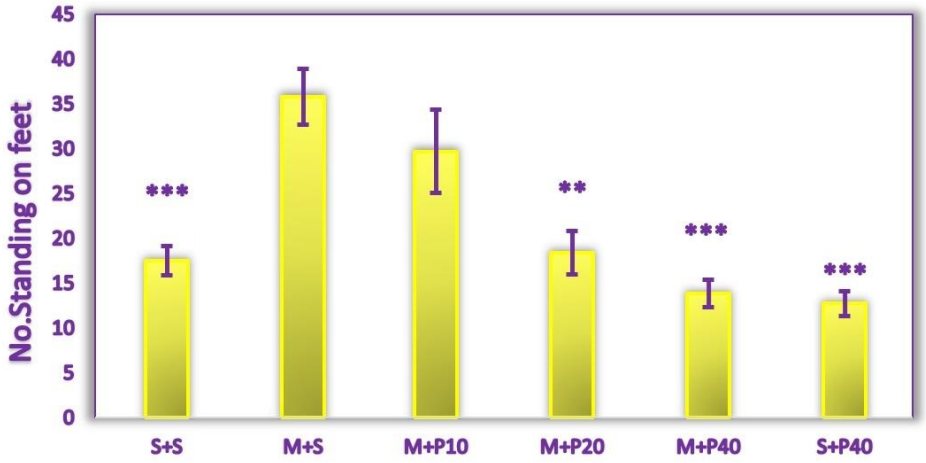

Figure 7. Effects of M. parviflorum on number of standing on feet. n = 8 in each group. **p<0.01, ***p<0.001 compared to M+S group (analyzed by ANOVA). S+S: The group treated by Saline and Saline, M+S: The group treated by Morphine and Saline, M+P10: The group treated by Morphine and The extract 10 mg.kg^{-1}, M+P20: The group treated by Morphine and The extract 20 mg.kg^{-1}, M+P40: The group treated by Morphine and The extract 40 mg.kg^{-1}, S+P40: The group treated by Saline and The extract 40 mg.kg^{-1}.

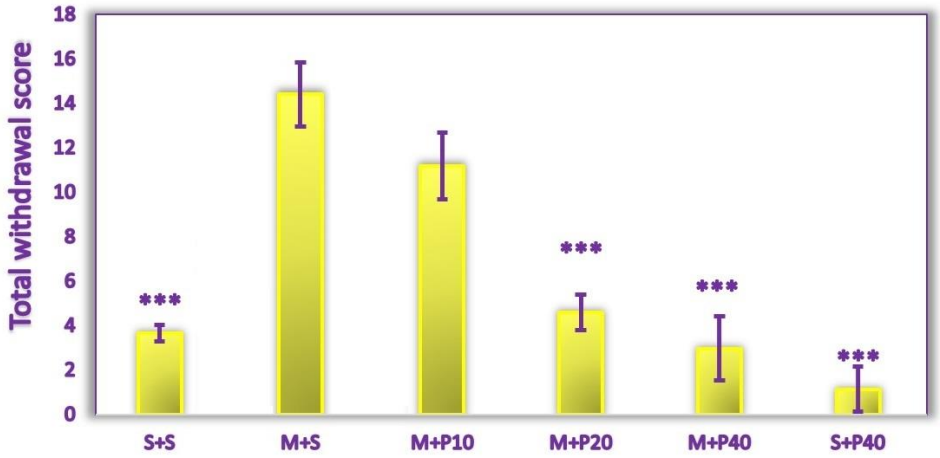

Figure 8. Effects of M. parviflorum on total withdrawal score (TWS). n = 8 in each group. ***p<0.001 compared to M+S group (analyzed by ANOVA). S+S: The group treated by Saline and Saline, M+S: The group treated by Morphine and Saline, M+P10: The group treated by Morphine and The extract 10 mg.kg^{-1}, M+P20: The group treated by Morphine and The extract 20 mg.kg^{-1}, M+P40: The group treated by Morphine and The extract 40 mg.kg^{-1}, S+P40: The group treated by Saline and The extract 40 mg.kg^{-1}.

Figure 9. Effects of M. parviflorum on locomotor activity. n = 8 in each group. *p<0.05 compared to M+S group (analyzed by Student's t-test). M+S: The group treated by Morphine and Saline, M+P40: The group treated by Morphine and The extract 40 mg.kg^{-1}.

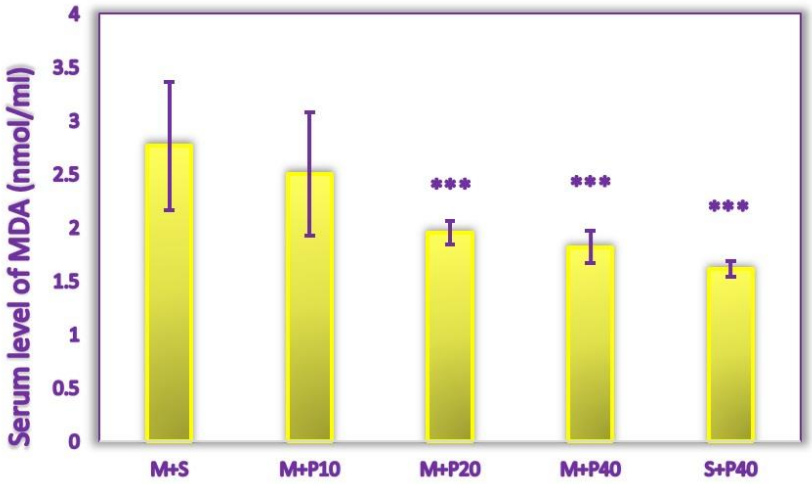

Figure 10. Effects of M. parviflorum on serum level of MDA (nmol.ml^{-1}). n = 8 in each group. ***p<0.001 compared to M+S group (analyzed by ANOVA). M+S: The group treated by Morphine and Saline, M+P10: The group treated by Morphine and The extract 10 mg.kg^{-1}, M+P20: The group treated by Morphine and The extract 20 mg.kg^{-1}, M+P40: The group treated by Morphine and The extract 40 mg.kg^{-1}, S+P40: The group treated by Saline and The extract 40 mg.kg^{-1}.

4. Discussion

Based on the data, administration of the increasing doses of morphine for 9 days would result in significant dependence and the subsequent administration of naloxone would lead to withdrawal syndrome in rats. Furthermore, the injections of M. parviflorum methanol extract would cause dose-independent alleviation of the withdrawal syndrome. Furthermore, alterations in MDA levels were clearly observed. Opioids would be defined as natural, semi-synthetic and synthetic derivatives of opium alkaloids that act through the main endogenous opioid receptors and are widely used as one of the choices to relieve different types of pain. (33) As noted above, one of the important challenges associated with the chronic use of the opioids would be addiction to these drugs (7). To date, numerous studies have been conducted on the mechanisms underlying opioid dependence and subsequent withdrawal syndrome, of which the neurotransmitter systems of glutamate, serotonin, dopamine, and norepinephrine have been found to be very important as well as inflammatory cytokines, nitric oxide and glucocorticosteroids which are considered to be other factors involved (34, 35). Among this group of drugs, morphine is the popular and natural opioid receptor agonist which is derived from the plant *Papaver somniferum* (36). By binding to the opioid receptors in various tissues and blocking voltage-dependent calcium channels, the alkaloid reduces the release of excitatory neurotransmitters such as glutamate, noradrenaline and substance P. (37) As a feedback, production of the mentioned neurotransmitters would be elevated, so that with long-term and chronic use of morphine, the release rate of excitatory neurotransmitters would increase (38). On the other hand, blocking opioid receptors with naloxone leads to changes in the synaptic levels of some neurotransmitters in the CNS. Just as the acute effects of opioids are exerted at multiple sites in the CNS, the development of physical dependence and withdrawal syndrome occurs through specific brain sites. It is generally accepted that several brain regions are responsible for some of the physical and excitatory symptoms of opioid withdrawal, and some others are mediated through visceral and peripheral receptors (39). It is clear that the management of the withdrawal syndrome is an important challenge for the medical personnel when prescribing opioids (40, 41), so the introduction of the suitable and effective approaches to attenuate this phenomenon would be a priority for the researchers. In this regard, many important achievements have been made in recent years. For example, in 2017, Burma et al. focused on microglial pannexin-1 channels and targeted them in their research, so that they could suggest Panx1 blockers as the novel agents for alleviating the syndrome (42). The other notable study in this area can be the research of Kourosh-Arami et al. which was focused on orexin-1 receptors. They showed that, antagonizing OXR1 receptors would significantly reduce the intensity of the syndrome to a significant extent (43). Moreover, the newly published reports have investigated the role of dopaminergic receptors in controlling morphine withdrawal syndrome. The results have shown that, blocking the mentioned receptors can be another approach to improve the relevant inconvenience and discomfort (44, 45). Based on the present results, M.parviflorum methanol extract contains remarkable amounts of phenols and flavonoids, the phytochemicals known for high free radical scavenging capacity (46, 47). On the other hand, studies have claimed that, oxidative stress occurs widely during opioid dependence. Therefore, the mitigating effects of antioxidants on morphine withdrawal syndrome seem to be important (7, 48). Supporting the fact, biochemical evaluations in our study showed that, serum samples of the rats treated with the extract contained lower levels of MDA. This is while our previous report had indicated an increase in the mentioned factor during the withdrawal syndrome (7). It is noteworthy that, elevated levels of MDA are known to be as an indicator for lipid peroxidation which underlies many pathologies and have been previously observed in morphine dependent patients (49, 50). Overall, the reduction in MDA levels we reported in the treatment group may be a key factor in future studies focusing on the lipid peroxidation process during morphine withdrawal syndrome. In conclusion, the data demonstrated that, M.parviflorum methanol extract would be able to significantly attenuate morphine withdrawal syndrome. Considering the phytochemical analyses on the one hand and the changes in MDA levels on the other hand, the alleviation would probably result from the antioxidant activity of the phenols and flavonoids in the plant's structure. However, despite obtaining clear and significant data, further mechanistic evaluations would be required to reveal various pharmacological and toxicological aspects of the herb.

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Authors' Contribution

Study concept and design: S.H., M.Ch. and A.P.
Acquisition of data: F.H.
Analysis and interpretation of data: S.H., M.Ch. and K.F.
Drafting of the manuscript: K.F.
Critical revision of the manuscript for important intellectual content: S.H
Statistical analysis: F.H., A.P. and K.F.
Administrative, technical, and material support: S.H., M.Ch. and A.P.
Study supervision: S.H. and M.Ch.
Except for the assigned tasks, all authors were involved throughout the whole procedures.

Ethics

The procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of Tabriz University of Medical Sciences, Tabriz, Iran (approval code: IR.TBZMED.VCR.REC.1398.043).

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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