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Research Article

Evaluation of the Effects of Chronic Administration of *Citrus aurantium* Essential Oil on the Development of Tolerance and Dependence to Morphine

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ABSTRACT

Background: Long-term exposure to opioids may lead to physical dependence and tolerance. The purpose of this study was to investigate the effects of *Citrus aurantium* essential oil (CEO) on the morphine-induced tolerance and dependence.

Methods: To evaluate morphine tolerance, the experiments were carried out in 6 rat groups (n=8) in the weight range of 225-275 g. The control group received morphine (10 mg/kg/day) and the test groups received morphine with the different doses of essential oil (CEO 20, 40 and 80 mg/kg/day) or 4 mL/kg of essential oil vehicle (Kolliphor® HS15 30% in normal saline that adjusted in pH=7.4 with phosphate buffer) intraperitoneally. The hot-plate test was carried out every other day, 90 minutes after the injections. To examine morphine withdrawal, male Wistar rats were divided into seven groups (n=8) randomly, including: morphine sulphate, CEO (20, 40 and 80 mg/kg) + morphine, vehicle of CEO + morphine. The rats were rendered morphine-dependent by injection of additive doses of morphine subcutaneously for 9 days. The procedure of the morphine administration was as following protocol: day1: 5 mg/kg/12h, day 2 and 3: 10 mg/kg/12h, day 4 and 5: 15 mg/kg/12h, day 6 and 7: 20 mg/kg/12h and day 8 and 9: 25 mg/kg/12h. In the 9th day, 2 hours after the last dose of morphine, naloxone (4 mg/kg) was injected intraperitoneally. Some withdrawal behaviors were counted for 60 minutes.

Results: Morphine tolerance was completed after 5 days in the control group. The vehicle group showed tolerance on the 9th day (p-value=0.991), 20mg group in the 13th day (p-value to control=0.010, to vehicle=0.049), 40 mg group on the 15th day (p-value to control and vehicle<0.001) and 80 mg group on the 13th day (p-value to control= 0.001, to vehicle= 0.007). The results showed that CEO could reduce the morphine withdrawal syndrome and total withdrawal score (TWS). Intraperitoneally injection of CEO in two doses (40 mg/kg with p<0.001 and 80 mg/kg with p<0.01) significantly reduced the TWS in comparison to the morphine+vehicle treated group.

Conclusion: The results indicated that chronic administration of *C. aurantium* essential oil extracted had beneficial effects in reducing morphine withdrawal syndrome and could significantly delay tolerance to morphine.

Introduction

Morphine and other opioids are widely used for thousands of years to control the moderate to severe pains. Tolerance and dependence are two main problems following chronic administration of morphine that restrict its therapeutic application. Extensive researches have been done to found the exact mechanisms underlying opioid's tolerance, dependence and withdrawal syndrome.

In this regard, numerous mechanisms have been recommended.[1-3]. In the induction of tolerance and dependence, inflammatory cytokines increases such as tumor necrosis factor alpha (TNFα), interleukin 12 (IL-12) and nitric oxide (NO).[4,5] Release of pro-inflammatory cytokines increases the neuronal excitability and induces glial activation, that is important in the opioid dependence and withdrawal.[6] Also the levels of glutamate and aspartate amino acids increase that affect the glia.[7]

The medicinal use of aromatic plants and their essential oils have been recognized thousands of years ago.[8] *C. aurantium* belongs to the Rutaceae family, also is identified as bitter orange or sour orange.[9]

Several studies have showed that *C. aurantium* is useful for medicinal goals due to the various compounds like flavonoids, essential oils, phenols and vitamins. In

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addition, CEO is worthful on the aromatherapy and cosmetic industry internationally.[10] Furthermore, the antibacterial, antifungal and anti-inflammatory effects of CEO have been demonstrated.[11-13] These effects are possibly related to the function of the main components of the CEO.

Previous studies showed that linalool and linalyl acetate were the major compounds in essential oil of the fruits epicarps (peel) of *C. aurantium*. Many evidences indicated that linalool and linalyl acetate had potentially anti-inflammatory effects.[14]

In this study, the effects of chronic administration of essential oil of *C. aurantium* peel on the development of morphine tolerance and withdrawal syndrome were evaluated in male rats.

Materials and Methods

Animals

Male Wistar rats, weighing 225-275 g were obtained from the laboratory animals of the Pasteur Institute. Rats were kept in cages with enough water and food in room with good air conditioning, constant temperature (23±2°C) and 12 hour light/dark cycle. The experiments to evaluate morphine tolerance were carried out in 6 groups of 8 rats that were divided randomly. Two days before beginning the test, rats were moved regularly to the lab environment, to minimize their stress which may affect the test results. After completion of the experiments, the rats were killed by intraperitoneal injection of pentobarbital (150 mg/kg). This study was in accordance with the ethics standards of "Principles of Laboratory Animal Care" and approved by ethics committee of Tabriz University of Medical Sciences (ethical code: IR.TBZMED.VCR.REC. 1395.575).

Drugs

Morphine sulfate and naloxone hydrochloride were obtained from Darupakhsh Company, Tehran, Iran.

Essential oil preparation

C. aurantium fruits were provided from Mazandaran, Iran. To prepare essential oil, fresh peels of fruits were subjected to hydrodistillation. The amount of essential oil from epicarp was almost 3%. The hydrodistillation was carried out using the clevenger apparatus. The essential oil was kept at 4 ^{oc} for further study. The essential oil was prepared as a stable emulsion by using Kolliphor® HS15 30% in normal saline (pH of vehicle adjusted in 7.4 by using phosphate buffer).

Thin layer chromatography of essential Oil

Quantitative evaluation of the essential oil was performed with TLC. Using pre-coated plate of silicagel 60F254 as stationary phase and Toluene–Ethyl acetate (93:7) as mobile phase. Detection was performed with spraying anisaldehyde–sulfuric acid reagent. The pale blue spot at retention factor (R_f)=0.57 indicated the presence of linalool in the essential oil.[15]

Evaluation of antioxidant activity of essential oil

For qualitative evaluation of the antioxidant properties of the essential oil, after dyeing on silicagel plate, 2,2-diphenyl-1-picrylhydrazyl (DPPH) spray in 8% methanolic solution was used. In this method, DPPH causes the plate to become violet and if the essential oil has antioxidant properties, it can change the color of the plate to yellow.[16]

Morphine Tolerance

Evaluation of the tolerance induction

The experiments were carried out in 6 rat groups in the weight range (225-275 g). The control group received morphine (10 mg/kg), second group received the same dose of morphine and 1ml of essence's vehicle (Kolliphor® HS15 30% in normal saline that adjusted in pH=7.4 with phosphate buffer) and the test groups received morphine with the different doses of essential oil (20, 40 and 80 mg/kg) intraperitoneally. Finally, using the results of the experimental groups, the most effective dose of essential oil was selected and the last group received this dose (40 mg/kg) without morphine, intraperitoneally.

Assessment of development of tolerance to the analgesic effects of morphine

Before starting the injections, rats were placed on hot plate (T= 55±0.5°C). The time that rats reacted to the pain (Hind-paw licking, freezing, vertical jumping) indicated acute pain, that represented base latency time (BL) and cut off time was 40 seconds. In the first day, half an hour after the injections, the hot plate test was repeated and this recorded time represented test latency time (TL). Every other day this test was performed as long as morphine tolerance was completed; i.e., when there were no statistically significant difference between the results of the hot plate test in baseline (BL) and the hot plate test after drug injections (TL). After development of tolerance, for more certainly we repeated the hot plate test for two other times. In order to compare the results obtained from the hot plate test, the MPE% (Maximum Possible Effect) relationship was used:[4]

$$MPE\% = \left[\frac{(TL-BL)}{(Cut\text{-}off\ Time-BL)}\right] \times 100 \qquad \text{Eq. (1)}$$

Assessment of the global analgesic effect

To evaluate global analgesic effect, the AUC (Area Under the Curve) of the MPE% was calculated. AUC (1-19 days) was also used to compare between groups.

Morphine Withdrawal

Experimental groups

56 male Wistar rats were distributed in 7 different experimental groups (n=8) randomly. The control group received additive doses of morphine (mentioned in paragraph 2-7-3). Saline group received only saline (1 ml/kg). 30 minutes after daily morphine injections, groups 3, 4 and 5 received 20, 40 and 80 mg/kg of essential oil with vehicle intraperitoneally twice a day, respectively. Group 6 (vehicle+morphine treated group) received 1 ml Kolliphor® HS15 30% in normal saline with morphine

every 12 h. Group 7 received only the most effective dose of essential oil (40 mg/kg/12h).

Locomotor activity test

The locomotor activity was evaluated in morphine+essential oil (40 mg/kg) and morphine+vehicle treated groups, on the ninth day. This test determined the number of crossing the lines drawing on the underside floor of the plexiglass behavioral cage (100×100 cm) by each rat.[17]

Induction of the morphine withdrawal and measurement of the withdrawal behaviors

Additive doses of morphine were administrated subcutaneously for 9 days in order to induce dependence. The performance procedure was as follows: day 1: 5 mg/kg/12h, days 2 and 3: 10 mg/kg/12h,days 4 and 5: 15 mg/kg/12h, days 6 and 7: 20 mg/kg/12h, and days 8 and 9: 25mg/kg/12h. This morphine administration protocol demonstrated a highly dependence in the rats.[18] The rats in saline group received only saline for the nine days and two hours after the morning saline injection on the ninth day, the rats received naloxone (4 mg/kg) intraperitoneally in order to induce the withdrawal signs.[4] The rats were examined in a clear plexiglass chamber and after naloxone injection, withdrawal signs were evaluated by an observer who was not aware of the nature of the treatments received by animals, during a 60 minute period and 11 distinct behaviors (jumping, wet dog shakes, standing on feet, genital grooming, abdomen writhing, body grooming, face wiping, head shakes, paw tremor, teeth chattering and swallowing) were recorded. The chamber was equipped with a digital camera to record the behaviors of the rats. The score of each behavior was divided by weighing factor attributed to it (Table 1), and the results were accumulated and total withdrawal score (TWS) was calculated for each animal. TWS was used as an index of the withdrawal intensity.[19]

Table 1. Weighting factors of morphine withdrawal symptoms.

Behavior signs	Weighting factor
Jumping	4
Wet-dog shake	5
Head shakes	5
Paw tremor	5
Abdomen writhing	5
Genital grooming	5
Body grooming	10
Face wiping	10
Teeth grinding	10
Swallowing	10
Standing on feet	20

Statistical analysis

The results obtained by recording the tolerance and withdrawal syndrome signs were expressed as (n=8) mean ± SEM. To compare the intergroup outcomes, one way ANOVA and the tukey post-test were used. In all analysis, p-values<0.05 represented a significant difference.

Results

Thin layer chromatography of essential oil

Using the TLC method, it was found that the essential oil has different components, especially Linalool with Rf=0.57, which was probably our effective ingredient.

Antioxidant activity of the essential oil

The essential oil of *C. aurantium* could change DPPH into its reduced form. The oil of the peel was able to change purple spot of DPPH on the TLC to the yellow spot. This radical scavenging activity was due to different antioxidant compounds.

Morphine tolerance

Tolerance was completed on the 5th day in control group that received 10mg/kg morphine.

The vehicle group showed tolerance on the 9th day (p-value to control group=0.991). Morhpine tolerance was completed in the 13th day (p-value to control group=0.010, to vehicle group=0.049) with 20 mg/kg of the essential oil. On the other hand tolerance was completed with 40 mg/kg of the essential oil (p-value to control and vehicle < 0.001) and 80 mg/kg of the essential oil (p-value to control group=0.001, to vehicle group=0.007) in the 15th and 13th days respectively. According to the results, it was found that the most effective dose was 40 mg/kg of essence. The group that received only 40 mg/kg of essential oil, showed tolerance on the 3rd day and compared to all the groups, the difference was statistically significant (p-value <0.001) (Figures 1a, 1b). Considering that the effective dose of essential oil showed tolerance on the third day, it could be concluded that the essential oil alone did not have analgesic effect.

The effect of the essential oil on the rat locomotor activity

The locomotor activity test was managed in the morphine+essential oil (40 mg/kg) and morphine+vehicle treated groups. The independent samples t-test (t test between morphine-effective dose of essence (40 mg/kg) and vehicle- morphine: t = 0.698; P = 0.495) showed no significant difference between the two groups. Thus, the effects of essential oil injection on the morphine withdrawal signs were not related to the motor activity in the rats.

Morphine withdrawal syndrome

The intraperitoneally injection of naloxone increased the TWS (14.093±0.428) in the control group (morphine+vehicle) significantly in comparison to the saline group (6.493 ±0.639, p<0.05) (Figure 2).

The comparison of control group and morphine with saline treated group indicated significant difference between them (p<0.05), thus we compared all of the groups with morphine+vehicle treated group.

C. aurantium essential oil administration (20, 40 and 80 mg/kg) reduced the naloxone-induced TWS in a dose independent manner and two doses (40 and 80 mg/kg) of

CEO, showed significant difference compared with the control group ($p<0.001$ for 40 mg/kg and $p<0.01$ for 80 mg/kg) (Figure 3). Data analysis showed that the most effective dose of essence was 40 mg/kg.

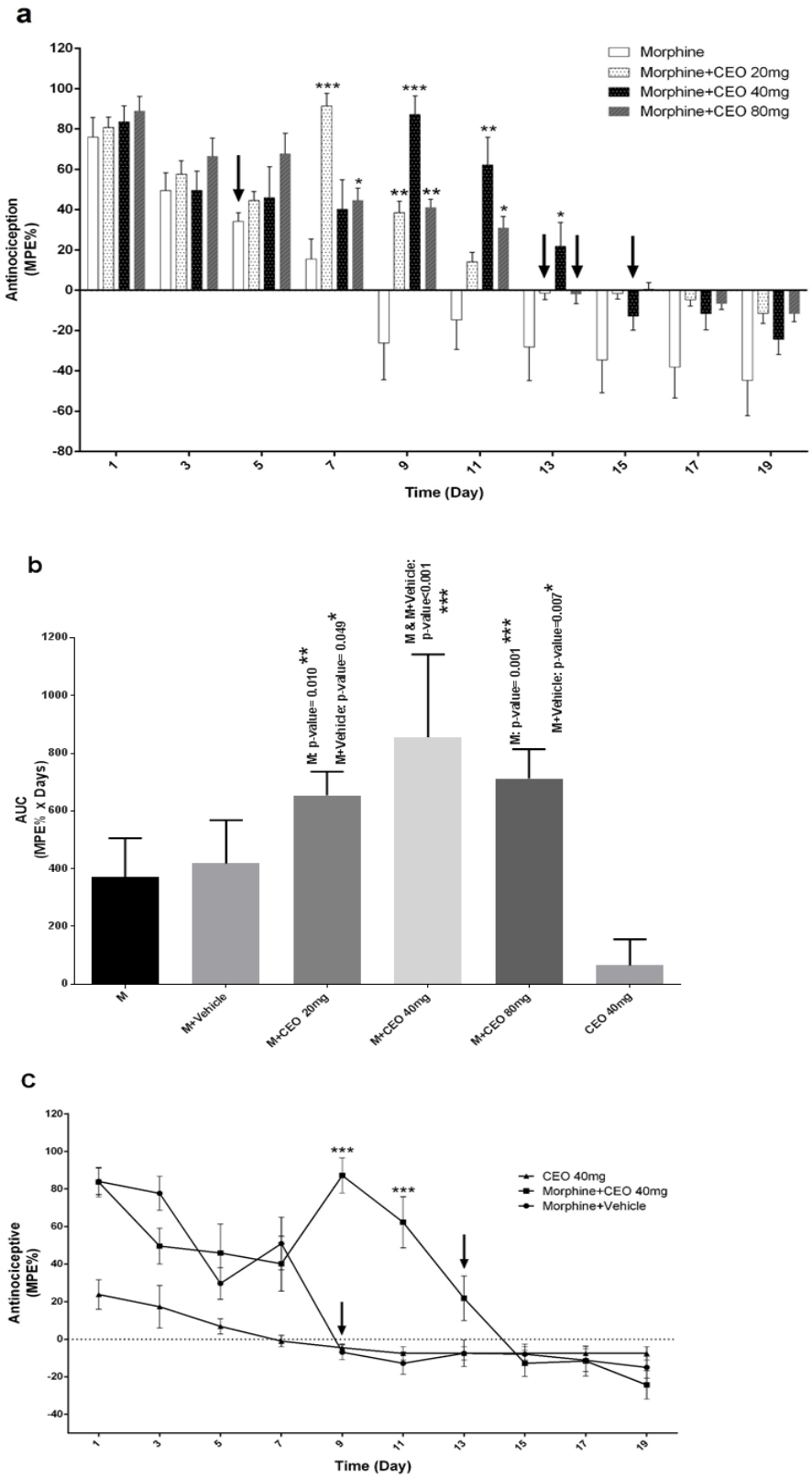

Figure 1. Analgesic effect (a) of morphine (10 mg/kg, i.p), vehicle (4ml/kg, i.p), CEO (20, 40 or 80 mg/kg, i.p). The AUC (b) of 19 days for %MPE were obtained from a. Comparison (c) of antinociceptive effect (MPE %) of 40mg/kg of CEO (the most effective dose) with the effect of 40mg/kg of CEO alone. All data points are expressed as mean ± SEM for eight rats. M morphine; CEO *Citrus aurantium*'s Essential Oil; MPE maximum possible effect; AUC areas under the curve. The arrows represent the day of morphine tolerance. *= p-value≤0.05, **= p-value ≤0.01, ***= p-value≤0.001

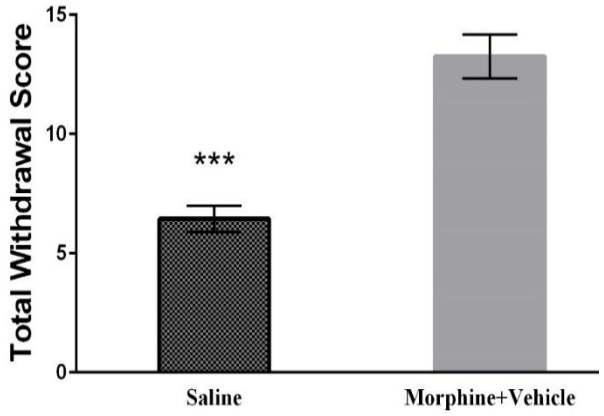

Figure 2. Naloxone (4mg/kg) – induced TWS in control group in comparison to saline group during 60 min of experiment. Data are showed as mean± S.E.M.

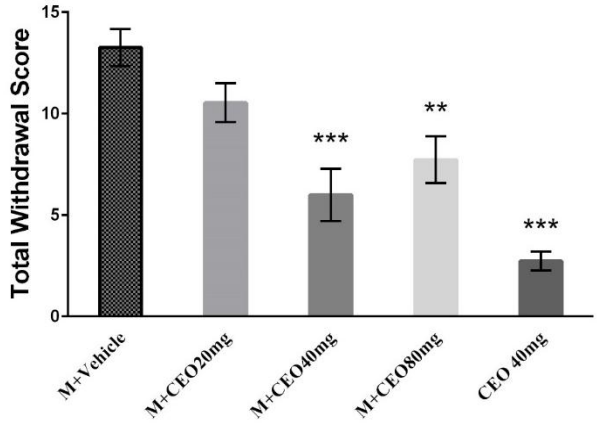

Figure 3. Effects of intraperitoneal injection of *Citrus aurantium* essential oil (CEO) on the expression of naloxone-induced TWS in morphine-dependent rats in comparison to control group (morphine+vehicle). Data are showed as mean± S.E.M. **: p<0.01 and ***: p<0.001.

The results of this study showed that CEO could attenuated the severity of morphine withdrawal syndrome. Table 2 depicts that attenuated effects of CEO on the withdrawal symptoms in comparison with the group that received morphine and vehicle.

Discussion

Repeated exposure to opiates like morphine showed addiction and this issue limited pharmacological use of morphine.

The results of this study showed that chronic administration of CEO, could attenuate morphine-induced tolerance and dependence in a dose independent manner impressively in the rat due to the fact that in both studies, moderate dose (40mg/kg) was more effective than lower and higher doses (20 and 80mg/kg) and significantly improved the tolerance and withdrawal syndroms.

According to the previous studies, morphine caused a neuroinflammatory response in developing the tolerance and dependence by unknown pathways.[20,21]

Linalool is a monoterpene compound that is found as a major ingredient of the many essential oils like *C. aurantium*.[22] Data showed that linalool reduced inflammation by suppression of TNF-α and IL-6 production,[23] reduction of intracellular oxidative stress and inhibition of microglial migration.[22]

Microglia and astrocytes react to chronic morphine administration, with up-regulation of activation markers.[24] In this process, expression of pro-inflammatory cytokines like IL-1, TNF and IL-6 were up regulated and these cytokines produced the other inflammatory derivatives such as nuclear factor-kappaB(NF-κB) and activator protein 1(AP-1).[21]

A study has shown that volatile constituents of *C. aurantium* prevented of the NO formation which produced by different nitric oxide synthase enzymes.[25,26]

In addition according to the data, linalool prevented Lipopolysaccharide (LPS)-induced TNF-α, IL-1, NO and PGE (Prostaglandin E) production. Linalool also inhibited NF-κB activation which promoted by LPS. On the other hand, due to intraperitoneal injection of naloxone methiodide, a peripherally acting μ-opioid receptor preferring antagonist, the analgesic effects of Linalool have been effectively antagonized.

Table 2. A comparison of the morphine withdrawal behaviors precipitated by by naloxone (4 mg/kg) between the experimental groups during the 60-min observation.

Groups		Mor	Sal	Mor + CEO (20 mg /kg)	Mor+ CEO (40 mg/kg)	Mor + CEO (80 mg/kg)	Mor + Veh	CEO (40 mg/kg)
Signs	**Jumping**	7.125±1.54	0±0*	2.857±0.80	3.143±1.033	4.571±1.02	5.429±1.571	0.25±0.25*
	Writhing	6.143±1.65	0±0	1.857±0.884*	0.143±0.143**	0.571±0.369**	7.429±2.707	0±0**
	Wet-dog shake	19.62±4.40***	0±0	4±0.707	0±0	0.375±0.183	1±0.26	0±0
	Swallowing	11.375±2.21	2±0.98***	10±1.722	0.75±0.25***	2.5±0.463***	12.25±0.959	0.25±0.164***
	Paw tremor	12.57±1.81	2.714±0.865***	10.14±1.883	3.857±0.884***	7.714±2.254	12.286±1.672	1±0.309***
	Genital grooming	3.429±1.30	1.857±0.459*	1.286±0.474**	0.429±0.202***	0.143±0.143***	5±0.655	0.286±0.184***
	Body grooming	7.143±2.01	9.714±1.358	4±1.069	3.143±0.80*	1.857±0.595**	8.857±1.10	7.143±1.503
	Face wiping	15±1.363	14.14±1.87	7.571±0.782	6.429±0.751*	6.286±1.375*	12.286±0.747	7±1.558
	Teeth chattering	10.28±1.71	1.571±0.649***	6.286±0.680	0.714±0.286***	3.714±0.714**	9.286±1.96	0±0***
	Head shakes	19±2.582***	10.143±2.415	6.286±0.944	2.429±0.896	4.286±1.190	6.571±0.685	0.143±0.143*
	Rearing	28±5.863	17±1.36	18.714±1.796	16.28±1.643	10.42±1.525**	29.28±3.822	9.429±1.925***

All data are showed as mean ± SEM. *Mor* morphine, *Sal* saline, *CEO Citrus aurantium essential oil, Veh* vehicle.
*p<0.05; **p<0.01; ***p<0.001 compared to the control group (morphine + vehicle)

In another group, the antinociceptive effects of intraperitoneal or on the other hand, the improved antinociceptive effects induced by simultaneous injection of morphine and Linalool were antagonized with previous injection of naloxone hydrochloride. The results of this study indicated that Linalool is likely to be involved in peripheral receptors of opioids.[27-29]

Other studies showed that Linalool injection could significantly reduce the carrageenin- induced oedema and acetic acid-induced writhing. This effect was completely antagonized by atropine (the muscarinic receptor antagonist) and naloxone. Considering the proven effects of formalin test and hot-plate, cholinergic, opioidergic and dopaminergic receptors were likely to be involved.[30,31]

Based on the researches, in addition to the mechanisms mentioned, linalool is likely to interact with adenosine and N-methyl-D-aspartate (NMDA) receptors that play an important role in morphine tolerance.[32-34]

According to the previous statements, the essential oil, due to its anti-inflammatory and antioxidant effects, is likely to have a decreasing effect on tolerance and withdrawal symptoms of morphine.

Further studies are necessary to found the exact mechanisms of morphine dependency.

Conclusion

According to our results, essential oil of *C. aurantium* peels, could reduce withdrawal syndrome and delay tolerance to morphine, significantly.

The inhibition of the inflammatory responses and glia activation induced by morphine and prevention of NO formation were the probable mechanisms for the CEO effect on the morphine dependence and tolerance.

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Conflict of interests

The authors claim that there is no conflict of interest.

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