

Effects of topical and systemic administration of *Eugenia caryophyllata* buds essential oil on corneal anesthesia and analgesia

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Abstract

Clinical studies suggest that essential oil of *Eugenia caryophyllata* (Clove) buds (EOEC) is efficacious in the treatment of dental pain. In the present study, we investigated the analgesic and local anesthetic effects of EOEC and its possible mechanisms of action in acute corneal pain in rats. EOEC was extracted by hydro-distillation in a Clevenger type apparatus from clove buds. The acute corneal pain was induced by applying a drop (40 μ l) of 5 M NaCl solution on the corneal surface, and the numbers of eye wipes were counted during the first 30 s. The mechanical sensation of the cornea was evaluated by calibrated Von Frey filaments. Systemic administration of EOEC (100 and 200 mg/kg, SC) and morphine (2.5 and 5 mg/kg, IP) produced a significant antinociceptive effect in acute corneal pain. Pretreatment with naloxone or atropine prevented the EOEC-induced analgesia. However, L-arginine and methylene blue did not change the suppressive effect of EOEC on corneal pain response. Topical application of EOEC, eugenol and lidocaine significantly decreased corneal sensitivity. Combination treatments of eugenol (25 μ g) with lidocaine (0.5%) and EOEC (50 μ g) with lidocaine (0.5%) also significantly suppressed corneal sensitivity. Systemic administration of EOEC produced analgesia in the acute corneal pain through mechanisms that involved both opioidergic and cholinergic systems. In addition, topical instillation of EOEC, eugenol, and lidocaine produced local anesthesia in the rat cornea. Sub-anesthetic doses of EOEC or eugenol produced a significant local anesthetic effect when concurrently used with the sub-anesthetic dose of lidocaine.

Keywords: *Eugenia caryophyllata*; Analgesic; Corneal pain; Lidocaine; Local anesthesia; Eugenol.

INTRODUCTION

Eugenia caryophyllata, well known as Clove, is a tree from the Myrtaceae family with a height ranging from 10 to 20 meters that is widely cultivated in Madagascar, Tanzania, Sri Lanka, Brazil and Indonesia (1). Clove oil and its essential oil are traditionally used in aromatherapy and for the relief of headaches, joint pains, toothaches and also as an oral antiseptic (2,3). In addition, eugenol as a major component of clove essential oil has different kinds of biological activities, including antifungal (4), anti-allergic (5), and antioxidant (6) properties. Essential oil of *E. caryophyllata* and its main constituent eugenol have been recognized as a safe, effective and inexpensive anesthetic for fishes and amphibians (7). Also, the analgesic effect of eugenol in different models of pain has been well documented (8-12).

Pain arises from the cornea would be very powerful and incapacitating. Corneal nociceptor density has been estimated to be 20-40 times greater than dental pulp and 300 to 600 times higher than skin (13). These polymodal nociceptors mostly respond to a range of noxious stimuli such as cold, heat, high threshold touch, chemicals, and protons. Moreover, there is a wide range of conditions including dry eye, post-herpetic neuralgia, trigeminal neuralgia, contaminated environments, contact lens wear and new surgical techniques for the correction of refractive defects that cause ocular discomfort and pain (14).

Due to lack of understanding and recognition of acute and chronic trigeminal pain mechanisms, there are some difficulties in the management of these kinds of pains (15).

Because of less adverse and more beneficial

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effects, herbal therapies have a great advantage over common painkillers like opioids and nonsteroidal anti-inflammatory drugs when side effects are taken into account (16).

The essential oil of *E. caryophyllata* buds (EOEC) is used in dental care as an analgesic, local anesthetic and oral antiseptic. Therefore, the present study was aimed to investigate the systemic antinociceptive and local anesthetic effects of EOEC in rat cornea. Also, to clarify the possible analgesic mechanisms of EOEC, we used morphine, naloxone (nonselective opioid receptor antagonist), atropine (nonselective muscarinic receptor antagonist), L-arginine (nitric oxide pathway precursor), and methylene blue (a non-specific inhibitor of NO/guanylyl cyclase) in the acute model of corneal chemical pain. Moreover, corneal touch thresholds and also duration of corneal local anesthesia were determined after topical instillation of EOEC and eugenol (as an active ingredient of EOEC), alone and in co-application with lidocaine.

MATERIAL AND METHODS

Animals

All study experiments and animal care procedures were approved by the Veterinary Ethics Committee of the Faculty of Veterinary Medicine, University of Tabriz (9 June 2014, Ref. No: D/2014.16) 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 (17).

Adult male Wistar rats weighing 230-260 g 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. All experiments were performed between 11:00 a.m. and 15:00 p.m. Each rat received one systemic and one topical administration of solution and seven days was allowed between each trial.

Drugs

Morphine sulfate was purchased from Tolid Darou Co (Tehran, Iran). Atropine sulfate and

naloxone hydrochloride, Tween 80, lidocaine and eugenol were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). L-arginine and methylene blue were purchased from Merck Chemicals (Darmstadt, Germany). For the systemic and topical studies all drugs and chemicals were dissolved in physiological saline. An emulsion of essential oil and eugenol were prepared using Tween 80 and saline (0.5%, v/v) as solvent. Polysorbate 80 (Tween 80) is a nonionic surfactant and emulsifier that enables EOEC and eugenol to be easily emulsified in the saline. All solutions were adjusted to pH 7.4 before use.

Plant material and essential oil extraction

The buds of *E. caryophyllata* were purchased from a Phyto-medical company (Parsi Teb Co, Tabriz, Iran) 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 dried buds of the plant were ground into a fine powder. The essential oil was extracted from powdered buds by hydro-distillation in a Clevenger type apparatus (Soham Scientific Co, Soham, UK) for 3 h and produced 8% (v/w) yield. Yielded essential oil was dried over anhydrous sodium sulfate until the last traces of water were removed, and then stored in dark glass bottles at 4 °C. Gas chromatography mass spectrometry (GC-MS) analysis of EOEC has already been performed in our laboratory and reported (18).

Acute corneal pain induction (Eye wiping test)

Each rat was placed on a 50 × 50 × 1 cm wooden table and after a 15 min habituation period, one drop (40 µl) of 5 M NaCl solution was topically applied onto the surface of the cornea using a sampler (Transferpette® S 10-100 µl Brand Co, Germany). After topical application of 5 M NaCl solution, rats always wiped their eyes with their forepaw and sometimes rapidly scratched their eyes with the hind paw. The numbers of the eye wipes performed with ipsilateral forepaw were counted over a period of 30 s. Each burst of hind paw scratches was counted as one wipe as well (19,20).

The first eye wiping test (pre drug wiping test) of each rat was measured 10 min before all chemicals administration. The second eye wiping test was performed 30 or 40 min after drug administration, depending on the type of treatment. The maximal possible effect (% MPE) of drugs and essential oil was calculated for eye-wipes according to the following formula:

$$\% \text{ MPE} = 100 \times [(\text{post drug wipes count} - \text{pre drug wipes count}) / (0 - \text{pre drug wipe count})]$$

Administration of chemicals for evaluation of acute corneal pain

The rats were injected with vehicle (Tween 80, 0.5% in saline, 1 mL/kg, subcutaneously (SC)), EOEC (50, 100 and 200 mg/kg, SC), and morphine sulfate (1.25, 2.5 and 5 mg/kg, intraperitoneally (IP) 30 min before induction of the pain. Naloxone (1 mg/kg, IP), atropine (1 mg/kg, IP), L-arginine (100 and 300 mg/kg, IP) and methylene blue (5 and 10 mg/kg, IP) were administrated 40 min before the last eye wiping test. Each animal was injected only once in the eye wiping test.

Von Frey test for assessing corneal touch threshold

The Von Frey test was employed to examine mechanical sensitivity thresholds of the cornea (21). Animals were gently held and restrained by hand during the testing period. Using a set of calibrated von Frey filaments (VFF) (Stoelting Co., Wood Dale, Illinois, USA), animal withdrawal responses to the corneal touch include blinking and head withdrawal (abrupt head movement) were counted as a positive response. The corneal touch threshold (CTT) was defined as the lowest force of the filaments that produced at least three withdrawal responses in five tests in control animals (21-23).

Local anesthetic assay using Von Frey filaments

Dose-dependent study of CTT

To assess behavioral corneal responses in the dose-dependent study, Von Frey filaments of ascending stiffness (0.008, 0.02, 0.04, 0.16, 0.4, 0.6, 1, and 2 g) were used to evaluate mechanical sensitivity of cornea (in the central cornea).

Vehicle (Tween 80, 0.5% dissolved in saline), EOEC (25, 50, 200 µg/eye), eugenol (25, 50 and 100 µg/eye), lidocaine (0.5% and 2%, 40 µl/eye) and combination of EOEC (25 µg) or eugenol (25 µg) with lidocaine (0.5%, 40 µl/eye) were topically applied to the cornea in two consecutive 20 µl volumes (total volume of 40 µl) and animals gently restrained by hand to prevent wiping of solution for 1 min in each application. Five to eight min after solution instillation to the cornea, Von Frey tests were started for evaluation of CTT (24).

Time-dependent study of local anesthesia duration

In the time-dependent study, we used only greatest filament (2 g) for assessment of the duration of corneal anesthesia induced by EOEC (200 µg), Eugenol (100 µl), lidocaine 2% and combination of EOEC (25 µg) or eugenol (25 µg) with lidocaine (0.5%). Touch test procedures were performed 5 min before and 5, 10, 15, 20, 25, 30, and 40 min after topical instillation of all solutions.

Statistical analysis

Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Tukey HSD post hoc test used for dose dependent studies and also two-way ANOVA with repeated measures and Bonferroni post hoc which used to assess the effects of time and drug in time course study 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.

RESULTS

Effect of EOEC and morphine administration on acute corneal pain

In the present study, subcutaneous administration of EOEC at doses of 100 and 200 mg/kg but not at a dose of 50 mg/kg showed an inhibitory effect on eye wiping response (33.84% and 39.91% respectively, $P < 0.05$, Fig. 1A) compared to vehicle treated group. In addition, morphine (1.25, 2.5 and 5 mg/kg, IP) produced an analgesic effect on eye wiping response (30.82%, 41.24% and 74.1% respectively, $P < 0.05$ and $P < 0.01$, Fig. 1B) compared to vehicle treated group.

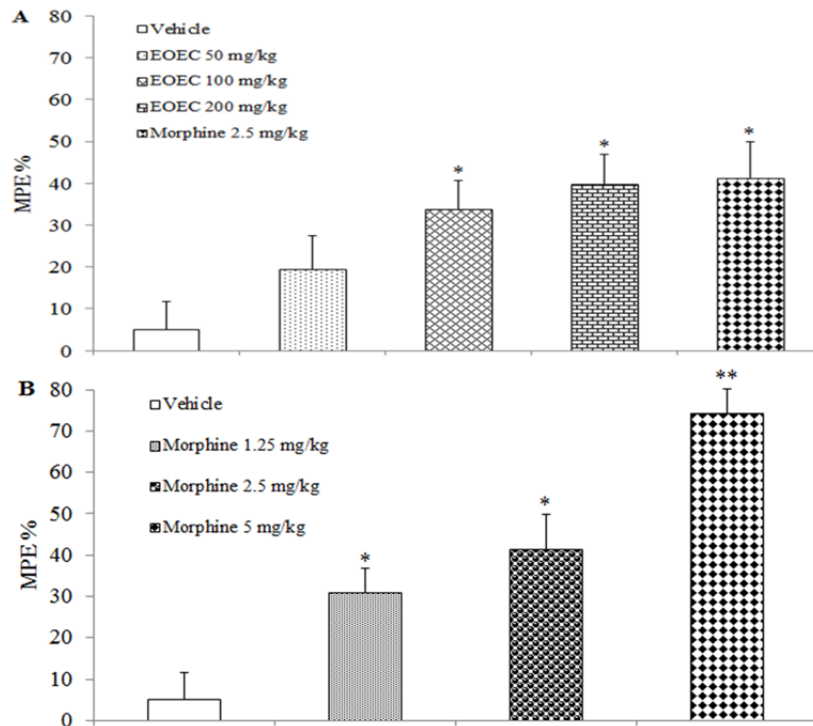


Fig. 1. A; Analgesic effect of subcutaneous administration of EOEC and B; intraperitoneal injection of morphine on corneal pain responses induced by 5 M NaCl solution applied to cornea surface in rats. % MPE is considered as an index for comparison between the results of two tests (for calculation of % MPE see the text). The values are expressed as Mean ± SEM (N = 6 per group). Data are compared with one-way analysis of variance (ANOVA) followed by Tukey HSD post hoc test; * $P < 0.05$, vs control. EOEC; essential oil of *Eugenia caryophyllata*.

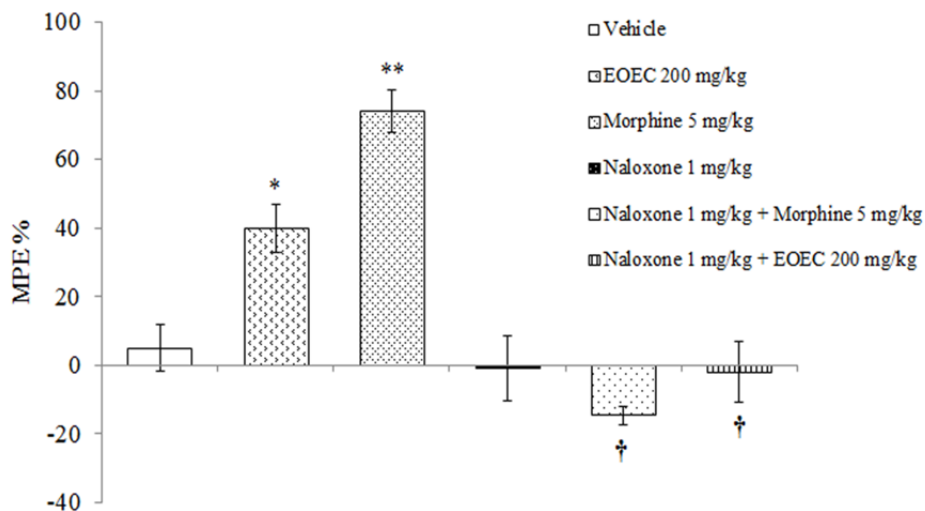


Fig. 2. Effect of pretreatment with naloxone on antinociceptive activity of EOEC and morphine in corneal pain response induced by 5 M NaCl solution applied to cornea surface in rats. % MPE is considered as an index for comparison between the results of two tests (for calculation of % MPE see the text). Values are expressed as Mean ± SEM (N = 6 per group). The data are compared with one-way analysis of variance (ANOVA) followed by Tukey HSD post hoc test; * $P < 0.05$ and ** $P < 0.01$ vs control. † $P < 0.01$ vs EOEC 200 mg/kg and morphine.

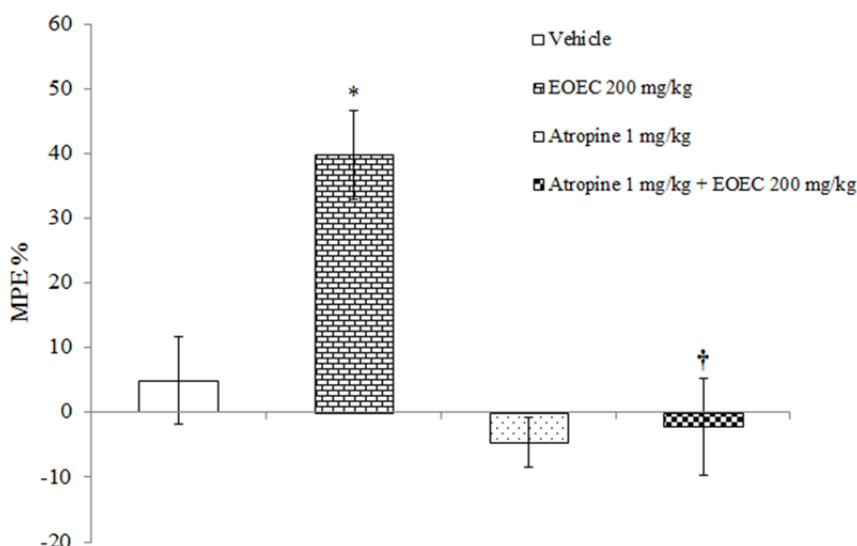


Fig. 3. Effect of pretreatment with atropine on the antinociceptive activity of EOEC in corneal pain response induced by 5 M NaCl solution applied to cornea surface in rats. % MPE is considered as an index for comparison between the results of two tests (for calculation of % MPE see the text). Values are expressed as Mean \pm SEM (N = 6 per group). The data are compared with one-way analysis of variance (ANOVA) followed by Tukey HSD post hoc test; * P <0.05, † P <0.01 vs EOEC 200 mg/kg.

Effect of naloxone, atropine, and L-arginine on EOEC-induced analgesia in the acute corneal pain

Administration of naloxone (1 mg/kg, IP) alone had no effect on eye wiping response, but pretreatment of animals with naloxone inhibited the antinociceptive effect of EOEC (200 mg/kg) and morphine (5 mg/kg) in the eye wiping response (-1.89% and 14.62% respectively, P <0.01, Fig. 2) in comparison with the EOEC 200 mg/kg and morphine 5 mg/kg groups respectively. On the other hand, administration of atropine (1 mg/kg, IP) alone had no effect on eye wiping response. However, pretreatment of animals with atropine inhibited the antinociceptive effect of EOEC (200 mg/kg) in the eye wiping response (2.22%, P <0.01, Fig. 3) in comparison with the EOEC 200 mg/kg.

Administration of L-arginine (100 and 300 mg/kg, IP) alone had no significant effects on eye wiping response. Pretreatment of animals with L-arginine failed to prevent the antinociceptive effect of EOEC at 200 mg/kg (38.65%, P <0.05, Fig. 4A). In addition, methylene blue (5 and 10 mg/kg, IP) alone had no significant effects on pain response and also pretreatment of animals with methylene blue (5 mg/kg, IP) did not produce any

significant effect on EOEC (200 mg/kg) induced antinociception (34.22%, P <0.05, Fig. 4B).

Local anesthetic activity of topical EOEC, eugenol, and lidocaine

The CTT was determined to be 0.4 g (calibrated force) in the vehicle treated cornea (Fig. 5A). In the dose-dependent study, EOEC at doses of 50 μ g/eye increased (P <0.05) corneal touch threshold to 0.6 g (calibrated force). EOEC at a dose of 200 μ g/eye significantly produced a local anesthetic effect (P <0.001) for all tested forces in comparison with the vehicle group (Fig. 5A).

Eugenol at doses of 50 and 100 μ g/eye significantly (P <0.05) increased corneal touch threshold to 1 and 2 g calibrated force respectively (Fig. 5B). Topical application of lidocaine at the dose of 2% (40 μ l/eye) but not 0.5% produced a potent local anesthetic effect (P <0.001) for all tested forces in comparison with the vehicle group (Fig. 5C). Moreover, co-administration of low (sub-anesthetic) dose of EOEC or eugenol with the low (sub-anesthetic) dose of lidocaine 0.5% produced significant (P <0.001) local anesthetic effect for all tested forces in comparison with the vehicle group (Fig. 5D).

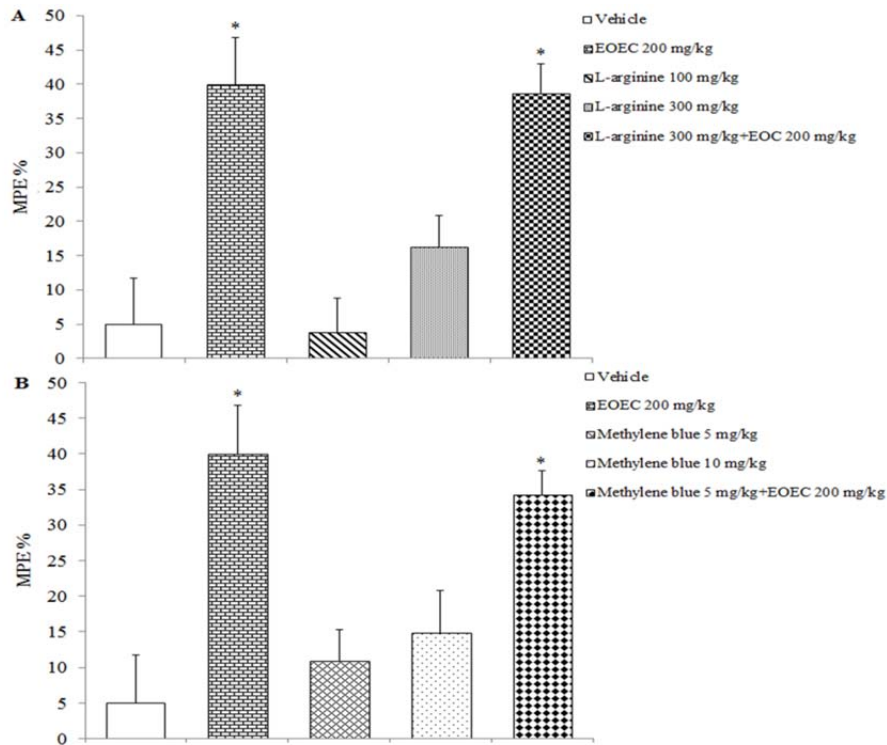


Fig. 4. Effects of pretreatment with A; l-arginine and B; methylene blue on the antinociceptive activity of EOEC (200 mg/kg) against 5 M NaCl-induced corneal pain response in rats. % MPE is considered as an index for comparison between the results of two tests (for calculation of % MPE see the text). The values are expressed as Mean \pm SEM (N = 6 per group). Data are compared with one-way analysis of variance (ANOVA) followed by Tukey HSD post hoc test; * $P < 0.05$.

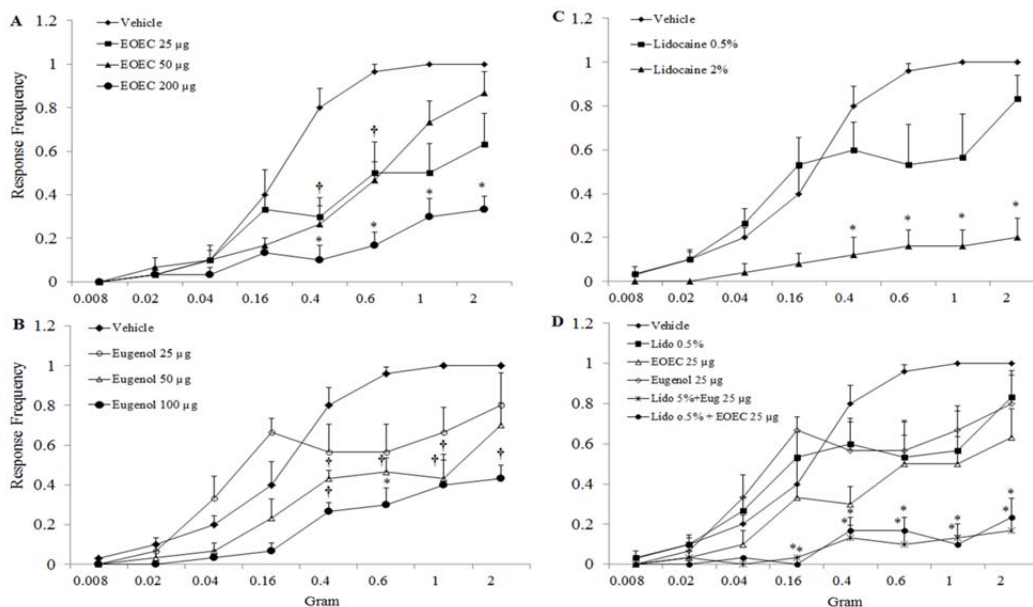


Fig. 5. Anesthetic effects of topically administered A; EOEC, B; Eugenol, C; lidocaine and D; combination of lidocaine with EOEC or Eugenol on the corneal touch sensitivity that was measured by the application of ascending series of Von Frey filaments against cornea in rats. The values are expressed as Mean \pm SEM (N = 6 per group). The data are compared with two way ANOVA with repeated measure, followed by Bonferroni post hoc test; * $P < 0.001$, † $P < 0.05$. vs vehicle. EOEC; essential oil of *Eugenia caryophyllata*, Lido; lidocaine, Eug: eugenol.

In time course study, the duration of effect was assessed using greatest filament (2 g) by comparing differences over time relative to the baseline for each solution. At baseline, there were no differences in corneal response values between any of the treatment groups. Topical administration of EOEC at 200 μ g and eugenol at 100 μ g significantly ($P < 0.001$ and $P < 0.05$, respectively) produced the local anesthetic effect against 2 g force produced by VFF until 15 min and 10 min after instillation, respectively, however, lidocaine 2% produced local anesthesia against the same force

persisting until 25 min after instillation ($P < 0.001$ and $P < 0.01$) (Figs. 6A and 6B).

Co-administration of low (sub-anesthetic) dose of EOEC (25 μ g) with the low (sub-anesthetic) dose of lidocaine (0.5%) produced significant ($P < 0.001$ and $P < 0.01$) local anesthesia until 30 min after instillation (Fig. 6C). Co-administration of low (sub-anesthetic) dose of eugenol (25 μ g) with the low (sub-anesthetic) dose of lidocaine (0.5%) produced significant ($P < 0.0001$ and $P < 0.001$) local anesthesia until 15 min after instillation (Fig. 6C).

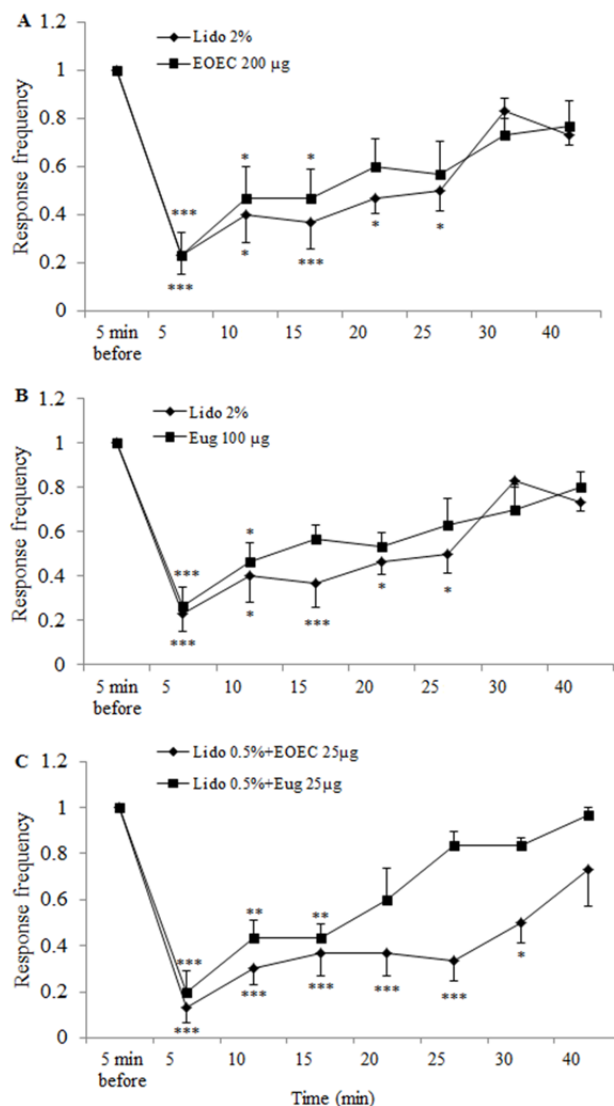


Fig. 6. Corneal response frequencies to maximum 2-g Von Frey filament force overtime after application of A; lidocaine or EOEC, B; eugenol and C; combination of lidocaine with EOEC or eugenol in rat. Values are expressed as the Mean \pm SEM (N = 6 per group). *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.01$ compared with 5 min before topical application of chemicals. (Two-way ANOVA with repeated measure, followed by Bonferroni post hoc test). Lido; lidocaine, EOEC; essential oil of *Eugenia caryophyllata*, Eug; eugenol.

DISCUSSION

Topical administration of one drop 5 M NaCl solution to the corneal surface produced acute chemical pain responses in this study. It has been shown that the application of NaCl, capsaicin and nicotine on the corneal surface produce a vigorous response in the nociceptive neurons in the trigeminal subnucleus caudalis in rat (25). Thin myelinated A-delta and unmyelinated C type fibers responds to chemical, mechanical and thermal noxious stimuli on the corneal surface (14). It has been reported that hyper-osmotic solutions like NaCl activating transient receptor potential vanilloid subtype 1 (TRPV₁) and transient receptor potential melastatin subtype 8 (TRPM₈) receptors in corneal nociceptors could induce chemical nociception (26,27). Using hypertonic saline to explore acute corneal pain was adapted by Frazifard *et al.* (2005) as a new model to assess acute pain in cornea and thereafter this test has been frequently used with success in several studies (19,20,28).

In the present study, IP injection of morphine suppressed eye wiping response in the acute corneal pain and also pretreatment with naloxone prevented this suppressive effect. These results showed that the analgesic effect of morphine is mediated by naloxone-sensitive mechanism in this model of pain.

Our results indicated the antinociceptive effect of EOEC (100 and 200 mg/kg) on hypertonic saline induced corneal pain in rats. In addition, these data provided some evidence for determination of possible mechanisms of antinociceptive action of EOEC in this model of nociception. The antinociceptive effect induced by the EOEC (200 mg/kg, SC) was significantly inhibited by both naloxone and atropine. Moreover, the administration of methylene blue and L-arginine did not alter EOEC-induced analgesia. These findings suggest that the analgesic effect of EOEC in this study may be mediated through opioidergic and cholinergic systems, but not via L-arginine/NO/cyclic GMP pathway.

It was reported that oral administration of eugenol produced naloxone-sensitive antinociception in the acetic acid-induced writhing test (8).

Analgesia produced by EOEC in the acute corneal pain appears to depend on its cholinergic activity, since EOEC effect was completely antagonized by the muscarinic cholinergic receptor antagonist atropine. *In vitro* studies of clove oil and eugenol have shown potent anti-acetylcholinesterase property (29,30). Since cholinergic system activation by muscarinic agonist or cholinesterase inhibitors have shown to produce analgesic effects, anti-acetylcholinesterase activity of EOEC and eugenol might also be in agreement with its mechanisms of their antinociception (31,32).

On the other hand, our previous experiments showed that SC administration of EOEC significantly reduced pain and inflammation in formalin-induced orofacial pain and xylene-induced ear edema, respectively (18).

The results of the present study showed that topical application of EOEC and eugenol like lidocaine (as a positive control) reduced corneal mechanical sensitivity in rats. The patterns of corneal anesthesia produced by application of high doses of EOEC and eugenol were similar to the anesthetic pattern of lidocaine. Co-administration of low (sub-anesthetic) dose of EOEC or eugenol with the low (sub-anesthetic) dose of lidocaine produced a significant local anesthetic effect. It seems that a concur effect between EOEC or eugenol with lidocaine is responsible for their local anesthetic effects when administered concomitantly. Co-administration of sub-anesthetic dose of lidocaine with EOEC produced a long lasting anesthesia in comparison with co-administration of lidocaine with a sub-analgesic dose of eugenol. This effect may be due to the coexisting of other components with eugenol in the *E. caryophyllata* essential oil.

We observed a high concentration of eugenol (54.86%) and β -caryophyllene (20.19%) in the EOEC composition (18). Eugenol is a phenylpropene derivative, well known as a local anesthetic (33), analgesic (8,11), and anti-inflammatory agent (34). The analgesic and local, anesthetic effects of eugenol may be modulated by its inhibitory effect on voltage-gated Na⁺ channels (35) and on high voltage-activated Ca²⁺ channels (36), also by weak activation of TRPV₁ receptors (37). As eugenol activates transient receptor

potential ankyrin subtype 1 (TRPA₁) receptors expressed in trigeminal ganglion neurons in rodents and humans, it is possible that activation of TRPA₁ by eugenol contributes to its antinociceptive activity (38). The inhibition of voltage-gated Na⁺ channels and activation of TRPV₁ produced by eugenol are very similar to the effects of local anesthetics such as lidocaine (39).

β-caryophyllene, a sesquiterpene with CB₂ cannabinoid receptor agonist activity, also exist in the *E. caryophyllata* essential oil (18,40). Cannabinoid receptor CB₂ and its selective agonists are accepted as a new pharmacological target for the treatment of painful disorders (41). Moreover, topical administration of β-caryophyllene produced a strong local anesthetic effect in the rabbit conjunctival reflex test (42).

The presence of another sesquiterpene, α-humulene, in EOEC was confirmed and reported by us (18). Fernandes *et al.* reported that oral treatment with α-humulene and (-)-trans-caryophyllene produced noticeable inhibitory effects in different inflammatory experimental models in mice and rats (43).

CONCLUSION

In conclusion, the present results suggest that the EOEC produced antinociception in the acute chemical corneal pain through mechanisms that involved both opioidergic and/or the cholinergic systems, but not via L-arginine/NO/cyclic GMP pathway, supporting the traditional usage of the plant to treat various painful processes in the trigeminal nerve territory. Topical application of EOEC, eugenol and lidocaine reduced corneal sensitivity, but maximal anesthesia and duration differed between chemicals. Sub-anesthetic doses of EOEC or eugenol produced a good local anesthetic effect when used concurrently with the sub-anesthetic dose of lidocaine.

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