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Original Article

Antinociceptive activity of *Cnicus benedictus L.* leaf extract: a mechanistic evaluation

Davoud Ahmadimoghaddam[1,2], Reihaneh Sadeghian[3,4], Akram Ranjbar[1,2], Zohreh Izadidastenaei[5], and Saeed Mohammadi[5,6,*]

[1]Department of Pharmacology and Toxicology, School of Pharmacy, Hamadan University of Medical Sciences, Hamadan, I.R. Iran.
[2]Medicinal Plants and Natural Products Research Center, Hamadan University of Medical Sciences, Hamadan, I.R. Iran.
[3]Student Research Committee, Hamadan University of Medical Sciences, Hamadan, I.R. Iran.
[4]Medical Plants Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, I.R. Iran.
[5]Neurophysiology Research Center, Hamadan University of Medical Sciences, Hamadan, I.R. Iran.
[6]Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, I.R. Iran.

Abstract

Background and purpose: *Cnicus benedictus*, a medicinal herb, traditionally had been used for the treatment of stomachache pain. In this study, the possible efficacy of *Cnicus benedictus* leaf methanolic extract (CBHE) and also cnicin, one of its major constituents, was measured on pain.
Experimental approach: In this study, pain assessment tests include writhing, tail-flick (TF), and formalin-induced paw licking test (FIPLT) were used. To understand the possible mediated anti-nociceptive mechanism of CBHE, the opioid mechanism(s), and involvement of the L-arginine/ nitric oxide/cGMP/ATP-sensitive potassium channel pathway (LNCaP) were scrutinized.
Findings/Results: In TF and writhing tests, CBHE (150 and 300 mg/kg, i.p) remarkably exhibited an anti-nociceptive effect compared to that of the control. Furthermore, CBHE (150 and 300 mg/kg, i.p) in comparison with the control showed a noteworthy anti-nociceptive effect ($P < 0.01$) in the tonic phase of FIPLT. In the writhing test, administration of selective opioid antagonist (naltrindole, nor-binaltorphimine, and naloxonazine) attenuated the anti-nociceptive effect of CBHE (300 mg/kg) in comparison with control. Moreover, pre-treatment with Nω-nitro-L-arginine methyl ester hydrochloride, L-arginine hydrochloride, and glibenclamide significantly blocked the CBHE (300 mg/kg) antinociception ($P < 0.05$) while administration of sodium nitroprusside remarkably potentiated ($P < 0.05$) the antinociception induced by CBHE in the tonic phase of the FIPLT. Besides, cnicin (30 mg/kg) showed noteworthy anti-nociceptive effects in writhing, TF, and FIPLT paradigms.
Conclusion and implications: Taken together, we elucidate that both CBHE and cnicin demonstrated anti-nociceptive effects in behavioral tests. The possible mechanisms of CBHE antinociception may involve in various neural signaling and modulatory pathways including LNCaP and opioidergic mechanisms.

Keywords: Cnicin; *Cnicus benedictus*; L-arginine/NO/cGMP/K (ATP); Opioid Receptor; Peripheral antinociception.

INTRODUCTION

Pain is not only one of the most important issues that humankind has been facing to it for since thousand years ago, but also it is a portent of inflammatory responses (1).

Nowadays, the drugs used for pain relief are mainly opioids. Non-opioid drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are also used for pain (2).

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*Corresponding author: S. Mohammadi
Tel: +98-8132518064, Fax: +98-8132518065
Email: s_mohammadi@srbiau.ac.ir

Administration of opioids over the transient or long-term can cause side effects, such as dependency (3). Consequently, there is a strong tendency toward the development of novel analgesic/antinociceptive drugs with better safety profiles (4). Medicinal plants are an important source of new chemicals with potent therapeutic effects (5). However, in most cases, the origin and mechanism of the therapeutic effects are unknown (6).

Cnicus benedictus L. popularly known as "blessed thistle", the sole species in the genus *Cnicus*, is a thistle-like medicinal plant in the family Asteraceae. It is an annual plant with leathery leaves. The leaf of the plant is astringent, diuretic, bitter, diaphoretic, and stomachic (7,8). An infusion of the whole plant has also been used for the treatment of arthritis, liver, and gallbladder problems (9). Blessed thistle leaves, flowers, and stems have been used in "bitter" tonic drinks and in other preparations taken orally to enhance appetite and digestion (10). This medicinal plant has been tested in laboratory investigations for its properties against cancer, infections, and oxidative stress (11,12).

One of the major constituents that exist in *Cnicus benedictus* is "cnicin" that belongs to the terpenes family (13,14). There are numerous documents concerning the effects of terpene on treating pain (15,16). It has been also demonstrated that cnicin (as a terpene) has antibacterial and anti-inflammatory effects (17,18).

Due to the chemical compounds that exist in this plant, such as terpene (i.e. cnicin), and the strong association of this components with tranquilizing and antinociceptive effects, and because the analgesic effect of this medicinal plant's referred in the "Canon of Medicine, an encyclopedia of medicine by Avicenna (19,20), we decided to evaluate the antinociceptive effects of *Cnicus benedictus* leaf methanolic extract (CBHE) and one of its major constituent, cnicin, in the rats. To elucidate the possible underlying mechanism(s) of CBHE antinociception, the involvement of the L-arginine (arg)/nitric oxide (NO)/cGMP/ ATP-sensitive K^+ channels (K_{ATP}) channel pathway (LNCaP) and opioids systems were analyzed.

MATERIALS AND METHODS

The extract of Cnicus benedictus L.

The fresh leaves of the CBHE (1 kg) were collected from the mount Alvand, Hamadan, Iran, and then approved by a botanist. A specimen (No. 2573) was retained in the herbarium of the Bu-Ali Sina University, Hamadan, Iran for identification. After drying in the shade, the powdered plant material (160 g) was macerated in 300 mL of methanol for three consecutive 48-h extractions each, at room temperature. The extract was separated by filtration (Whatman No.1 filter paper) and the filtrates were combined and concentrated on a rotary evaporator to obtain 34.176 g of methanolic extract of *Cnicus benedictus.*

Animals

Male Wistar rats (220-250 g) were purchased from Elm-Bavarian Institute, Iran, and were kept in animals' room under standard conditions 12/12-h dark/light cycle and temperature conditions of 22 ± 1 °C. The rats had free access to water and nourishment in their enclosures. The present research project has been approved by The Local Ethics Committee of Hamadan University of Medical Sciences, Hamadan, I.R. Iran (HUMSS, No. IR.UMSHA.REC.1396.390).

Administration routes and reagents

The agonist and antagonist drugs and the dosage were selected according to the previous studies and on pilot experiments in our laboratory (21-23). For all tests, the CBHE (50, 150, and 300 mg/kg, i.p.) and cnicin (8, 20, and 30 mg/kg, i.p.) were dissolved in dimethyl sulfoxide (DMSO). Morphine sulfate (Morph; 1 mg/kg, i.p.), naloxone (NLX; 1 mg/kg, i.p.), diclofenac (Diclo; 10 mg/kg, i.p.), and xylazine were purchased from Daroopakhsh (Iran). Acetic acid and formalin were purchased from Merck (Germany). Cnicin isolated from *Cnicus benedictus* L., L-arg hydrochloride (L-arg HCL; 25, 50, 100 μg/paw), Nω-nitro-L-arg methyl ester hydrochloride (L-NAME; 25, 50, 100 μg/paw), sodium nitroprusside (SNP; 125, 250, 500 μg/paw), methylene blue (MB; 100, 200, 400 μg/paw), glibenclamide (Gli; 25, 50, 100 μg/paw), naltrindole (NAL; 0.99 mg/kg,

i.p.), nor-binaltorphimine (NBT; 1.03 mg/kg, i.p.), naloxonazine (NAX; 3.5 mg/kg, i.p.), and glutamate all were purchased from Sigma Aldrich Company; USA.

Determination of median lethal dose

The median lethal dose (LD_{50}) was determined based on the previous report (24).

Tail-flick test

This test was carried out by a tail-flick meter (LE7106 Harvard apparatus, USA) and in accordance with the previously published method (25).

Writhing test

Rats were placed in a translucent plastic box and the doses of CBHE were i.p. administrated 20 min before to i.p. injection of acetic acid 0.6% (in a dose volume of 10 mL/kg body-weight) for the ability to suppress abdominal constriction responses (24,26).

Formalin induced paw licking test

Formalin induced paw licking test (FIPLT) was operated according to the method previously reported (27).

Involvement of L-arg/NO pathway

To determine whether CBHE antinociception effects or reduce the sensitivity of pain had related to L-arg/NO pathway or not, animals were pre-treated with different doses of L-arg HCL (25-100 µg/paw), L-NAME (25-100 µg/paw) and SNP (125-500 µg/paw) and/or their vehicles, 10 min before CBHE (300 mg/kg, i.p) in rats utilizing FIPLT and nociceptive feedbacks were assessed 20 min later (28,29).

Involvement of cGMP pathway

To assess the probable involvement of cGMP in the reduced sensitivity of pain action caused by CBHE, animals were pre-treated with varying doses of MB (100-400 µg/paw) or its vehicle, 10 min before CBHE (300 mg/kg, i.p) administration (21).

Involvement of K_{ATP} channel pathway

The possible contribution of the potassium channel in the antinociceptive effect of CBHE was evaluated using the method previously described (21).

Involvement of selective opioid receptors (μ, κ, and δ) in the antinociceptive action of CBHE

The roles of specific opioid receptor subtypes (μ, κ, and δ) were checked using pretreatment of NAL (0.99 mg/kg, i.p.), NBT (1.03 mg/kg, i.p.), and NAX (3.5 mg/kg, i.p.) 15 min before administration of DMSO, CBHE (300 mg/kg i.p.) or cnicin (30 mg/kg, i.p.) (30,31).

Motor performance (rotarod test)

Motor performance was evaluated as previously described (32).

Statistical analysis

Data were presented as mean ± SEM and one-way ANOVA analysis of variance followed by Tukey post hoc test has been used to indicate statistically significant differences. $P < 0.05$ was determined as an index of significance. SPSS software was used as data analysis.

RESULTS

Acute toxicity test

In this experimental model, 72 h after oral administration of various doses of CBHE or cnicin no fatalities were recorded (data not shown).

Tail-flick test

According to Fig. 1A, in the 60 and 90 min, CBHE treatment at 150 ($P < 0.05$) and 300 mg/kg ($P < 0.01$) made a marked difference in comparison with the control (DMSO) group.

According to the Fig. 1B, administration of NLX completely reversed the antinociceptive effects of CBHE as well as morphine.

Writhing test

As can be seen in Fig. 2A, CBHE at 150 and 300 mg/kg of ($P < 0.05$ and $P < 0.01$, respectively) decreased writhing numbers compared to the control group. Furthermore, it was revealed that the co-administration of NLX with a high dose of the CBHE (300 mg/kg) reversed the antinociceptive effects of the CBHE when used alone (Fig. 2B).

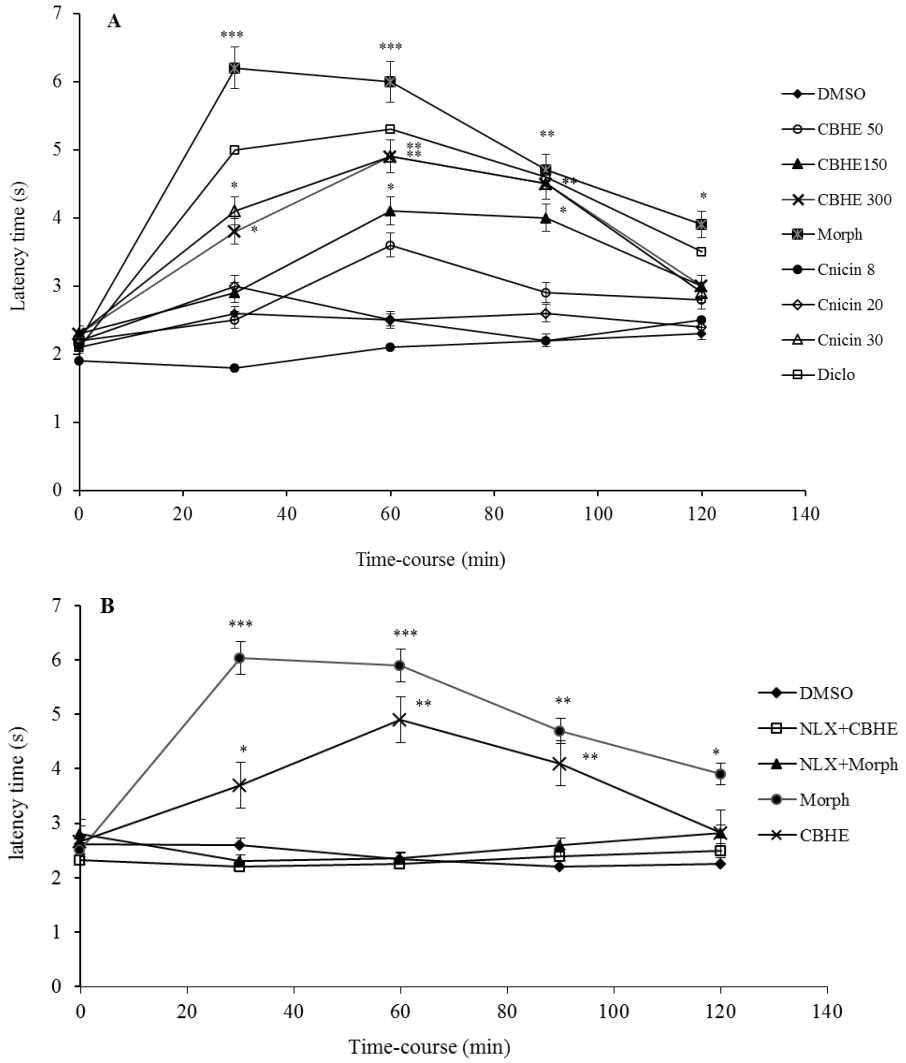

Fig. 1. (A) Evaluation of the antinociceptive effect of CBHE at 50, 150, and 300 mg/kg and cnicin at 8, 20, and 30 mg/kg in the tail-flick test. Morph at 1 mg/kg and diclofenac at 10 mg/kg were used. (B) The influence of the pre-treatment with NLX (3.5 mg/kg) on the antinociceptive effect of CBHE at 300 mg/kg in the tail-flick test. Data represent means ± SEM; n = 5, in each group. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001; indicate significant differences in comparison with the control group (DMSO). CBHE, *Cnicus benedictus* methanolic extract; DMSO, dimethyl sulfoxide; Morph, morphine; Diclo, diclofenac; NLX, naloxone.

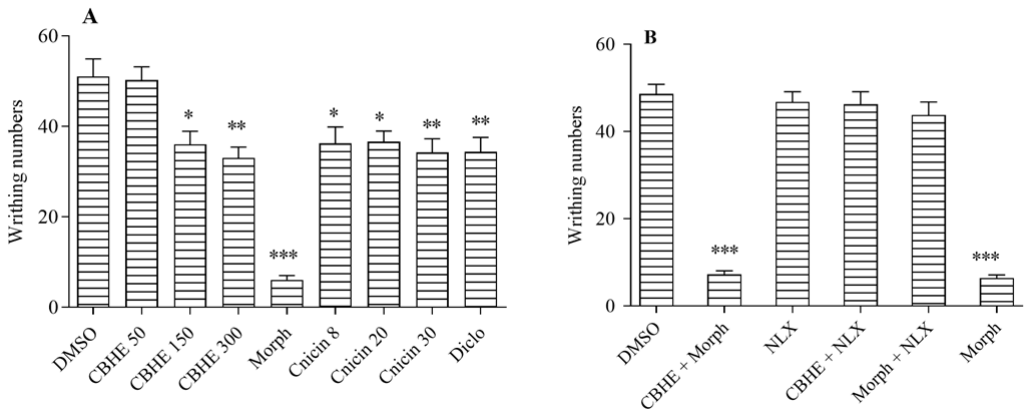

Fig. 2. (A) The dose-related effect of CBHE at 50, 150, and 300 mg/kg and included cnicin at 8, 20, and 30 mg/kg in the writhing test. Morph at 1 mg/kg and diclofenac at 10 mg/kg were used. (B) The pre-treatment influence of NLX (3.5 mg/kg) on the antinociceptive effect of CBHE at 300 mg/kg in writhing test. Data represent means ± SEM; n = 5, in each group. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001; indicate significant differences in comparison with the control group (DMSO). CBHE, *Cnicus benedictus* methanolic extract; DMSO, dimethyl sulfoxide; Morph, morphine; Diclo, diclofenac; NLX, naloxone.

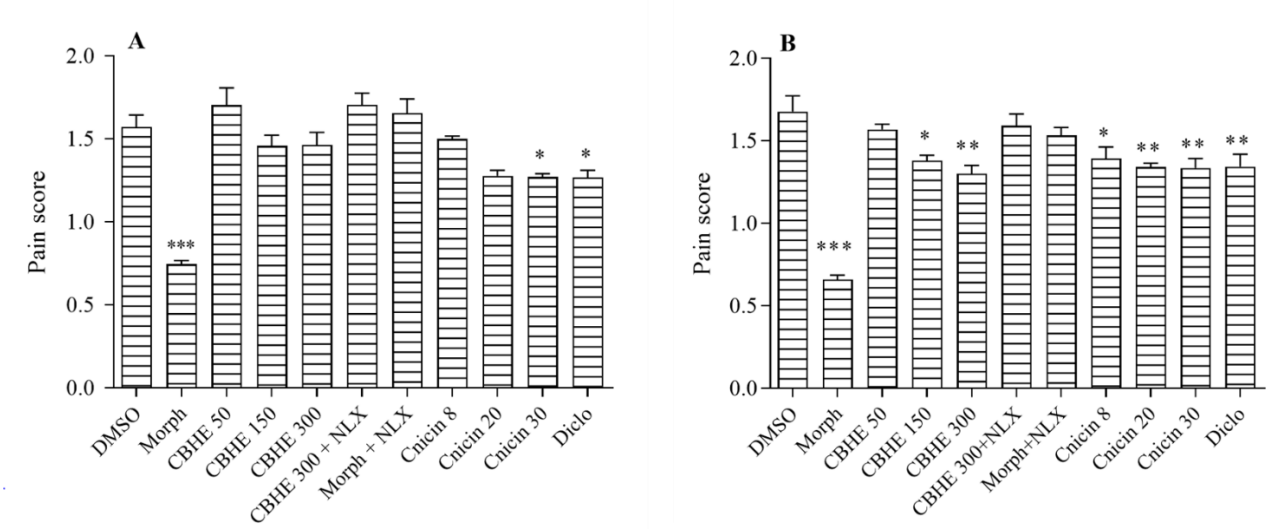

Fig. 3. Comparing the effects of CBHE at 50, 150, 300 mg/kg, Morph (1 mg/kg), Diclo (10 mg/kg), CBHE 300 mg/kg + NLX, morphine + NLX, cnicin at 8, 20, and 30 mg/kg on (A) nociceptive phase and (B) tonic phase of formalin test in rats. Data represent means ± SEM; n = 5, in each group. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001; indicate significant differences in comparison with the control group (DMSO). CBHE, *Cnicus benedictus* methanolic extract; DMSO, dimethyl sulfoxide; Morph, morphine; Diclo, diclofenac; NLX, naloxone.

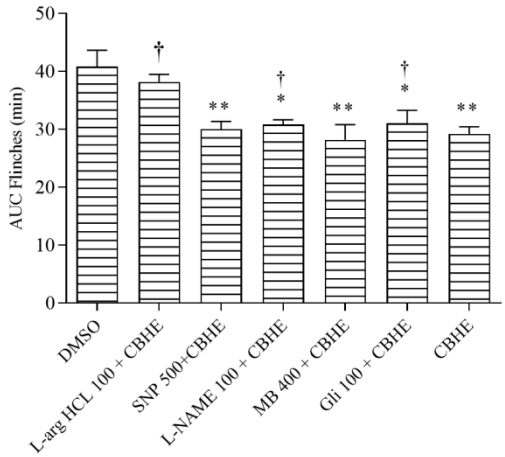

Fig. 4. The Effect of maximum doses of L-arg HCL, L-NAME, SNP, MB, and Gli on CBHE (300 mg/kg)-induced peripheral antinociception during the early phase in the rat formalin test. Rats were pre-treated with a local injection of L-arg HCL, L-NAME, SNP, MB and Gli before CBHE into the right paw. Data are expressed as the area under the number of flinches against time curve (AUC) corresponding to the first phase of the formalin test. The bars represent means ± SEM; n = 5, in each group. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001; indicate significant differences in comparison with the control group (DMSO); †*P* < 0.05 statistically different from CBHE-treated group. L-arg HCL, L-argininehydrochloride; L-NAME, Nω-nitro-L-arginine methyl ester hydrochloride; SNP, sodium nitroprusside; MB, methylene blue; Gli, glibenclamide; CBHE, *Cnicus benedictus* methanolic extract.

Formalin-induced paw licking test

According to the results from Fig. 3A, in the nociceptive phase (phase I), the injection of cnicin (30 mg/kg) had a significant antinociceptive effect (*P* < 0.05) compared to the control group.

In the tonic phase (phase II), CBHE at 150 and 300 mg/kg (*P* < 0.05 and *P* < 0.01, respectively), had significant antinociceptive effect compared to the control group (Fig. 3B).

Involvement of the LNCaP in CBHE-induced antinociception in the early phase of FIPLT

The peripheral pre-treatment with SNP (500 μg/paw, i.p.) had a significant effect (*P* < 0.01) on the antinociceptive response of CBHE (300 mg/kg, i.p.) in comparison to the control (Fig. 4).

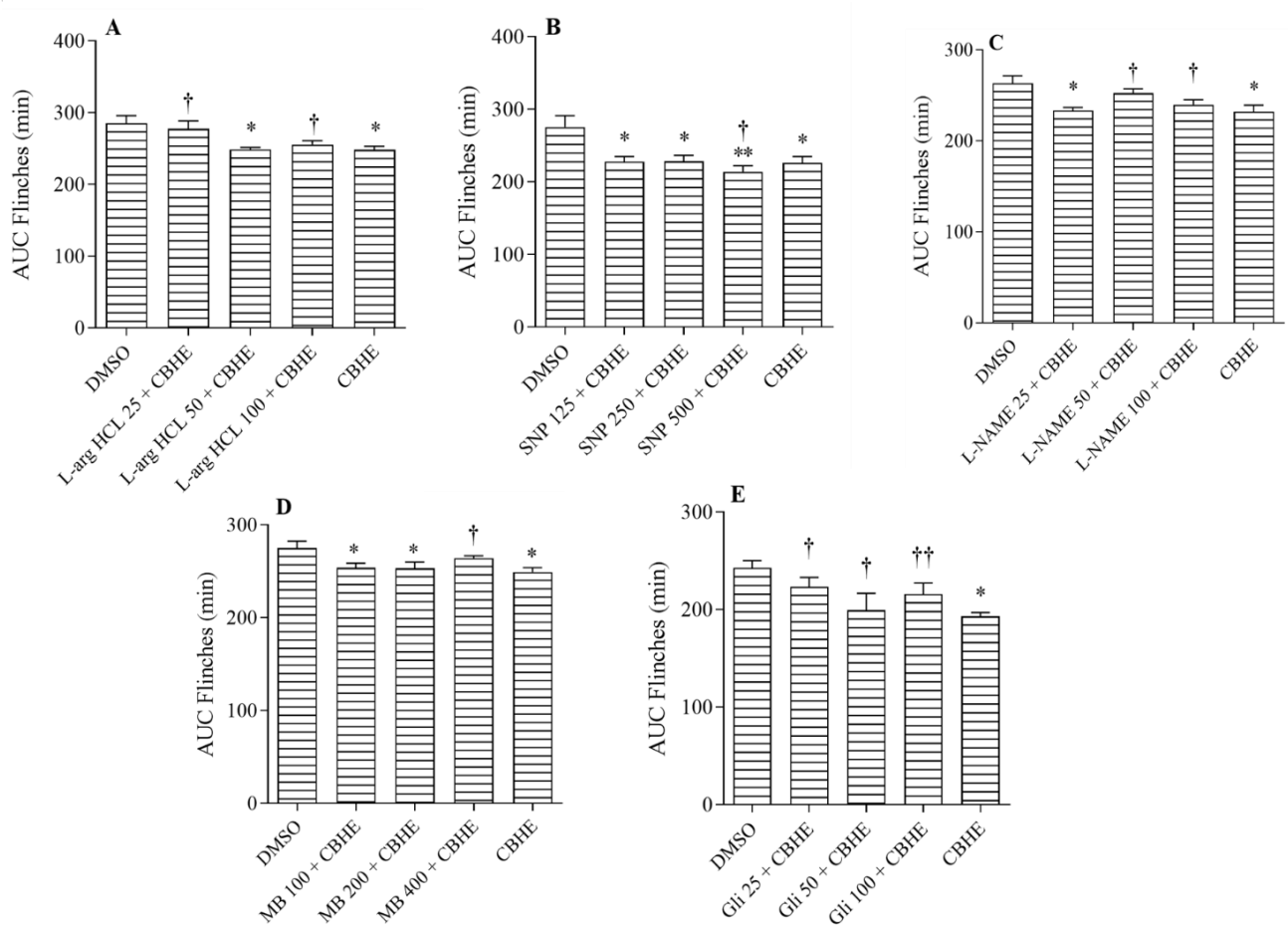

Fig. 5. Effect of (A) L-arg HCL, (B) SNP, (C) L-NAME, (D) MB, and (E) Gli on CBHE (300 mg/kg)-induced peripheral antinociception during the late phase of formalin test. Rats were pre-treated with a local injection of L-arg HCL, L-NAME, SNP, MB, and Gli then CBHE (i.p.) into the right paw. Data are expressed as the area under the number of flinches against time curve (AUC) corresponding to the first phase of the formalin test. The bars represent means ± SEM; n = 5, in each group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; indicate significant differences in comparison with the control group (DMSO); $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ statistically different from CBHE-treated group. L-arg HCL, L-argininehydrochloride; L-NAME, Nω-nitro-L-arginine methyl ester hydrochloride; SNP, sodium nitroprusside; MB, methylene blue; Gli, glibenclamide; CBHE, *Cnicus benedictus* methanolic extract.

Involvement of the L-arg-NO pathway in CBHE-induced antinociception in the late phase of FIPLT

We observed that co-administration of L-arg HCL (50 µg/paw) and CBHE (300 mg/kg, i.p.) decreased pain score in comparison to the control ($P < 0.05$; Fig. 5A). Moreover, co-administration of SNP (125, 250, and 500 µg/paw) plus CBHE (300 mg/kg) attenuated pain score in comparison to the control ($P < 0.05$; Fig. 5B). However, co-administration of L-NAME (25 µg/paw) plus CBHE (300 mg/kg) could decrease pain score in comparison to the control ($P < 0.05$; Fig. 5C).

Involvement of the guanylyl cyclase inhibition in CBHE-induced antinociception in the late phase of FIPLT

The analysis showed that concomitant administration of MB (100 and 200 µg/paw) plus CBHE (300 mg/kg) attenuated pain score in comparison to the control ($P < 0.05$; Fig. 5D).

Involvement of the K_{ATP} channels in CBHE-induced antinociception in late phase FIPLT

As shown in the Fig. 5E, concomitant administration of Gli (25, 50, and 100 µg/paw, i.p.) plus CBHE (300 mg/kg) reversed the antinociception effect produced by CBHE (300 mg/kg, i.p.) when used alone ($P < 0.05$, $P < 0.05$, $P < 0.01$, respectively).

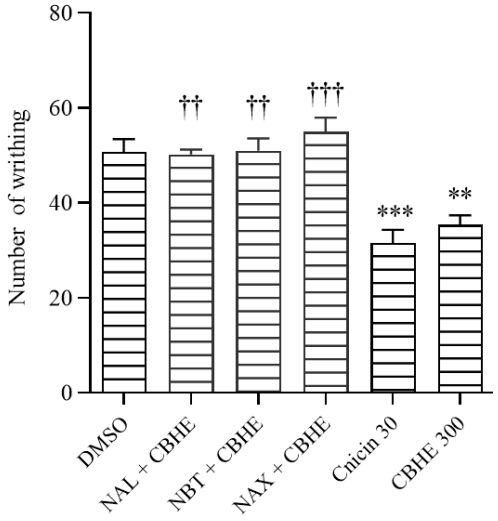

Fig. 6. Effects of pre-treatment of NAL (0.99 mg/kg, i.p.), NBT (1.03 mg/kg, i.p.) and NAX (3.5 mg/kg, i.p.) on the anti-nociceptive effect of CBHE (300 mg/kg) in the acetic acid-induced writhing test. Data represent means ± SEM (n = 5, in each group). Data represent means ± SEM; n = 5, in each group. ***P* < 0.01, and ****P* < 0.001; indicate significant differences in comparison with the control group (DMSO); ††*P* < 0.01 and †††*P* < 0.001 statistically different from CBHE-treated group. NAL, naltrindole; NBT, nor-binaltorphimine; NAX, naloxonazine; CBHE, *Cnicus benedictus* methanolic extract.

Role of selective opioid receptors in the antinociceptive effect of CBHE

Co-administration of NAL, NBT (*P* < 0.01), and NAX (*P* < 0.001) plus CBHE reversed the antinociception produced by CBHE (300 mg/kg, i.p.; Fig. 6) when used alone.

Motor performance activity

Administration of different doses of CBHE (50, 150, and 300 mg/kg) and cnicin (8, 20, and 30 mg/kg) did not show any significant changes in motor performance compared to xylazine (data not shown).

DISCUSSION

The results of this study report the analgesic activity of CBHE and one of its constituents, cnicin. We also demonstrated a possible role of the LNCaP and opioid receptors in the antinociceptive effects of CBHE.

The data obtained from the tail-flick test indicate that the infusion of CBHE inhibited the nociception induced by thermal light. In the study carried out by Zarei *et al*. it is well established that *Inula britannica*, a medicinal plant from the Asteraceae family at 100 mg/kg (i.p) has an antinociceptive effect when evaluated by tail-flick test. In our study, administration of CBHE attenuated pain which revealed that CBHE probably has a direct effect on both spinal and supraspinal levels (29,33).

The second pain model used for evaluating the effect of CBHE and cnicin on acute pain was an acetic acid-induced writhing test. The administration of acetic acid triggers the release of considerable mediators such as substance-p and prostaglandins (34). These mediators by increasing vascular absorption can induce nociceptors that are sensitive to opioids and/or non-opioid compounds (35). In the study carried out by Schneider *et al*. it has been proven that the administration of cnicin could decrease the number of writhing in the acetic acid-induced writhing test (12). In this study, both CBHE and cnicin remarkably decreased the number of constrictions, induced by acetic acid injections, in the abdomen of the animals. The reduction in the number of constrictions after cnicin administration proposes that this terpene may exert its analgesic activity indirectly by decreasing inflammatory mediators and/or directly by inhibition of their receptors.

In the previous study, it is demonstrated that borneol, one of the important monoterpene constituent of CBHE, reduces nociceptive behavior in both phases of the formalin test (36). We founded that the administration of CBHE in formalin test can inhibit pain in the tonic phase. The inhibitory effect on the tonic phase of pain in the FIPLT could be *via* blockade of the inflammation process that causes the release of prostaglandins E_2 and $F_{2\alpha}$ which can lead to the sensitization of central neural pain (37).

The LNCaP plays an important role in the analgesic activity of several constituents in the FIPLT (38). NO boosts the concentration of cGMP levels, leading to the activation of potassium channel which in turn induces analgesia (39). The previous study demonstrated that SNP produces significant

peripheral antinociception in the FIPLT (40). In our research, the administration of SNP (500 mg/kg) potentiated the analgesic activity of CBHE in phase II which is in accordance with the previous study.

Oghbaei *et al.* reported that the administration of L-NAME elicits antinociception evaluated by FIPLT (41). In contrast with this view, our findings indicated that co-administration of L-NAME (100 mg/kg) and CBHE in both phases of the FIPLT could reverse antinociception of CBHE. It can be suggested that L-NAME causes the antinociceptive effect by stimulation of L-arg/NO/cGMP pathway since the antinociceptive effect of L-NAME can be antagonized by L-NMMA and abolished by the guanylate cyclase inhibitors (MB).

K_{ATP} participates in the antinociception induced by many drugs that activate them in the peripheral nervous system. It was shown that the co-administration of Gli and *Urtica circularis* extract did not reverse the antinociceptive effect of *Urtica circularis* extract alone (42). In contrast, our findings indicated that co-administration of Gli and CBHE could reverse the antinociception of CBHE. It can be suggested that KATP has been involved in the antinociceptive effect of CBHE.

We showed that pretreatment with L-arg HCL, L-NAME, and Gli before formalin injection reversed the analgesic activity of CBHE in phase I of the FIPLT. This indicates the LNCaP did not participate in the analgesic activity of CBHE in the early phase of FIPLT. Therefore, it looks that the analgesic activity of CBHE in the early phase of FIPLT might be related to the opioidergic pathway (43).

To evaluate the interaction between the opioid system and the analgesic activity of CBHE, naloxone, a non-selective opioid antagonist, was used (44). The results showed that naloxone reversed the analgesic effect of CBHE, indicating the role of opioid receptors in the analgesic effect of CBHE (45). We used opioid receptors subtype-specific antagonist-NAX, NAL, and NBT to evaluate the potential involvements of particular opioid receptors subtypes to the antinociceptive activity (46). In the acetic acid-induced writhing test, the antinociceptive effect of CBHE was antagonized by pretreatment with NBT, NAL, and NAX. These findings indicated that all opioid receptors (δ, κ, and μ) may play a role in the analgesic effect of CBHE.

CONCLUSION

Taken together, we elucidate that both CBHE and cnicin have antinociceptive effects in behavioral tests. The possible antinociception mechanisms of CBHE may involve various neural signaling and modulatory pathways including LNCaP and opioidergic mechanisms. The present findings may offer a new approach for clinical trials using *Cnicus benedictus* as an herbal remedy for the treatment of nociceptive and inflammatory pain.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest in this study.

AUTHORS' CONTRIBUTION

S. Mohammadi performed the majority of the experiments and data collection. D. Ahmadimoghaddam designed the experiments, conducted data analysis, and interpretation. A. Ranjbar provided technical support. Z. Izadidastenaei drafted the manuscript.

REFERENCES

1. Williams ACdC, Craig KD. Updating the definition of pain. Pain. 2016;157(11):2420-2423. DOI: 10.1097/j.pain.0000000000000613.
2. Fallahzadeh AR, Mohammadi S. An investigation of the antinociceptive and anti-inflammatory effects of hydroalcoholic extract of Inula helenium on male rats. J Babol Univ Med Sci. 2016;18(12):57-63. DOI: 10.22088/jbums.18.12.57.

3. Zarei M, Mohammadi S, Jabbari S, Shahidi S. Intracerebroventricular microinjection of kaempferol on memory retention of passive avoidance learning in rats: involvement of cholinergic mechanism (s). Int J Neurosci. 2019;129(12):1203-1212. DOI: 10.1080/00207454.2019.1653867.
4. Mahmoodi M, Mohammadi S, Zarei M. Antinociceptive effect of hydroalcoholic leaf extract of *Tribulus terrestris* L. in male rat. J Babol Univ Med Sci. 2013;15(6):36-43.
5. Mohammadi S, Golshani Y. Neuroprotective effects of rhamnazin as a flavonoid on chronic stress-induced cognitive impairment. J Adv Neurosci Res. 2017;4:30-37. DOI: 10.15379/2409-3564.2017.04.02.03.
6. Aghababaei R, Javadi I, Nili-Ahmadabadi A, Parsafar S, Ahmadimoghaddam D. Occurrence of bacterial and toxic metals contamination in illegal opioid-like drugs in Iran: a significant health challenge in drug abusers. DARU. 2018;26(1):77-83. DOI: 10.1007/s40199-018-0205-5.
7. Durdun C, Papuc C, Crivineanu M, Nicorescu V. Antioxidant potential of *Lycopodium clavatum* and *Cnicus benedictus* hydroethanolic extracts on stressed mice. Sci Works. C Series. Vet Med. 2011;57(3):61-68.
8. Toscano LT, Gonçalves GA, Shahidi F. Phenolics from purple grape juice increase serum antioxidant status and improve lipid profile and blood pressure in healthy adults under intense physical training. J Funct Foods. 2017;33:419-424. DOI: 10.1016/j.jff.2017.03.063.
9. Farzaei MH, Farzaei F, Abdollahi M, Abbasabadi Z, Abdolghaffari AH, Mehraban B. A mechanistic review on medicinal plants used for rheumatoid arthritis in traditional Persian medicine. J Pharm Pharmacol. 2016; 68(10):1233-1248. DOI: 10.1111/jphp.12606.
10. Briskin DP. Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. Plant physiol. 2000;124(2):507-514. DOI: 10.1104/pp.124.2.507.
11. Can Z, Baltaş N, Keskin S, Yıldız O, Kolaylı S. Properties of antioxidant and anti-inflammatory activity and phenolic profiles of şevketi bostan (*Cnicus benedictus* L.) cultivated in aegean region from Turkey. TURJAF. 2017;5(4):308-314. DOI: 10.24925/turjaf.v5i4.308-314.1054.
12. Horn G, Kupfer A, Rademacher A, Kluge H, Kalbitz J, Eißner H, *et al*. *Cnicus benedictus* as a potential low input oil crop. Eur J Lipid Sci Tech. 2015;117(4):561-566. DOI: 10.1002/ejlt.201400158.
13. Kumar NS, Paul D, Allakonda L. Liquid Chromatography-Mass Spectrometry (LC-MS): approaches to adulterant detection in herbal products. In: Medicinal plants-recent advances in research and development. 3rd ed. New York: Springer; 2016. pp. 73-95. DOI: 10.1007/978-981-10-1085-9_3.
14. Ulbricht C, Basch E, Dacey C, Dith S, Hammerness P, Hashmi S, *et al*. An evidence-based systematic review of blessed thistle (*Cnicus benedictus*) by the natural standard research collaboration. J Diet Suppl. 2008;5(4):422-437. DOI: 10.1080/19390210802519754.
15. Guimarães AG, Serafini MR, Quintans-Júnior LJ. Terpenes and derivatives as a new perspective for pain treatment: a patent review. Expert Opin Ther Pat. 2014;24(3):243-265. DOI: 10.1517/13543776.2014.870154.
16. Bak CW, Yoon SJ, Chung H. Effects of an α-blocker and terpene mixture for pain control and spontaneous expulsion of ureter stone. Korean J Urol. 2007;48(5):517-521. DOI: 10.4111/kju.2007.48.5.517.
17. Bach SM, Fortuna MA, Attarian R, de Trimarco JT, Catalán C, Av-Gay Y, *et al*. Antibacterial and cytotoxic activities of the sesquiterpene lactones cnicin and onopordopicrin. Nat Prod Commun. 2011;6(2):163-166.
18. Erel SB, Karaalp C, Bedir E, Kaehlig H, Glasl S, Khan S, *et al*. Secondary metabolites of *Centaurea calolepis* and evaluation of cnicin for anti-inflammatory, antioxidant, and cytotoxic activities. Pharm Biol. 2011;49(8):840-849. DOI: 10.3109/13880209.2010.551538.
19. Aziz E, Nathan B, McKeever J. Anesthetic and analgesic practices in Avicenna's *Canon of Medicine*. Am J Chin Med. 2000;28(1):147-151. DOI: 10.1142/S0192415X00000180.
20. Heydari M, Hashem Hashempur M, Zargaran A. Medicinal aspects of opium as described in Avicenna's Canon of Medicine. Acta Med Hist Adriat. 2013;15;11(1):101-112. DOI:10.1016/S1474-4422(20)30263-5.
21. Ortiz MI, Castro-Olguín J, Peña-Samaniego N, Castañeda-Hernández G. Probable activation of the opioid receptor-nitric oxide-cyclic GMP-K+ channels pathway by codeine. Pharmacol Biochem Behav. 2005;82(4):695-703. DOI: 10.1016/j.pbb.2005.11.011.
22. Nili-Ahmadabadi A, Borzouee Z, Ahmadimoghaddam D, Firozian F, Dastan D. The occurrence of acetaminophen/codeine as an adulterant in herbal analgesic supplements in Hamadan, Iran: A pilot study. Complementary therapies in medicine. 2019;42:223-5. DOI: 10.1016/j.ctim.2018.11.018.
23. Zarei M, Mohammadi S, Shahidi S, Fallahzadeh AR. Effects of *Sonchus asper* and apigenin-7-glucoside on nociceptive behaviors in mice. J Pharm Pharmacogn Res. 2017;5(4):227-237.
24. Golshani Y, Zarei M, Mohammadi S. Acute/chronic pain relief: is althaea officinalis essential oil effective? Avicenna J Neuro Psycho Physiol. 2015;2(4):e36586,1-7. DOI: 10.17795/ajnpp-36586.
25. Hajhashemi V, Ghannadi A, Mousavi S. Antinociceptive study of extracts of *Platanus orientalis* leaves in mice. Res Pharm Sci. 2011;6(2):123-128.

26. Bagheri SM, Dashti-R MH, Morshedi A. Antinociceptive effect of *Ferula assa-foetida* oleo-gum-resin in mice. Res Pharm Sci. 2014;9(3):207-212.
27. Dubuisson D, Dennis SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain. 1978;4:161-174. DOI: 10.1016/0304-3959(77)90130-0.
28. Ortiz MI, Medina-Tato DA, Sarmiento-Heredia D, Palma-Martínez J, Granados-Soto V. Possible activation of the NO-cyclic GMP-protein kinase G-K+ channels pathway by gabapentin on the formalin test. Pharmacol Biochem Behav. 2006;83(3):420-427. DOI: 10.1016/j.pbb.2006.03.002.
29. Zarei M, Mohammadi S, Komaki A. Antinociceptive activity of *Inula britannica* L. and patuletin: *in vivo* and possible mechanisms studies. J Ethnopharmacol. 2018;219:351-358. DOI: 10.1016/j.jep.2018.03.021.
30. de los Santos-Arteaga M, Sierra-Domínguez SA, Fontanella GH, Delgado-García JM, Carrión ÁM. Analgesia induced by dietary restriction is mediated by the κ-opioid system. J Neurosci. 2003;23(35):11120-11126. DOI: 10.1523/JNEUROSCI.23-35-11120.2003.
31. de Freitas Pires A, Assreuy AMS, Lopes ÉAB, Celedônio NR, Soares CEA, Rodrigues NVFC, *et al*. Opioid-like antinociceptive effects of oral administration of a lectin purified from the seeds of *Canavalia brasiliensis*. Fundam Clin Pharmacol. 2013;27(2):201-209. DOI: 10.1111/j.1472-8206.2011.00987.x.
32. Kikuchi Y, Yasuhara T, Agari T, Kondo A, Kuramoto S, Kameda M, *et al*. Urinary 8-OHdG elevations in a partial lesion rat model of parkinson's disease correlate with behavioral symptoms and nigrostriatal dopaminergic depletion. J Cell Physiol. 2011;226(5):1390-1398. DOI: 10.1002/jcp.22467.
33. Bannon AW, Malmberg AB. Models of nociception: hot-plate, tail-flick, and formalin tests in rodents. Current Protoc Neurosci. 2007;41(1):8.9.1-8.9.16. DOI: 10.1002/0471142301.ns0809s41.
34. Gawade SP. Acetic acid induced painful endogenous infliction in writhing test on mice. J Pharmacol Pharmacother. 2012;3(4):348. DOI: 10.4103/0976-500X.103699.
35. Ji RR, Chamessian A, Zhang YQ. Pain regulation by non-neuronal cells and inflammation. Science. 2016;354(6312):572-577. DOI: 10.1126/science.aaf8924.
36. Almeida JRGdS, Souza GR, Silva JC, Saraiva SRGdL, Júnior RGdO, Quintans JdSS, *et al*. Borneol, a bicyclic monoterpene alcohol, reduces nociceptive behavior and inflammatory response in mice. ScientificWorldJournal. 2013;2013:808460,1-5. DOI: 10.1155/2013/808460.
37. Meymandi MS, Sepehri G, Izadi G, Zamiri Z. Evidence for antinociceptive effects of combined administration of vitamin E and celecoxib in tail-flick and formalin test in male rats. Pharmacol Rep. 2019;71(3):457-464. DOI: 10.1016/j.pharep.2019.02.005.
38. Lugo-Lugo DE, Pozos-Guillén AdJ, Zapata-Morales JR, Rodríguez-Chong A, Rangel-López AdJ, Saavedra-Leos MZ, *et al*. Antinociceptive local activity of 4-allyl-1-hydroxy-2-methoxybenzene (*eugenol*) by the formalin test: an anti-inflammatory effect. Braz J Pharm Sci. 2019;55:e18022,1-7. DOI: 10.1590/s2175-97902019000118022.
39. Nguelefack TB, Dutra RC, Paszcuk AF, Andrade EL, Tapondjou LA, Calixto JB. Antinociceptive activities of the methanol extract of the bulbs of *Dioscorea bulbifera* L. var sativa in mice is dependent of NO-cGMP-ATP-sensitive-K+ channel activation. J Ethnopharmacol. 2010;128(3):567-574. DOI: 10.1016/j.jep.2010.01.061.
40. Oghbaei H, Mohaddes G, Hamidian G, Keyhanmanesh R. Sodium nitrate preconditioning prevents progression of the neuropathic pain in streptozotocin-induced diabetes Wistar rats. JDMD. 2020;19:105-113. DOI: 10.1007/s40200-019-00481-4.
41. Tolou-Dabbaghian B, Delphi L, Rezayof A. Blockade of NMDA receptors and nitric oxide synthesis potentiated morphine-induced anti-allodynia via attenuating pain-related amygdala pCREB/CREB signaling pathway. J Pain. 2019;20(8):885-897. DOI: 10.1016/j.jpain.2019.01.329.
42. Gorzalczany S, Marrassini C, Mino J, Acevedo C, Ferraro G. Antinociceptive activity of ethanolic extract and isolated compounds of *Urtica circularis*. J Ethnopharmacol. 2011;134(3):733-738. DOI: 10.1016/j.jep.2011.01.025.
43. Miclescu A, Gordh T. Nitric oxide and pain: Something old, something new. Acta Anaesthesiol Scand. 2009;53(9):1107-1120. DOI: 10.1111/j.1399-6576.2009.02054.x.
44. Moeller FG, Cunningham KA. Innovative therapeutic intervention for opioid use disorder. Neuropsychopharmacology. 2018;43(1):220-221. DOI: 10.1038/npp.2017.192.
45. Fallahzadeh AR, Zarei M, Mohammadi S. Preliminary phytochemical screening, analgesic and anti-inflammatory effect of *Eryngium pyramidale* Boiss. & Husson essential oil in male rat. EASL. 2016;3(5):140-147.
46. Galligan JJ, Akbarali HI. Molecular physiology of enteric opioid receptors. Am J Gastroenterol Suppl. 2014;2(1):17-21. DOI: 10.1038/ajgsup.2014.5.