



## Anti-nociceptive mechanisms of *Melilotus officinalis* Linn. ethanoic extract in mice: Involvement of opioidergic, nitrenergic and muscarinic receptors

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### Abstract

**Background and Objective:** Pain is a physiologic protective function with regard to external or internal harmful stimulus or tissue damage. The aim of the current study was to determine anti-nociceptive activity of *Melilotus officinalis* (Linn.) extract on formalin-induced pain in mice.

**Materials and Methods:** In experiment 1, adult male mice were injected (i.p) with saline, ethanoic extract of *Melilotus officinalis* (Linn.) (EEMO) (100, 200 and 400 mg/kg) or morphine (5 mg/kg). In experiment 2, mice were injected with saline, EEMO (400 mg/kg), naloxone (2 mg/kg) and co-injection of EEMO (400 mg/kg) + naloxone (2 mg/kg). In experiment 3, animal received i.p injection of saline, EEMO (400 mg/kg), L-NAME (10 mg/kg) and EEMO (400 mg/kg) + L-NAME (10 mg/kg). In experiment 4, mice were injected (i.p) with saline, EEMO (400 mg/kg), atropine (1 mg/kg) and co-administration of EEMO (400 mg/kg) + atropine (1 mg/kg). Then, the time spent for paw licking was determined in first and second phase after formalin injection. Then, licking and biting time of the injected paw was recorded after formalin injection in first and second phases.

**Results:** According to the results, EEMO in a dose dependent manner significantly diminished licking and biting time of injected paw (pain response) in comparison with the control group ( $P < 0.05$ ). Co-injection of the naloxone + EEMO significantly amplified pain response compared to the EEMO group ( $P < 0.05$ ). Co-injection of the L-NAME + EEMO significantly decreased pain response in comparison with the EEMO group ( $P < 0.05$ ). Pretreatment with atropine significantly enhanced pain response in comparison with the EEMO group ( $P < 0.05$ ).

**Conclusion:** These findings suggest that anti-nociceptive activity of the EEMO is mediated via opioidergic, nitrenergic and muscarinic systems in mice.

**Keywords:** Anti-nociceptive, *Melilotus officinalis* (Linn.), Mice

### 1. Introduction

Pain is a physiologic protective response to external or internal harmful stimulus or tissue damage (1). Activation of nociceptors in viscera leads to visceral pain including angina, colic, dyspepsia, pancreatitis, appendicitis and dysmenorrhea (2). Visceral tissue injury and inflammation can activate nociceptive primary afferent fibers and leads to central

sensitization or hyperexcitability of nociceptive neurons in the spinal cord dorsal horn (3). Current analgesic therapies such as non-steroidal anti-inflammatory drugs (NSAIDs) and opiates are effective in pain relief, but side effects are an important challenge to drug research (4). Medicinal plants are believed to be important sources of new chemical substances with potential therapeutic efficacy. Numerous plant-derived pharmaceutical products used in folk medicine because of their

analgesics properties (5). Identification of bioactive compounds from plants has become a highly active area of pharmaceutical research which can use for treatment of different conditions, such as anxiety, pain, and inflammation (6).

*Melilotus officinalis* (Linn.) (yellow sweet clover) is a member of the Fabaceae family. It is a biennial aromatic herb, native to Europe and Asia and famous since folk medicine. This herb contains high level of 7-hydroxycoumarin, 6,7-dihydroxycoumarin, flavonoids, steroids and saponins, phenolic acids, volatile components, fats, alcohols and uric acid which are used in the treatment of phlebitis, thrombosis, vascular fragility and treatment of varicose veins and hemorrhoids (7). Also, it has positive effect against cancer, atherosclerosis, nephritis and diabetes mellitus (8). Sweet clover has sedative and antispasmodic effects towards the smooth muscles of the digestive tract, respiratory and excretory systems (Jasicka-Misiak et al. 2017). *Melilotus officinalis* (Linn.) extract has anti-inflammatory effects against lipopolysaccharide induced nitric oxide (NO) and prostaglandin E2 (PGE2) production. Also, most of the compounds from *Melilotus officinalis* (Linn.) has anti-oxidant activity (9). Methanolic extract of *Melilotus officinalis* (50-300 mg/kg) had analgesic effect in mice and this effect is mediated via opioid receptors (10).

The main metabolite of coumarin, a molecule presents in a variety of edible fruits and plants, is 7-hydroxycoumarin which has immunomodulatory, antioxidant, antitumor and anti-nociceptive properties (11). Daily administration of 7-hydroxycoumarin decreases the mechanical hyper nociception and anti-nociceptive effect of 7-hydroxycoumarin was also observed in acetic acid-induced writhing and the formalin test, but not in the tail-flick test mice (11). Based on the reports, there is limit information about the bioactivity of *Melilotus officinalis* (Linn.). So, the first aim of current research was to investigate anti-nociceptive effects of the EEMO on acute and chronic inflammatory pain models, which were induced by formalin. Also, the secondary outcome was to evaluate the possible interaction of the anti-nociceptive activity of the EEMO with opioidergic, nitrenergic and muscarinergic systems.

## 2. Materials and Methods

### 2.1. Animals

In this study, 170 adult male albino Naval Medical Research Institute (NMRI) mice (25-30 g) were prepared (Pasteur Institute, Tehran, Iran) and kept in groups of 8–10 per cage (45 cm × 30 cm × 15 cm) (at 23°C ± 1°C ambient temperature, 12-hour dark/light cycle, and 55–56% relative humidity). During the study, mice had ad libitum access to chow pellets and fresh water. After one week of acclimatization, formalin test was used to assess the anti-nociceptive effect of *Melilotus officinalis* (Linn.). Experimental procedures were done according to the guide for the care and use of laboratory animals to investigate experimental pain in animals (12, 13), approved by the ethical committee of the Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran. To avoid the possible effects of the animals' circadian rhythm, all experiments were done from 08:00 a.m. to 02:00 p.m. All experiments were performed blind to experimental condition.

### 2.2. Extraction and Drugs

*Melilotus officinalis* (Linn.) was purchased from local market and taxonomic identification of plant was done at the division of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Iran. The gathered samples were dried at ambient temperature and under sunlight for 5 days. Thirty gram of plant material was soaked with 150 mL of the ethanol for 24 hours at room temperature. Then sample was filtered twice through Whatman filter paper, until a clear extract was obtained. The filtrates obtained were combined and then evaporated to dryness using a rotary evaporator at 40°C. The obtained extracts were stored in sterile sample tubes at -20°C (14). Phenolic acid constituents of *Melilotus officinalis* (Linn.) were identified by high performance liquid chromatography (HPLC) and presented in Table 1. Morphine, naloxone, L-NAME and atropine were purchased from Sigma (St. Louis, MO, USA). Also, formalin was purchased from Merck (Darmstadt, Germany). Drugs were dissolved in saline. All drugs were injected intraperitoneally (i.p.) and the injection volume was 0.5 mL (1).

**Table 1.** Bioactive compounds of *Melilotus officinalis* (Linn.) compounds by High Performance Liquid Chromatography

<b>Coumarin compounds</b>	<b>Levels (mg/ml)</b>
Cinnamic acid	546.87
Coumarin	288.45
p-coumaric acid	131.25
Carvacrol	-
Rutin	-
<b>Phenolic acid</b>	
Catechin	100.24
Gallic acid	10.05
Caffeic acid	30.66
Chlorogenic acid	0.95

### 2.3. Formalin test

In experiment 1, animals were injected (i.p) with saline, EEMO (100, 200 and 400 mg/kg), morphine (5 mg/kg), and 30 minutes later received 10 µl of the 1% formalin solution into the plantar surface of the right paw. The test was performed according to Hunskaar and Hole (15). To minimize the possible effect of stress during the study, mice were placed inside a Plexiglas observation chamber (30×30×25 cm<sup>3</sup>) equipped with a mirror angled at 45° below the chamber for 30 minutes per day for 3 days (16). During the test, 30 minutes adaptation periods were applied to the animals, then the test was performed. Briefly, 30 min after formalin injection into the subplantar space of the right hind paw, the time spent for paw licking was determined 0-5 minutes (first phase) and 15-45 minutes (second phase) (17). In experiment 2, animals were injected (i.p) with saline, EEMO (400 mg/kg), naloxone (2 mg/kg) and EEMO (400 mg/kg) + naloxone (2 mg/kg). In the co-injection group, first, the mice were pretreated with the antagonist and 15 minutes later received betaine (10 mg/kg) followed by formalin (10 µl of the 1% solution) after 15 minutes. Then, the time spent for paw licking was determined in the first and second phase after formalin injection. In experiment 3, injections consisted of saline, EEMO (400 mg/kg), L-NAME (10 mg/kg) and a mixture of EEMO (400 mg/kg) + L-NAME (10 mg/kg). In experiment 4, mice were injected (i.p) with saline, EEMO (400 mg/kg), atropine (1 mg/kg) and a mixture of EEMO (400 mg/kg) + atropine (1 mg/kg). Then, the time spent for paw licking was determined in first and second phases after formalin injection. The doses of the drugs used were chosen based on the literature review and a preliminary pilot study (18, 19).

### 2.4. Statistical analysis

Data was analyzed by one-way analysis of variance (ANOVA) using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA) and presented as mean ± SE (standard error). For treatments showing a main effect by ANOVA, the analysis was followed by a Tukey post-hoc test. P <0.05 was considered to indicate significant difference.

## 3. Results

### 3.1. Evaluation of anti-nociceptive effects of the EEMO

According to figure 1, EEMO in a dose dependent manner significantly decreased the time spent for licking and biting in injected paw (pain response) compared to the control group in phases I and II (P<0.05). Morphine, as a reference drug, significantly decreased licking and biting time of injected paw in comparison with the control group in phases I and II (P<0.05).

### 3.2. Effect of naloxone on anti-nociceptive activity of the EEMO

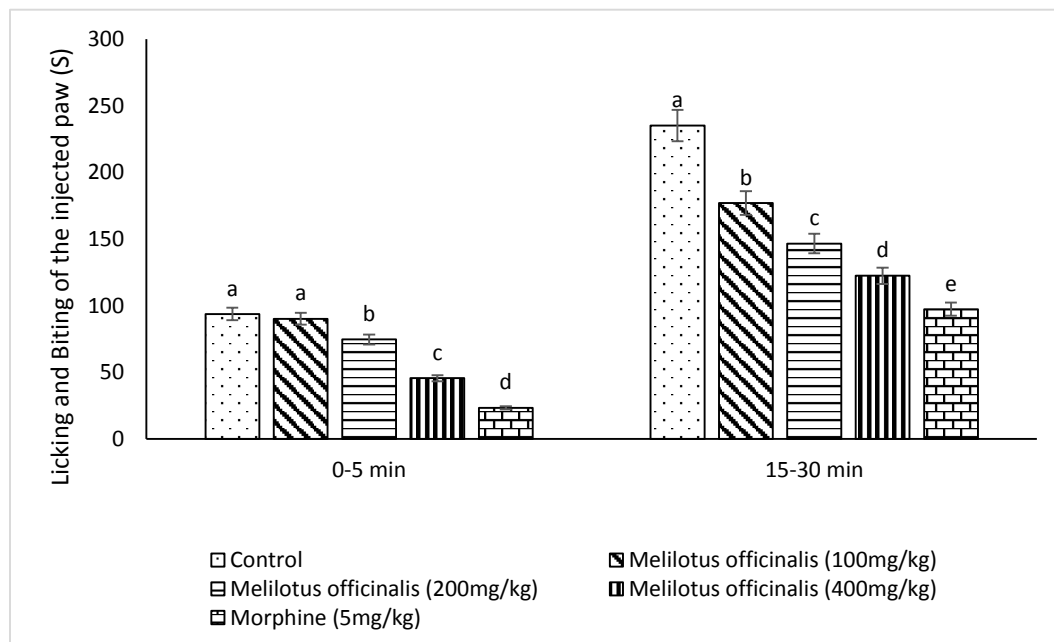
EEMO (400 mg/kg) significantly decreased the time spent for licking and biting in injected paw in phases I and II (P<0.05). Naloxone (2 mg/kg) had no significant anti-nociceptive effect using the formalin test in phases I and II (P>0.05). Pre-treatment of naloxone + EEMO significantly increased the time spent for licking and biting in injected paw in comparison with the EEMO group in phases I and II (P<0.05; figure 2). These results suggest that blockade of opioid receptors using naloxone had an effect on the anti-nociceptive effects of the EEMO. It seems that anti-nociceptive activity of EEMO is mediated by opioid receptors.

### 3.3. Effect of L-NAME on anti-nociceptive activity of the EEMO

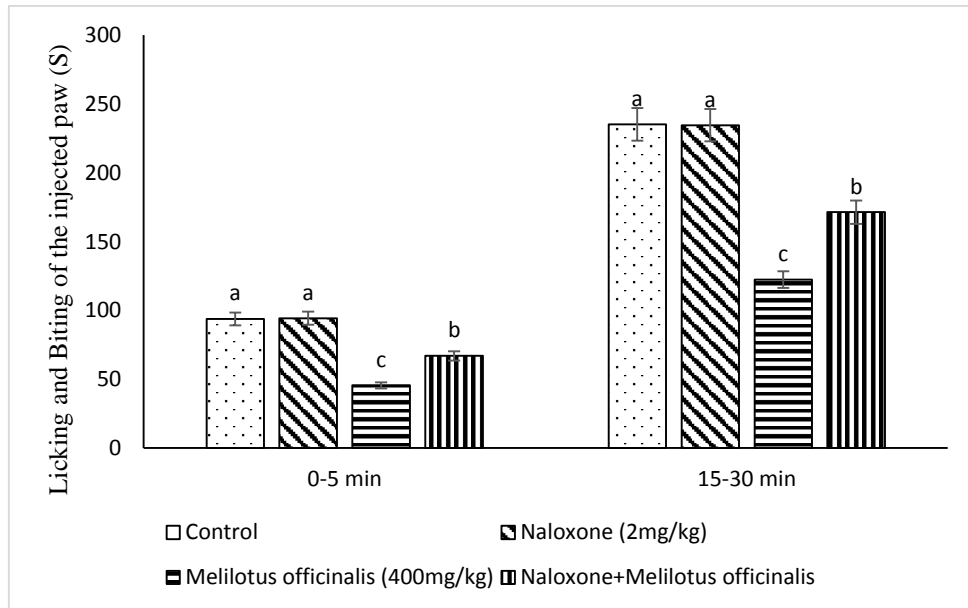
As shown in figure 3, EEMO (400 mg/kg) significantly decreased the time spent for licking and biting as compared to the control group in phases I and II ( $P < 0.05$ ). L-NAME (10 mg/kg) had no significant effect in phases I and II ( $P > 0.05$ ). Pretreatment with L-NAME + EEMO significantly decreased the time spent for licking and biting in injected paw in comparison with the EEMO group in phases I and II ( $P < 0.05$ ). These results suggest that injection of the nitric oxide inhibitor L-NAME had an effect on the anti-nociceptive effects of the EEMO. Perhaps, the anti-nociceptive activity of the EEMO is mediated through nitric system.

### 3.4. Effect of atropine on anti-nociceptive activity of the EEMO

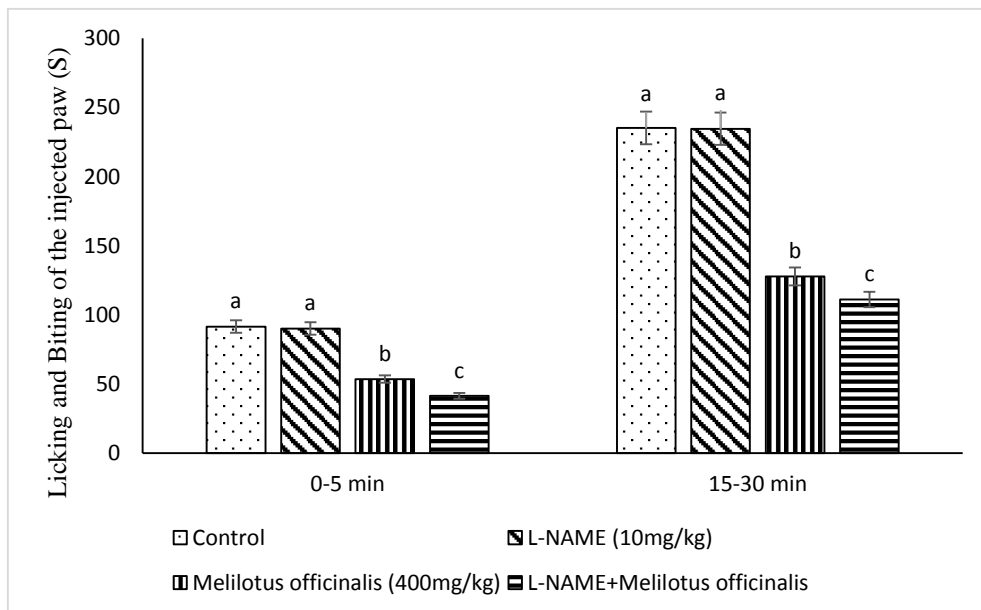
The effect of pretreatment with atropine on the anti-nociceptive effects of the EEMO is shown in figure 4. Injection of the EEMO (400 mg/kg) significantly decreased the time spent for licking and biting as compared to the control group in phases I and II ( $P < 0.05$ ). Atropine (1 mg/kg) had no anti-nociceptive effect in phases I and II ( $P > 0.05$ ). Co-administration of the atropine + EEMO significantly increased the time spent for licking and biting in comparison with the EEMO group in phases I and II ( $P < 0.05$ ). These results suggest that blockade of muscarinic receptors using atropine had an effect on the anti-nociceptive effects of the EEMO. It is assumed that muscarinic receptors play a role in anti-nociceptive activity of the EEMO.



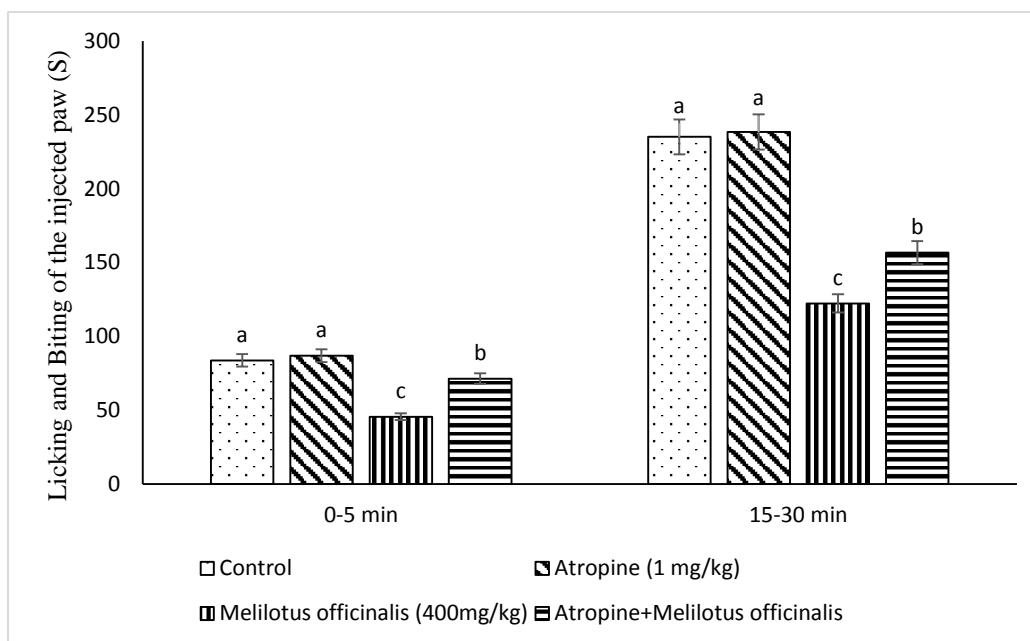
**Figure 1.** Effect of the *Melilotus officinalis* (Linn.) on licking and biting time of the injected paw in male mice (n=50). Data are expressed as mean  $\pm$  SE. Different superscripts (a-d) indicate significant differences between groups ( $P < 0.05$ ).



**Figure 2.** Effect of the *Melilotus officinalis* (Linn.), naloxone and their co-injection on licking and biting time of the injected paw in male mice (n=40). Naloxone: opioid receptor antagonist. Data are expressed as mean ± SE. Different superscripts (a-c) indicate significant differences between groups (P< 0.05).



**Figure 3.** Effect of the *Melilotus officinalis* (Linn.), L-NAME and their co-injection on licking and biting time of the injected paw in male mice (n=40). L-NAME: L-N<sup>G</sup>-Nitro arginine methyl ester, nitric oxide inhibitor. Data are expressed as mean ± SE. Different superscripts (a-c) indicate significant differences between groups (P< 0.05).



**Figure 4.** Effect of the *Melilotus officinalis* (Linn.), atropine and their co-injection on licking and biting time of the injected paw in male mice (n=40). Atropine: muscarinic receptors antagonist. Data are expressed as mean  $\pm$  SE. Different superscripts (a-c) indicate significant differences between groups ( $P < 0.05$ ).

#### 4. Discussion

To date, several studies have been performed to investigate possible anti-nociceptive and anti-inflammatory activities of medicinal plants. Also, there is a growing interest in the use of plants for the search for new therapeutic agents (19). Based on our information, this is the first report on interaction of anti-nociceptive effect of the EEMO. As observed, EEMO in a dose dependent manner decreased licking and biting time of injected paw. It is possible 7-hydroxycoumarin has anti-nociceptive effects against chronic pain conditions (11). Pain acts via several chemical mediators released during this process and leads to nociceptive sensitization. The mechanism underlying formalin-induced pain behavior involves series of events including peripheral and central biphasic responses (20). Acute pain serves as a warning device that indicates imminent tissue damage. Chronic pain has no protective role and persists long time after injury without reflecting lesion or disease (21). It is reported 7-hydroxycoumarin had an anti-nociceptive effect both in the primary and late phases of the formalin test (22). Anti-nociceptive activity of the 7-hydroxycoumarin on the late phase of the formalin test suggests for its anti-inflammatory action (11) and because of the same metabolite as the main metabolite of the EEMO, our finding was similar to previous report. However, no anti-nociception revealed by oral administration of 7-hydroxycoumarin in the tail-flick test. Tail-flick test is thermal model of pain which considered as spinal reflex, but might involve higher neural structures and this method mainly identifies central analgesics (23). So, 7-

hydroxycoumarin induced anti-nociception is related to its anti-inflammatory activity.

As observed, co-administration of the opioid receptor antagonist + EEMO increased pain response. Controversial reports exist for nociceptive effect of the coumarin. Park et al. (24) reported orally administration of the coumarin (1-10 mg/kg) had an anti-nociceptive effect in the acetic acid-induced writhing test. Pretreatment with naloxone (an opioid receptor antagonist) attenuated anti-nociceptive effect induced by coumarin in the writhing test. However, no nociceptive effect of coumarin was observed in the formalin, substance P or glutamate pain models (24) which our result was dissimilar to their report. The nociceptive behaviors in acute phase mediates by direct effect on peripheral nociceptors activating primary afferent fiber while chronic phase mediates from inflammatory nociceptive response (25). Although scarce information exists for observed differences in anti-nociception activity of coumarin in pain models in previous and our results, it seems, coumarin-induced anti-nociception mediates by activation of differential neuronal circuits via visceral pain and other pain modalities. Also, different fractions of the coumarin (7-hydroxycoumarin, 6,7-dihydroxycoumarin) and other bioactive metabolites in the EEMO might explain differences (24).

The roles neurotransmitters such as opioidergic, serotonergic and adrenergic receptors in the regulation of modulation of nociceptive processing play have been demonstrated in many previous studies. However, determining role of these systems on

nociceptive properties of medical plants is important for prepare new drug and medications (26). Based on findings of current study, co-injection of the nitric oxide inhibitor + EEMO significantly decreased pain response. Nitric oxide (NO) is an important mediator of nociception in acute and chronic (27) pain in central and peripheral nervous system (28). Nitric oxide can induce analgesia and play key role in the modulation of nociceptive processing. Subplantar injection of formalin increase the NO level in injected site and pretreatment with L-NAME (non-selective NOS inhibitor), 7-nitroindazole (selective nNOS inhibitor) and aminoguanidine hydrochloride (selective iNOS inhibitor) decreased pain response in mice (27). Also, pretreatment with L-NAME or L-arginine (NO processor) potentiated or inhibited the anti-nociceptive effect of *S. micranthum* extract in formalin test (29, 30) and or result was in agreement with previous reports. Despite direct mechanism of action for observed results is not fully elicited, but it seems L-arginine induced NO production in the spinal cord decrease anti-nociceptive effect of the extract. It is assumed extracts can decrease NO concentration at the spinal level (28). Nitric oxide pathway has important role in the carrageenan-induced inflammatory response and paw edema test. It seems the anti-nociceptive response of EEMO is mediated via nitrenergic system. However, based on the limitation of the current study we were not able to determine NO levels following EEMO injection in formalin-induced pain mice (27). Additionally, co-administration of the muscarinergic receptor antagonist + EEMO

significantly increased pain response in mice. Role of the muscarinergic receptors in pain is well documented (20). Atropine is a competitive nonselective antagonist of central and peripheral muscarinic acetylcholine receptors which antagonized the analgesic and anti-inflammatory effects in acute and chronic phases of formalin- and Writhing tests induced pain (19, 20). Perhaps, anti-nociceptive response of the EEMO is mediated via muscarinergic receptors.

Based on limitation of this study to find previous report to compare for antioxidant activity of EEMO interacts with its analgesic effect. The novel findings of current study suggest that anti-nociceptive activity of the EEMO mediates via opioidergic, nitrenergic and muscarinergic systems in mice. Based on the limitations of this study, we were not able to determine molecular events following EEMO injection in formalin-injected mice. Also, it is suggested to determine NO levels following EEMO in formalin-injected mice.

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### Conflict of Interest:

There is no conflict of interest

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