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Title: Chemical Composition and Interaction of Antinociceptive Effect of Ziziphora clinopodioides Essential Oil and Opioidergic System in Male Rats

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A B S T R A C T

Introduction: *Ziziphora clinopodioides* has been used widely for various therapeutic purposes in Iranian folk medicine. The aim of the current study was to determine interaction of antinociceptive effect of the essential oil of *Ziziphora clinopodioides* (EOZC) and opioidergic system in male rats using formalin test.

Methods: Sixty-four male Wistar rats were distributed into eight groups. The groups 1-7 were injected with saline, vehicle (Tween-80, 0.5%), 10, 20, 40 mg/kg of the EOZC, morphine (5 mg/kg) and naloxone (2 mg/kg), respectively. Afterward, 30 min later, the formalin test was performed by intraplantar injection of formalin (50 µl, 2%). In group 8, naloxone (2 mg/kg) was injected 15 min before injection of EOZC (20 mg/kg), then 15 min later, followed by formalin. The formalin test was done as time spent for licking and biting of the injected paw. Formalin induced a biphasic pain reaction. The chemical composition of EOZC was identified using gas chromatography-mass spectrometry (GC-MS).

Results: EOZC (10, 20 and 40 mg/kg) dose dependently and morphine (5 mg/kg) reduced pain responses in the both phases of pain ($P<0.05$). Naloxone (2 mg/kg) alone had no effect on the severity of pain ($P>0.05$) but pretreatment with naloxone inhibited EOZC-induced antinociception activity ($P<0.05$). Based on the GC-MS results, 65.22% carvacrol, 19.51% thymol, 4.86% p-Cymene and 4.63% γ-terpinene were found in the EOZC.

Conclusion: These results demonstrate that EOZC has antinociceptive effect and this effect might mediate via opioidergic pathways.

Key Words: Ziziphora clinopodioides, Antinociceptive, Opioidergic, Rat

1. Introduction

Pain is a displeasing sensation which related to tissue damage (Karimi, Monajemi, & Amjad, 2014). Pain is a physiologic protective function which happens via an external or internal harmful stimulus (Ashok & Upadhyaya, 2013). Activation of nociceptors in viscera leads to visceral pain including angina, colic, dyspepsia, pancreatitis, appendicitis and dysmenorrhea (de Oliveira Júnior et al., 2017). Visceral tissue injury and inflammation can activate nociceptive primary afferent fibers, which results in central sensitization or hyperexcitability of nociceptive neurons in the spinal cord dorsal horn (Grace, Hutchinson, Maier, & Watkins, 2014). Recently, attainable analgesic drugs like non-steroidal anti-inflammatory drugs (NSAIDs) and opioids are not prescribed because of their adverse effects (Zendehdel, Torabi,

& Hassanpour, 2015). Recently, variety of plant-derived pharmaceutical products used in folk medicine because of their positive properties (Yama et al., 2011). Plants are a rich source of a wide variety of secondary metabolites such as flavonoids, Thymol, Carvacrol, terpenoids, alkaloids among others (Hassanpour, Sadaghian, MaheriSis, Eshratkhah, & ChaichiSemsari, 2011). Nowadays, worldwide interest on traditional medicine has increased. People desire to consume much more medicinal plants due to their therapeutical properties (Yousif, Ashoush, Donia, & Hala Goma, 2013). 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 (de Oliveira et al., 2012). The evaluation of pharmacological effects can be used as a strategy for discovering new plant-deprived drugs (Zendehdel, Taati, Jadidoleslami, & Bashiri, 2011). *Ziziphora clinopodioides* belongs to the Lamiaceae family and in persian name popularly known as Kakuti-e-kuhi or Taramoshk which is spread worldwide especially in Iran, Afghanistan, Iraq and Turkey. It is important medicinal and aromatic plants that traditionally are used for the treatment of various diseases (Khodaverdi-Samani, Pirbalouti, Shirmardi, & Malekpoor, 2015). In Iranian folk medicine, the fresh leaves and stem prescribed for wound healing, sedation, stomach tonic, antiseptic, expectorant, antifungal, antibacterial and antiseptic (Shahbazi, 2015). EOZC contains a diversity of biologically active compounds such as monoterpenes and sesquiterpenes (Shahbazi, 2015). Most studies have demonstrated that some of these terpenes have analgesic effects using rats and/or mice in experimental models of pain (Almeida, Navarro, & Barbosa-Filho, 2001). Therefore, the aim of the current study was to determine interaction of antinociceptive effect of EOZC and opioidergic system in male rats.

2. Methods

2.1. Preparation of essential oil

The fresh leaves of *Ziziphora clinopodioides* were collected from Gilan-e-Gharb suburb (Kermanshah Province, Iran) during March-July 2016 and specimen identification was done in Faculty of Agriculture, Razi University, Kermanshah, Iran. Voucher specimen (No. 6816) of the plant was deposited in the herbarium of the Research Center of Natural Resources of Tehran, Iran.

2.2. Isolation of essential oil

The *Ziziphora clinopodioides* leaves (100g) shade dried at room temperature (25±2°C). Samples were hydro-distilled using a Clevenger-type apparatus for 3.5 hours till full of essential oil, then supernatant was collected and dried over 0.5g anhydrous sodium sulfate

(Merck, Darmstadt, Germany). The essential oil was stored in dark glass bottle, covered with aluminum foil at 4±1°C (Shahbazi, 2015).

2.3. Analysis of the essential oil of *Ziziphora clinopodioides*

The chemical compounds of EOZC was obtained by Gas chromatography–mass spectrometry (GC-MS) (Thermo Quest Finningan, UK) and given in table 1. The GC-MS instrument was 5% phenyl methyl silicone and 95% dimethylpolysiloxane and equipped with DB5 capillary column (30m, 0.25mm, film thickness 0.25μm). An electron ionization mode with ionization energy of 70eV was used for determination of the EOZC constituents (Shahbazi, 2015). The carrier gas was helium at constant flow rate 1.2 ml/min, with linear velocity of 29.6 cm/s and split ratio was1:20. The initial oven temperature was held at 50°C for 3 min, then raised to 265ºC at program ramp rate 2.5°C/min. The final temperature was 265ºC and maintained for 6°C. The temperature of the injector was 250°C. To improve accuracy of the results, the GC-MS analysis was done in triplicate.

2.4. Drugs

Morphine (as an opioid receptor agonist), naloxone hydrochloride (as an opioid receptors antagonist) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tween-80 and formaldehyde (37%) were purchased from Merck Co. (GERBU, Germany). All drugs were dissolved in normal saline. The various doses of the EOZC were prepared in Tween-80 (0.5%). Distilled water was used in order to dissolve Tween-80 in to 1% (v/v) and diluted with the same volume of saline. The control group injected with vehicle. All drugs were prepared before use.

2.5. Animals

Sixty-four male Wistar rats (200–220 g) were supplied from the Laboratory Animal Facility of the school of Veterinary Medicine, Razi University, Kermanshah, Iran. The animals were randomly divided into 8 groups (n=8 in each group). Rats were kept under standard laboratory conditions according to European community guidelines for laboratory animals (ambient temperature of 22 ± 1 ºC, 12 h lighting period) and had ad libitum access to chow pellets and fresh water. In group 1, animals were intraperitoneally (i.p.) injected with saline 30 min before intraplantar injection of formalin. In group 2, rats were i.p. injected with vehicle (Tween-80, 0.5%) 30 min before intraplantar injection of formalin. In groups 3, 4 and 5, i.p. injection of EOZC at doses of 10, 20 and 40 mg/kg, respectively, were performed 30 min before induction of formalin pain. In groups 6 and 7, animals i.p. treated with morphine (5 mg/kg) and naloxone (2 mg/kg), respectively, 30 min before intraplantar injection of formalin. In group 8, animals received naloxone (2 mg/kg), then 15 min later received EOZC

(20 mg/kg) followed by formalin solution after 15 min. Drug solutions were i.p. injected in a volume of 1 ml/kg using a 25-gauge injection needle. To reduce the possible effect of circadian rhythm on the nociceptive susceptibility, all experiments were done at 9 a.m until 12 a.m. (Borowicz, Kleinrok, & Czuczwar, 2003). All experimental procedures were carried out in accordance with the guidelines for the care and use of laboratory animals to investigate experimental pain in conscious animals (Zimmermann, 1983).

2.6. Estimation of Acute Toxicity

In order to identify the acute toxicity of the essential oil with few animals, a limit test was conducted according to OECD 425 guideline. The animals were kept in their cages for at least 5 days prior to dosing to allow for adaptation to the laboratory conditions. The EOZC at dose of 1000 mg/kg was administered i.p. to one animal followed by 24 h of observation. If the animal survived, four additional animals sequentially were administered with EOZC (1000 mg/kg, i.p.) under the identical conditions. A total of five animals were tested. Observation was carried on for 14 days.

2.7. Formalin test

The formalin test as a valid model of pain is commonly used (Erami, Azhdari-Zarmehri, Imoto, & Furue, 2017). To minimize possible effect of stress during the study, rats were placed inside a Plexiglas observation chamber (30×30×25 cm^3) equipped with a mirror angled at 45° below the chamber for 30 min on three consecutive days (Abbott & Bonder, 1997). In the test day, 30 min adaptation period applied to the animals, then the test was done. Formalin (50 µl, 2%) was injected subcutaneously via a 30 gauge needle into the plantar surface of the right hind paw (Sofiabadi et al., 2014). Immediately following the formalin injection, rats were returned to the observation chamber. The time spent in licking and biting of the injected paw determined as a nociceptive behavior. The formalin-induced behavioral responses were biphasic: 0-5 min (first phase, neurogenic phase) and 15-45 min (second phase, inflammatory phase) (Tamaddonfard & Hamzeh-Gooshchi, 2010).

2.8. Statistical analysis

Data were prepared in excel and analyzed with analysis of variance (ANOVA) using SPSS version 19.0 for Windows (SPSS, Inc., Chicago, IL, USA) followed by Tukey's HSD post-hoc test. Student t-test was further employed to determine the differences between two control groups of formalin test. The results are expressed as means ± SEM. P value of < 0.05 was defined to denote significant differences between groups.

3. Results

3.1. Analysis of *Ziziphora clinopodioides* essential oil

Table 1 lists the composition of the *Ziziphora clinopodioides* essential oil. In total, 24 components were fully identified, covering 99.65% of the total composition. Regarding the chemical constituents, carvacrol (65.22%), thymol (19.51%), p-Cymene (4.86%) and γ-terpinene (4.63%) were the main components of the EOZC (Table 1).

3.2. Acute Toxicity Testing

Single dose acute toxicity of the essential oil of *Z. clinopodioides* was done intraperitoneally through a limit test of 1000 mg/kg. EOZC produced no animal mortality in a period of 14 days. Hence, LD_{50} of the EOZC was considered to be more than 1000 mg/kg.

3.3. Effect of essential oil *Ziziphora clinopodioides* on formalin-induced pain behaviors

The intraplantar injection of formalin 2% produced a biphasic pattern of pain behavior. Effect of saline and vehicle (Tween-80, 0.5%) on licking and biting time of the injected paw in male rats is presented in figure 1. No significant difference was observed on first phase of pain in control (72.16 ± 6.39 sec) and tween-80 (0.5%) (66.50 ± 5.02 sec) groups ($P>0.05$). Also, no significant difference was observed on second phase of pain in control (208.50 ± 15.21 sec) and tween-80 (0.5%) (196.66 ± 14.43 sec) groups ($P>0.05$) (figure 1).

Therefore, the obtained data from experimental groups were compared with vehicle treated group.

EOZC at dose of 10 mg/kg did not show any significant effect on both first and second phases of formalin pain in comparison with vehicle treated group ($P>0.05$) but 20 and 40 mg/kg of EOZC induced a significant reduction in the pain response compared to the vehicle group in a dose dependent manner in the both first (39.16 ± 3.80 sec and 31.33 ± 3.76 sec, respectively) and second (121.66 ± 10.44 sec and 108.50 ± 11.87 sec, respectively) phases ($p<0.05$). As expected, the standard drug morphine (5 mg/kg) significantly decreased the nociceptive response in the both first (10.33 ± 2.61 sec) and second (87.00 ± 8.05 sec) phases of formalin test compared to vehicle treated group ($p<0.05$) (figure 2).

Based on the findings, naloxone (2 mg/kg) alone had not significant effect on both phases of formalin test ($P>0.05$). In addition, pre-treatment with naloxone (2 mg/kg) significantly reversed antinociception by EOZC (20 mg/kg) in the first (58.50 ± 3.77 sec) and second (176.00 ± 10.25 sec) phase of formalin test ($p<0.05$) (figure 3).

4. Discussion

The present study was designed to investigate the antinociceptive effect of essential oil of *Ziziphora clinopodioides* (EOZC) and possible involvement of opioidergic system on EOZC-

induced antinociception in male rats using formalin test. In the traditional medicine, herbs and plants due to their potentially positive action are widely used for improvement of numerous ailments (Sofiabadi et al., 2014). To date, numerous experiments have been carried out to determine analgesic effects of medicinal plants (Riedel, Marrassini, Anesini, & Gorzalczany, 2015). To our knowledge, this is the first report on interaction of antinociceptive effect of the EOZC and opioidergic system in the formalin test in rats. Two phases of pain evokes by formalin injection into the hind paw of rodents. Each phase of formalin test has different mechanisms of nociception. The first phase consists of neurogenic nociception, by direct stimulation of nociceptors via C fibers to the dorsal horn of the spinal cord after substance P is secreted and acts as a neurotransmitter. The second phase consists of inflammatory-induced pain because of release of various inflammatory mediators such as serotonin, histamine, bradykinin, prostaglandins (PGs) and excitatory amino acids from the tissue that is damaged by formalin (Akbari, Mirzaei, & Shahabi Majd, 2013; Abbott, Franklin, & Westbrook, 1995). Formalin test is known as a suitable method for generating and quantifying the chemical pain in the rat model. The formalin test, has long been used as a well characterized method to evaluate antinociceptive and anti-inflammatory properties of new substances and novel drugs continue to be developed from plants (Vissers, Hoffmann, Geenen, Biermans, & Meert, 2003; Dubuisson & Dennis, 1977). Several studies suggested that the centrally acting analgesic drugs such as opioids can inhibit both phases of formalin test (Shibata, Ohkubo, Takahashi, & Inoki, 1989) while peripherally acting drugs exert an inhibition only in the second phase (Elisabetsky, Amador, Albuquerque, Nunes, & Carvalho Ado, 1995). In the current study, i.p. injection of EOZC revealed a dose-dependent antinociceptive effect on both phases of formalin-induced nociception in rats. Since essential oil of *Z. clinopodioides* was effective in suppression of paw licking time in both phases of the formalin test, it seems that the analgesic activity of *Z. clinopodioides* is mediated by both peripheral and central antinociceptive mechanisms. Based on the GC-MS results, EOZC contained high concentrations of phenolic compounds including carvacrol (65.22%), thymol (19.51%), p-Cymene (4.86%) and γ-terpinene (4.63%). Recently, several studies have reported the chemical composition of EOZC (Aghajani et al., 2008; Behravan et al., 2007; Ozturk & Ercisli, 2007).

It has been shown that flavonoids and polyphenolic compounds possess a great variety of pharmacological properties including antioxidant activity (Altiok, Altiok, & Tihminlioglu, 2010), immunomodulatory activity (Lima et al., 2010), inhibition of histamine release from mast cells (Amresh, Reddy, Rao, & Singh, 2007a) and suppression of prostaglandin synthesis

(Amresh, Zeashan, Rao, & Singh, 2007b). In this regard, previous reports have demonstrated antinociceptive activity for carvacrol (Guimarães et al., 2010), thymol (Beer, Lukanov, & Sagorchev, 2007), p-Cymene (De Sousa, 2011) and γ-terpinene (Hajhashemi, Sajjadi, & Zomorodkia, 2011) in model of formalin induced licking. Besides, it has been previously shown that thymol partially blocks voltage-operated Na^+ channels and directly activates Cl^- currents via $GABA_A$ receptors (Haeseler et al., 2002; Mohammadi et al., 2001). On the other hand, it was expressed that thymol reversibly inhibited prostaglandin synthesis; probably related to the analgesic effect of thymol in endodontic therapy (Sarmento-Neto, do Nascimento, Felipe, & de Sousa, 2016). There is also evidence that the antinociceptive effects of carvacrol are presumably to be related at some degree to antioxidant activity and its scavenging activity on NO and other reactive oxygen species (ROS) (Guimarães et al., 2010). Consequentially, based on the above-mentioned findings and since these four compounds are among the predominant components of EOZC, it can be concluded that the antinociceptive property of EOZC might be due, at least in part, to the presence of these chemical compounds.

In the present study, morphine was an effective inhibitor of both phases of formalin pain. Morphine and other opioid analgesics have been used for alleviate pain. The opioidergic system consists of three receptors including μ, δ and κ which are located in the central nervous system (CNS) as well as throughout the peripheral tissues (Trescot, Datta, Lee, & Hansen, 2008). Studies have shown that endogenous opioidergic system and their receptors take part in many functions for example behavior, pain and analgesia, stress, tolerance and dependence, learning and memory, alcohol and drugs of abuse, respiratory control, locomotion, seizures, neurologic disorders and neuroendocrine physiology (Bodnar, 2016).

In order to reveal the possible participation of endogenous opioidergic system on the antinociception mechanism exerted by EOZC, naloxone (an opioid receptor antagonist) was evaluated in the formalin test. Naloxone is a competitive antagonist of mu, kappa, and delta receptors, with a high affinity for the mu receptor (Trescot, Datta, Lee, & Hansen, 2008).

Our findings showed that the antinociception caused by the EOZC was significantly attenuated by pre-treatment of rats with naloxone (2 mg/kg), thus it reverses analgesic activity of EOZC to some extent.

Taken together, these results suggest that the constituents in the essential oil of *Z. clinopodioides* may be acting through opioidergic pathway to produce antinociceptive activity. However, further investigation is required to elucidate the underlying cellular and molecular signaling pathways.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1. The chemical composition of *Ziziphora clinopodioides* essential oil.

Compound	Composition (%)	Retention Time (min)	Kovats Index

Carvacrol	**65.22**	**30.57**	**1315**
Thymol	**19.51**	**29.61**	**1293**
p-Cymene	**4.86**	**16.62**	**1030**
γ-Terpinene	**4.63**	**18.31**	**1063**
E-Caryophyllene	1.07	35.47	1427
α-Terpinene	0.79	16.11	1021
Borneol	0.61	24.36	1183
Myrcene	0.51	14.62	992
Terpinene-4-ol	0.48	24.7	1190
Caryophyllene oxide	0.31	42.30	1595
α-Pinene	0.27	11.71	934
α-Thujene	0.26	11.33	927
Linalool	0.13	20.5	1105
Camphene	0.13	12.61	952
α-Phellandrene	0.13	15.58	1010
Spathulenol	0.12	42.10	1590
β-Phellandrene	0.11	16.89	1036
Limonene	0.1	16.77	1033
α-Terpineol	0.08	25.49	1206
Terpinolene	0.08	19.69	1089
1-Octen-3-ol	0.08	14.32	986
cis-Sabinene hydrate	0.07	19.02	1077
β-Pinene	0.06	14.06	981
Carvacrol, methyl ether	0.04	27.38	1246
Total	**99.65**		

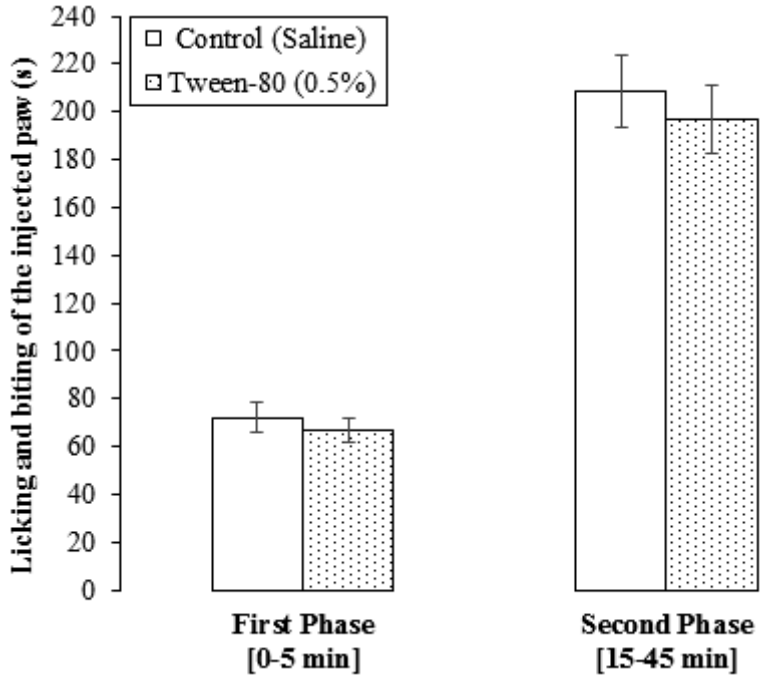

Figure 1. Effect of normal saline and vehicle (Tween-80, 0.5%) on the formalin induced pain response in rats. Data are expressed as mean ± SEM (n=8).

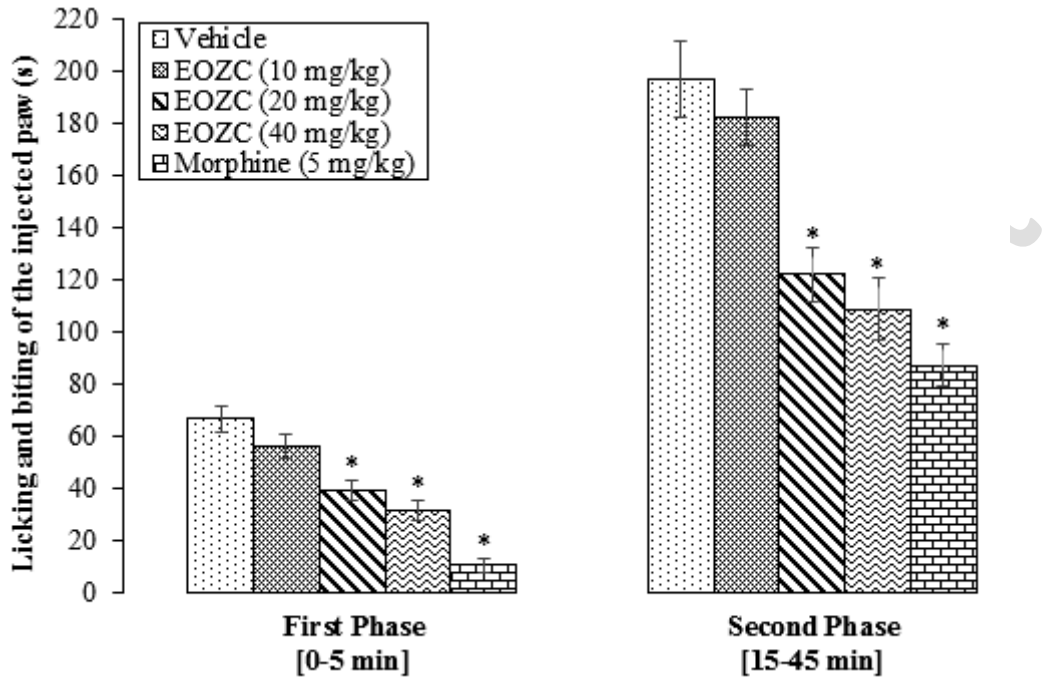

Figure 2. Effect of different doses of the EOZC and morphine on the formalin induced pain response in rats. Data are expressed as mean ± SEM (n=8). * P<0.05 as compared with vehicle treated group. EOZC: Essential oil *Ziziphora clinopodioides*.

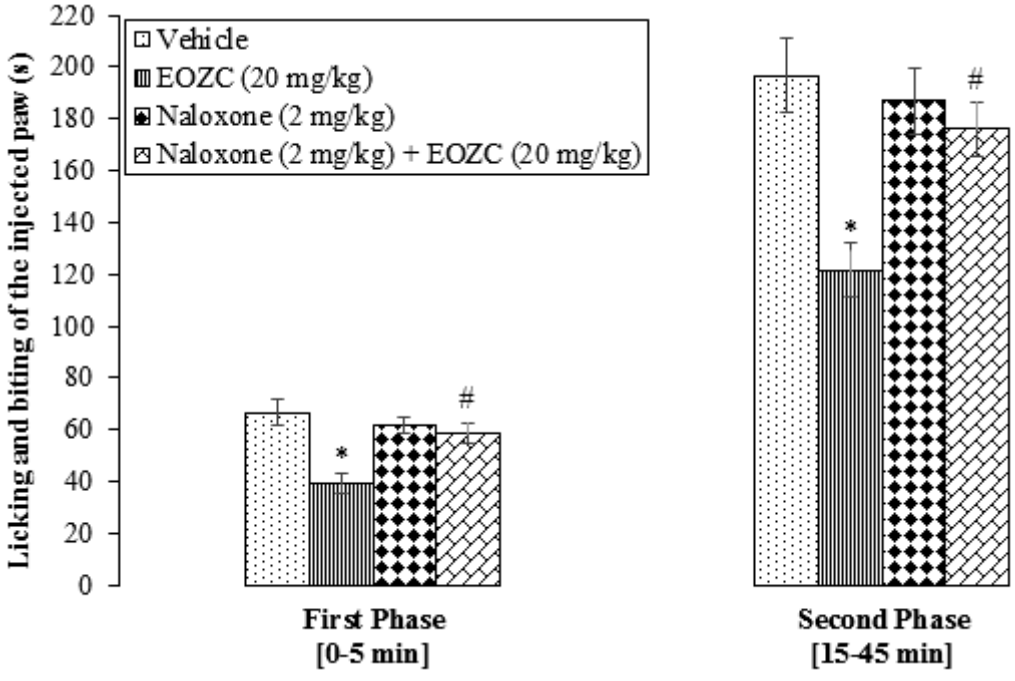

Figure 3. Effect of EOZC, naloxone and pretreatment with naloxone on the antinociceptive activity induced by administration of EOZC in formalin pain response in rats. Data are expressed as mean ± SEM (n=8). * $P<0.05$ as compared with vehicle treated group. # $P<0.05$ as compared with EOZC (20 mg/kg) treated group. EOZC: Essential oil *Ziziphora clinopodioides*.