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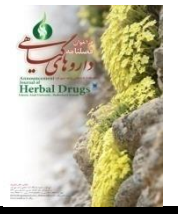

Evaluation of chemical composition, anti-inflammatory and anti-nociceptive effects of *Eugenia caryophyllata* buds essential oil

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ABSTRACT

Background & Aim: *Eugenia caryophyllata* well known as Clove is a tree from Myrtaceae family that several parts of this plant traditionally used in dental care as an analgesic. This study aimed to assess the chemical composition, anti-inflammatory and anti-nociceptive activities of the essential oil extracted from Clove buds.

Experimental: The essential oil of Clove buds (EOC) was extracted by Clevenger type apparatus and its chemical composition determined by gas chromatography-mass spectrometry (GC-MS). Analgesic activities of EOC were measured by formalin-induced orofacial pain and tail immersion test in rat. Also anti-inflammatory effect of the EOC was evaluated by using xylene induced ear edema test in mice.

Results: EOC (100, 200 mg/kg, SC) and ketoprofen (80 and 160 mg/kg, IP) inhibit only the second phase of orofacial pain. Morphine (5 mg/kg) as a positive control significantly ($p < 0.05$) reduced pain response in the both phases of pain. Pre-treatment of animals with naloxone did not prevent the EOC (200 mg/kg) analgesic activity. Co-administration of sub-analgesic doses of EOC (50 mg/kg) and ketoprofen (40 mg/kg) significantly ($p < 0.05$) reduced nociceptive behavior in second phase. Also EOC (100 and 200 mg/kg) failed to increase nociceptive response latency in the tail immersion test. Meanwhile, EOC (100 and 200 mg/kg) and ketoprofen (80 mg/kg) significantly ($p < 0.001$) attenuated xylene-induced ear edema in mice. Also according to GC-MS results the major components of the EOC were eugenol (54.86%), β-Caryophyllene (20.19%), α-Humulene (7.11%), eugenol acetat (4.85%) and Chavibetol (2.23%).

Recommended applications/industries: These data showed that EOC possessed potent anti-inflammatory activity and produced non-opioid mediated analgesia in the second phase of orofacial pain without any effect on tail immersion response.

1. Introduction

Eugenia caryophyllata well known as Clove is a tree from Myrtaceae family with a height ranging from 10 to 20 meters that widely cultivated in Indonesia, Sri Lanka, Madagascar, Tanzania and Brazil (Arung *et al.*, 2011). Clove buds and leaves are used in cooking and food industries as a food flavoring agent, pharmaceutical company, perfumery and cosmetic products (Daniel *et al.*, 2009). Clove oil and its essential oil traditionally used in aromatherapy (Schiller and Schiller, 2008), relief of headaches, joint pain, toothaches and also as an oral antiseptic (Shelef, 1983; Soto and Burhanuddin, 1995; Chaieb *et al.*, 2007) Eugenol, as major component of clove essential oil has many of biological activities including antifungal, (Pinto *et al.*, 2009) antiallergic, (Kim *et al.*, 1998) antioxidant (Gülçin *et al.*, 2012) and insecticidal (Park *et al.*, 2000) properties. Essential oil of Eugenia caryophyllata has been recognized as an effective, safe and inexpensive anesthetic for fishes and amphibians (Kildea *et al.*, 2004). It was reported that clove essential oil has cytotoxic and anti-cancerogenic properties (Kouidhi *et al.*, 2010). Also, analgesic effect of eugenol in different models of pain has been well documented (Park *et al.*, 2011; Lionnet *et al.*, 2010; Kurian *et al.*, 2006; Peana *et al.*, 2004; Okhubo and Shibata, 1997). It has also been reported that, eugenol activates calcium channels and inhibits of Na^+ currents in rat dorsal root ganglion cells (Okhubo and Kitamura, 1997; Cho *et al.*, 2008) blocks calcium and potassium channels in guinea pig cardiac muscles (Sensch *et al.*, 2000). Topical application of eugenol in small quantities to dental cavity could produce anti-inflammatory and local analgesic effects (Markowitz *et al.*, 1992).

The orofacial region is one of the most densely innervated (by trigeminal nerves) areas of the body, which wide range of conditions like trigeminal neuralgia, post-herpetic neuralgia, temporomandibular joint disorders, periodontal inflammation and pathological states of the teeth produce acute and chronic painful sensation in this region (Raboisson and Dallel, 2004). Because of the lack of recognition and understanding of orofacial pain mechanisms, there are many difficulties in management of this kind of pain (Miranda *et al.*, 2009; Belmonte *et al.*, 2004) Natural therapies, because of less adverse effects and also more beneficial effects have a great advantage over common pain killers like opioids and non-steroidal anti-inflammatory drugs when side effects are taken into account (Petrović *et al.*, 2008; Zdunić *et al.*, 2009).

Therefore, we investigated the effects of EOC in two models of nociception (tail immersion test and formalin induced orofacial pain) as well as xylene induced ear edema as a model of inflammation to determine its possible anti-nociceptive and anti-inflammatory activities. Naloxone pretreatment was performed to clarify the involvement of opioidergic system in EOC-induced analgesia in the orofacial test. The possible contribution of cyclooxygenase system was assessed using ketoprofen (as positive control with peripheral analgesic and anti-inflammatory actions) with and without EOC.

2. Materials and Methods

2.1. Plant material and essential oil extraction

The buds of *Eugenia caryophyllata* was purchased from local market in Tabriz and were subsequently authenticated by Dr A. Ebrahimi, a botanist at the Herbarium of Faculty of Pharmacy (Tbz-Fph), Medical University of Tabriz, Tabriz, Iran. The buds were ground into a fine powder. The essential oil of Eugenia caryophyllata buds were extracted from powdered plant by hydro-distillation in a Clevenger type apparatus for 3 h and produced 8% (v/w) yield. Obtained EO was dried over anhydrous sodium sulfate until the last traces of water were removed, and then stored in dark glass bottles at 4 °C.

2.2. Animals

Adult male Wistar rats, weighing 270–300 g and adult male mice, weighing 32-36 g were used in this study. They were randomly housed in polyethylene cages with *ad libitum* access to food and water in a room with controlled temperature (22±1 °C) and under a 12 h light-dark cycle (lights on from 07:00 a.m.) Six rats were used in each group of orofacial formalin test and tail immersion test. All experiments were performed between 11:00 h and 15:00 h. All research and animal care procedures were approved by the Veterinary Ethics Committee of the Faculty of Veterinary Medicine, University of Tabriz and were performed in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983).

2.3. Drugs and chemicals

Morphine sulfate was purchased from Toliddarou Co (Tehran, Iran). Atropine sulfate and naloxone hydrochloride, Tween 80 and ketoprofen were purchased from Sigma Chemical Co (St. Louis, MO, USA) and formalin solution 37% and NaCl from Merck chemicals (Darmstadt, Germany). Xylene was purchased from Dr. Mojalali Chemical Co (Tehran, Iran). All drugs and chemicals were dissolved in physiological saline. Xylene is used in pure form in the xylene-induced ear edema test. An emulsion of essential oil was prepared using Tween 80 and saline (0.5%, v/v) as solvent.

2.4. Essential oil GC-MS analyze

The essential oil was analyzed by gas chromatography – mass spectrometry (GC-MS). The chromatograph instrument (Agilent 6890 UK) was equipped with an HP-5MS capillary column (30 × 0.25 mm ID × 0.25 mm film thickness) and the data were taken under the following conditions: initial temperature 50°C, temperature ramp 5°C/min, 240°C/min to 300°C (holding for 3 min), and injector temperature at 290°C. The carrier gas was helium and the split ratio was 0.8 mL-1/min. For confirmation of analysis results, EOs were also analyzed by gas chromatography–mass spectrometry (Agilent 6890 gas chromatograph equipped with an Agilent 5973 mass-selective detector; Agilent UK) and the same capillary column and analytical conditions as above. The MS was run in electron-ionization mode with ionization energy of 70 eV.

2.5. Xylene-induced ear edema in mice

Ear edema was induced by topical application of pure xylene (20 µl) to inner and outer surfaces of the left ear of each mouse and right ear was considered as control. Mice were divided into four groups (n=5/group). Group 1 served as control administered with Tween 0.5% (100 µl, s.c.). Group 2 served as positive control and was dosed with 80 mg/kg, IP ketoprofen 30 min before induction of ear edema. Groups 3 and 4 were treated with 100 and 200 mg/kg, SC of EOC 30 min prior to induction of ear edema. Mice were sacrificed by cervical dislocation 1 h after xylene application. The plug (7mm in diameter) was removed with a stainless steel punch from both xylene treated ear and untreated ear then weighted by a sensitive digital balance (Zhang *et al*., 2008). The ear edema percent was calculated according to the following formula:

Ear edema percent =100 × (left ear punch weight - right ear punch weight) / (right ear punch weight).

2.6. Orofacial formalin test

For reduction of possible effects of stress during the test, on two successive days prior to the orofacial formalin test, animals were placed 30 minute inside a Plexiglas observation chamber (30×30×25 cm) that equipped with a mirror angled at 45° below the chamber. In the test day after 30-min adaptation period, formaldehyde solution (1.5% in saline; 50µl) was injected subcutaneously into the left side of the upper lip (narrow area between upper lip and vibrissal pad) just lateral to the nose using a 31-gauge injection needle (insulin syringe 0.5 ml, BD® USA) (Raboisson and Dallel, 2004).

Following formalin injection, the rat was immediately put back in the observation chamber. The time spent in face rubbing with the forepaws determined as a nociceptive behavior and it was recorded in uninterrupted 3-min blocks over a period of 45 min (Erfanparast *et al.*, 2010). The first 3 min measured as the first phase (phasic pain, neurogenic phase) while the period between 18 - 36 min was considered as the second phase (inflammatory pain). Vehicle (Tween 80, 0.5% in saline; 1ml/kg, SC), EOC (50, 100 and 200 mg/kg), morphine sulphate (5 mg/kg, IP) and ketoprofen (40, 80, 160 mg/kg; IP) were administered 30 min prior to formalin injection. Naloxone (1 mg/kg, IP) was administered 40 min before formalin injection. In combination treatment, 50 mg/kg EOC was co-administered with 40 mg/kg ketoprofen.

2.7. Tail Immersion Test

The tail of rats was immersed (on 5 cm) in a water bath at noxious temperature of 55± 0.5 C until the tail was withdrawn or when the whole body was recoiled. The cut-off time was fixed at 15 seconds to prevent any tissue damage to the tail (Le Bars *et al.*, 2001). Reaction latencies at the 0, 15, 30, 45 and 60 min after chemicals administration were used as a parameter reflecting of the pain experienced.

2.8. Statistics

To evaluate significant differences among treated groups in the orofacial pain test as well as xylene-induced ear edema test, one-way analysis of variance

(ANOVA) were applied followed by Tukey HSD test. Also two way ANOVA with repeated measure and Bonferroni post hoc were used to assess the effects of time and drug in tail immersion test using IBM® SPSS® software version 19 (IBM company, USA). In figures, all values are expressed as Mean±SEM. A value of P <0.05 was considered statistically significant**.**

3. Results and discussion

3.1. GC-MS Analysis

The chemical composition of EOC was determined by GC-MS, which identified 9 compounds, representing 93.66% of total oil compounds. According to these results, the major components of the oil were Eugenol (54.86%), β-Caryophyllene (20.19%), α-Humulene (7.11%), eugenol acetat (4.85%) and Chavibetol (2.23%). The main percentage compositions of EOC are represented in table1.

3.2. Orofacial pain

The SC injection of formalin into the upper lip region in the normal saline and vehicle (Tween 80, 0.5% 200µl) treated animals without any significant differences produced pain responses in the first and 6^{th} – 12^{th} three min blocks. Therefore, formalin produced a biphasic pattern (first phase: 0-3 min and second phase: 18-36 min) of pain response in orofacial region (Figure 1).

The results are presented in Figure 2 showed that essential oil of *Eugenia caryophyllata* at dose of 50 mg/kg did not produce any significant effects in the both first and second phases of pain. The SC injection of EOC at doses of 100 and 200 mg/kg induced significant (p<0.05) anti-nociceptive activities only in the second phase when compared to control group. Morphine (5 mg/kg) significantly (p<0.05) inhibited behavioral responses in the both phases of the orofacial formalin test (Figure 2).

The IP injection of ketoprofen at doses of 80 and 160 mg/kg but not 40 mg/kg induced significant (p<0.05) antinociceptive activities only in the second phase of orofacial formalin test when compared to the control group in a dose dependent manner (Figure 3).

Naloxone (1 mg/kg) alone had no any significant effect. Pre-treatment of animals with naloxone did not prevent the EOC (200 mg/kg) analgesic activity (p<0.05) (Figure 4).

Co-administration of sub-analgesic doses of EOC (50 mg/kg) and ketoprofen (40 mg/kg) significantly (p<0.05) reduced nociceptive behavior in the second phase of formalin induced pain when compared with control group (Figure 5).

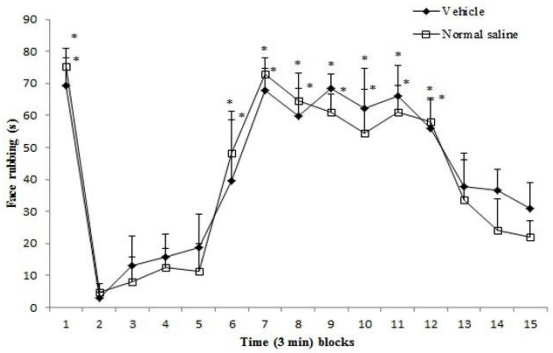

Fig. 1. Three-min blocks of face rubbing induced by injection of formalin into the upper lip in normal saline and Tween 80 0.5% treated groups. Values are expressed as the mean ± SEM (n=6/group). * p < 0.05 compared with other 3-min blocks (Two way ANOVA followed by Bonferroni post hoc test).

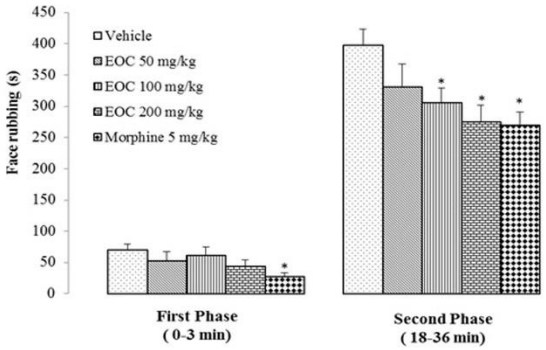

Fig. 2. Effects of P injection of essential oil of *Eugenia caryophyllata* (EOC) and Morphine on formalin-induced orofacial pain. Values are expressed as the mean ± SEM (n = 6/group). * p < 0.05 compared with vehicle (Tween 80 0.5%) group (One way ANOVA followed by Tukey HSD post hoc test).

3.3. Tail immersion test

As is shown in Figure 6, morphine (5 mg/kg) analgesic activity was started 30 min after IP injection (p<0.0001) and last until the end of observation period (60 min). SC injection of EOC at doses of 100 and 200 mg/kg failed to prolong withdrawal latency during the observation period.

3.4. Xylene-induced ear edema

Subcutaneous injection of EOC at doses of 100 and 200 mg/kg as well as ketoprofen at dose of (80 mg/kg) significantly ($p<0.01$, $p<0.001$ and $p<0.001$ respectively) attenuated xylene-induced ear edema in mice (Figure 7).

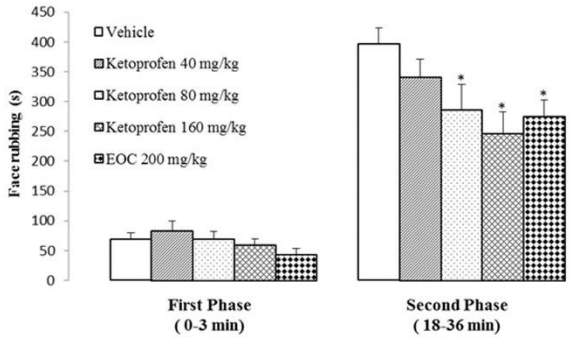

Fig. 3. Effects of IP injection of Ketoprofen on formalin-induced orofacial pain. Values are expressed as the mean ± SEM (n = 6/group). * p < 0.05 compared with vehicle (Tween 80 0.5%) group (One way ANOVA followed by Tukey HSD post hoc test).

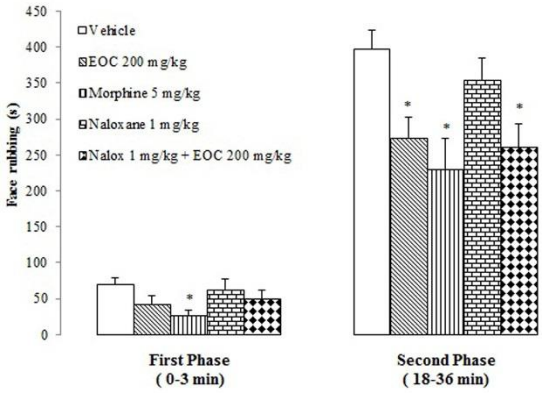

Fig. 4. Effects of IP injection of naloxone alone and before essential oil of *Eugenia caryophyllata* on on formalin-induced orofacial pain. Values are the means ± SEM (n = 6/group). * p < 0.05 compared with vehicle (Tween 80 0.5%) group (One way ANOVA followed by Tukey HSD post hoc test). EOC: Essential oil of *Eugenia caryophyllata*, Nalox: Naloxone.

Topical administration of Clove essential oil widely and traditionally used for relief of tooth pain and also it is well known in the dentistry because of its local analgesic and local anesthetic effects (Daniel *et al.*, 2009). Nonetheless, there aren't any published data about its systemic effects and mechanisms of action in the different kinds of pain arise from orofacial region and trigeminal nerve territory.

In this experiment chemical composition of EOC was analyzed by GC-MS, which identified 9 compounds, representing 93.66% of total oil compounds. The major components of the oil were Eugenol (54.86%), β-Caryophyllene (20.19%), α-Humulene (7.11%), Eugenol acetat (4.85%) and Chavibetol (2.23%).

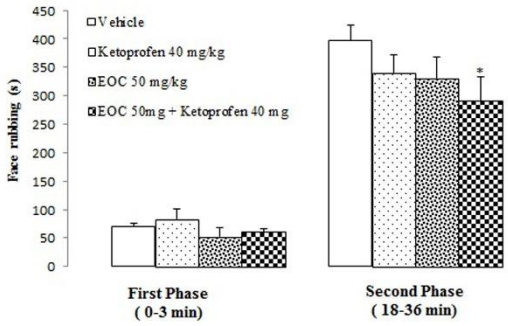

Fig. 5. Effects of combination treatments with sub-analgesic dose of essential oil of *Eugenia caryophyllata* (EOC) with Ketoprofen on formalin-induced orofacial pain. Values are expressed as the mean ± SEM (n = 6/group). * p < 0.05 compared with vehicle (Tween 80 0.5%) group (One way ANOVA followed by Tukey HSD post hoc test).

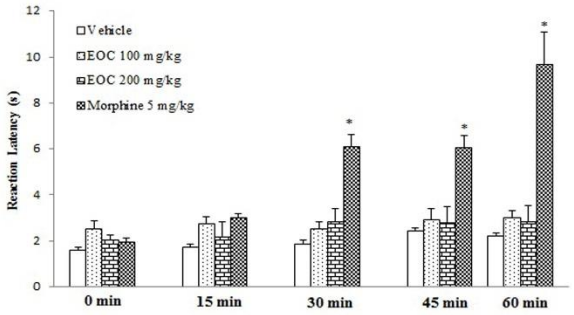

Fig. 6. The effects of essential oil of *Eugenia caryophyllata (EOC)* and morphine on tail immersion test response. * p < 0.0001 compared with other time points of vehicle. (n=6/group). (Two way ANOVA with repeated measure followed by Bonferroni post hoc test) EOC: Essential oil of *Eugenia caryophyllata*.

We identified high concentration of Eugenol and β-Caryophyllene in the extracted essential oil. Eugenol is a phenylpropene derivative, well known as local anesthetic (Park *et al.*, 2009), analgesic (Okhubo and Kitamura, 1997; Park *et al.*, 2006) and anti-

inflammatory agent (Hashimoto et al., 1988) that widely used in dentistry. The analgesic effects of eugenol may be modulated by its inhibitory effect on voltage-gated Na^+ channels (Park *et al.*, 2006) and on high voltage-activated $Ca2^+$ channels (Lee *et al.*, 2005). β-Caryophyllene is another component exists in the Eugenia caryophyllata essential oil, this sesquiterpene has a functional non-psychoactive CB_2 cannabinoid receptor agonistic activity (Gertsch *et al.*, 2008). Cannabinoid receptor CB_2 and its selective agonist accepted as a new pharmacological target for treatment of pain (Anand *et al*., 2009).

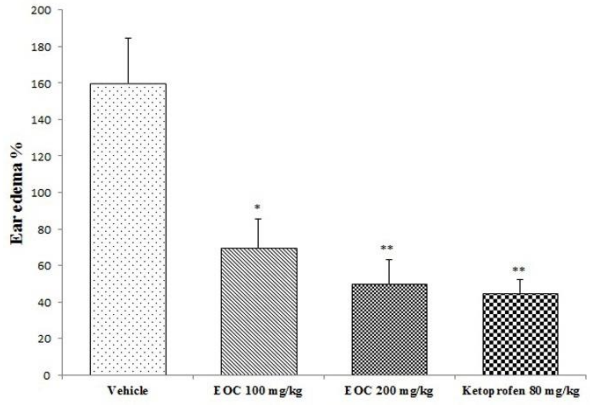

Fig. 7. Effect of essential oil of *Eugenia caryophyllata* (EOC) and ketoprofen on xylene induced ear edema in mice. Values are expressed as the mean ± SEM (n = 5/group). * $p < 0.01$ and **$p < 0.001$ compared with vehicle (Tween 80, 0.5%) group (One way ANOVA followed by Tukey HSD post hoc test).

Table1. The main components (%) of the *Eugenia caryophyllata* buds essential oil.

NO	Compound	RT[1]	%
1	Eugenol	12.99	54.86
2	β- Caryophyllene	14.00	20.19
3	α -Humulene	14.54	7.11
4	Iso-eugenol	13.49	1.23
5	Chavibetol	13.61	2.23
6	O- egunol	14.17	1.70
7	cis- Ocimene	15.51	1.41
8	Eugenol acetat	16.08	4.85
9	Caryophyllene oxide	17.29	0.08

[1]Retention Time.

Topical application of xylene to ear leads to an acute inflammatory response and is known to cause severe vasodilation and oedematous changes of skin (Kim *et al*., 2007). This method is used for the evaluation of anti-inflammatory steroids as well as non-steroidal antiphlogistic agents especially those inhibiting phospholipase A_2 (Zaninir *et al*., 1992).

Our data obtained from ear edema test revealed that EOC and ketoprofen can modulate inflammatory response induced by xylene which suggests it might peripherally reduce the release of inflammatory mediators.

Our results in the present study indicate antinociceptive effects of clove essential oil in the formalin induced orofacial nociception in rats, and also provide some evidence for determination of possible mechanisms of antinociceptive action in this model of pain.

In the orofacial formalin test, morphine (5 mg/kg) reduced pain response in both phases of orofacial pain. Also the results (Fig.2 and Fig. 4) showed that the essential oil of clove at doses of 100 and 200 mg/kg considerably inhibited second phase (inflammatory phase) of formalin induce orofacial pain response but this analgesic effect was not antagonized with non-selective opioid receptors antagonist, naloxone. Moreover high dose of EOC (200 mg/kg) reduced pain response in the first phase of formalin induce orofacial pain but this effect was not statistically significant. These findings suggest that analgesic effect of EOC is not mediated through opioidergic mechanisms and it's containing active analgesic properties acting predominantly with peripheral mechanisms (anti-inflammatory action) in this model of nociception. In addition, ketoprofen (non-selective cyclooxygenase and lipoxygenase inhibitor) at doses of 80 and 160 mg/kg significantly reduced pain response only in the second phase of orofacial pain and co-administration of sub-analgesic doses of EOC (50 mg/kg) and ketoprofen (40 mg/kg) significantly ($p<0.05$) reduced nociceptive behavior only in the second phase when compared with control group (Fig. 3 and 5). This potentiated analgesic effect may indicates that EOC can inhibits cyclooxygenase pathway and reduce inflammatory mediators of pain in the second phase of this test.

Two different phases of orofacial formalin test have different properties and are very useful tools, not only for assessing the potency of analgesics, but also for

elucidating the mechanisms of pain and analgesia (Chen *et al.*, 1995). Analgesic drugs that inhibit both phases of formalin pain like morphine primarily act centrally but peripherally acting drugs such as anti-inflammatory drugs as the cyclooxygenase inhibitors only inhibit the second phase of formalin-induced nociception (Chen *et al.*, 1995).

In the tail immersion test SC injection of EOC failed to prolong withdrawal latency during the observation period. The tail immersion test well known as a proper method to evaluate the central mechanisms of pain relief as observed in the case of opioids like morphine (Halder *et al.*, 2009).

Our results in formalin induced orofacial pain as well as tail immersion test revealed that EOC has a relatively more potent peripheral action than central action in causing pain relief.

4. Conclusion

In conclusion, the results of present study indicated that EOC possesses potent anti-inflammatory activity in xylene induced ear edema and also EOC and ketoprofen exhibited antinociceptive activities only in the inflammatory phase of formalin induced orofacial pain and opioidergic system may not be involved in the antinociceptive activity of Clove essential oil in this model of nociception.

5. Acknowledgments

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6. Conflicts of interest

The authors declare that they have no conflict of interest.

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