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Research Article

OPIOIDERGIC AND CHOLINERGIC BUT NOT NITRIC OXIDE PATHWAYS ARE INVOLVED IN ANTINOCICEPTIVE ACTIVITY OF *VITEX AGNUS-CASTUS* ESSENTIAL OIL IN THE ACUTE TRIGEMINAL MODEL OF PAIN IN RAT

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ABSTRACT

Objective: *Vitex agnus-castus* (VAC) and its essential oil traditionally used to treat many conditions and symptoms such as premenstrual problems, mastalgia, inflammation, sexual dysfunction and pain. This study was conducted to investigate the analgesic effect of essential oil extracted from VAC (EOVAC) leaves in acute trigeminal model of nociception in adult male Wistar rats. Furthermore, possible involvements of opioidergic, cholinergic and L-arginine/NO/cyclic GMP pathway in analgesic activity of EOVAC were investigated.

Methods: The EOVAC was extracted from powdered plant material by hydro-distillation in a Clevenger type apparatus. Acute trigeminal pain was induced by putting a drop of NaCl 5 M solution (40 µl) on the corneal surface of the eye and the numbers of eye wipes counted during the first 30 seconds.

Results: EOVAC at doses of 100 and 200 mg/kg subcutaneous and morphine 2.5 and 5 mg/kg i.p. produced a significant anti-nociceptive effect in this model of corneal pain. Naloxone (1 mg/kg i.p.) and atropine (1 mg/kg i.p.), L-arginine (100 and 200 mg/kg i.p.) and methylene blue (5 and 10 mg/kg i.p.) alone had no any effect in the acute corneal pain. Pretreatment with naloxone or atropine significantly reversed the EOVAC-induced analgesia, but L-arginine (300 mg/kg) and methylene blue (5 mg/kg) did not change the suppressive effect of EOVAC on pain response.

Conclusion: The present results suggest that the EOVAC produced anti-nociception in the acute corneal pain through mechanisms that involved both opioidergic system and/or the cholinergic system, but not via L-arginine/NO/cyclic GMP pathway, supporting the folkloric usage of the plant to treat various painful processes.

Keywords: Acute trigeminal pain, *Vitex agnus-castus* essential oil, Opioidergic system, Cholinergic system, Rat.

INTRODUCTION

The cornea is one of the most densely innervated (by the trigeminal nerves) organs in the body with a nerve density 300-600 times that of skin, which wide range of conditions such as dry eye, trigeminal neuralgia, post-herpetic neuralgia, contaminated environments, contact lens wear and the extended use of new surgical techniques for the correction of refractive defects, such as photorefractive keratectomy or laser-assisted *in situ* keratomileusis, cause ocular discomfort and pain often described as eye dryness [1,2]. Because of the lack of recognition and understanding of acute trigeminal pain mechanisms, there are many of difficulties in management of this kind of pain [2,3]. Natural therapies, because of less adverse effects and also more beneficial effects have a great advantage over common painkillers like opioids and non-steroidal anti-inflammatory drugs when side-effects are taken into account [4,5].

Essential oil is a volatile aromatic compound from plants that have been used medicinally in history [6]. *Vitex agnus-castus* (VAC) is a small deciduous shrub commonly known as monk pepper, or chaste tree belongs to the *Lamiaceae* family of plants that widely distributed in the Middle East and Mediterranean region [7]. VAC traditionally used as a treatment for menstrual problems, inflammation, sexual dysfunction and pain [8]. In the Iranian folk medicine VAC used for treatment of epilepsy [9,10], sedation, constipation, and bloat [11,12].

Essential oil extracted from VAC (EOVAC) contains some important active pharmacological ingredients' like α-pinene, α-bisabolol, 1,8-cineol, β-caryophyllene and limonene [7]. Previous studies have indicated that these terpenes have anti-inflammatory and anti-nociceptive effects in different models of pain and inflammation [13,14]. Furthermore, essential oil of VAC has shown anti-microbial and anti-fungal activities [15].

Different kind of extracts from VAC and other *Vitex* species have been reported to produce anti-nociceptive and anti-inflammatory effects [12,16], enhance female fertility [17], suppress tumor growth activity [18], reduce moderate to severe symptoms of premenstrual syndrome (PMS) like mastalgia, headache, fatigue, anxiety and depression [19-21]. Beneficial effect of VAC extracts in the treatment of PMS symptoms causes to increasing interest for the determination of possible mechanisms of action of VAC in PMS symptoms treatment.

Despite the demonstration of the efficacy of VAC extracts in the treatment of PMS symptoms and reduction of pain perception, nothing has been published about the effects of EOVAC in acute trigeminal pain modulation. Therefore, the present study was aimed to investigate the anti-nociceptive activity of EOVAC on the acute trigeminal model of pain and also we used morphine, naloxone (nonselective opioid receptors antagonist), atropine (nonselective muscarinic receptors antagonist), L-arginine (nitric oxide pathway precursor) and methylene blue (a non-specific inhibitor of NO/guanylyl cyclase), to determine its possible opioidergic, cholinergic and L-arginine/NO/cyclic GMP pathway mechanisms of action respectively.

METHODS

Plant material and essential oil extraction

The leaves of VAC was collected during August-September in 2012 from vicinity of Maragheh county in the East Azerbaijan-Iran and were subsequently authenticated by Dr Fatemeh Khoshbakht Koolagh, a botanist at the Herbarium of Faculty of agriculture, University of Tabriz, Tabriz, Iran. A voucher specimen (16697) has been deposited at the Herbarium of Faculty of agriculture. The leaves were dried in room temperature avoiding from direct sunlight and then ground into a fine powder. The essential oil of VAC leaves were extracted from powdered plant by hydrodistillation in a Clevenger type apparatus for 4 hrs and

produced 0.7% (v/w) yield. Obtained 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.

Animals

Adult male Wistar rats, weighing 250-280 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 hrs light-dark cycle (lights on from 07:00 hrs). Six rats were used in each group of test. All experiments were performed between 11:00 hrs and 15:00 hrs. 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 [22].

Drugs and chemicals

Morphine sulfate was purchased from Toliddarou Co (Tehran, Iran). Atropine and naloxone hydrochloride and Tween 80 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). L-arginine, Methylenblue and NaCl were purchased from Merck chemicals (Darmstadt, Germany). All drugs and chemicals were dissolved in physiological saline only Nacl dissolved in distilled water. An emulsion of essential oil was prepared using Tween 80 and saline (0.5%, v/v) as solvent.

Acute trigeminal test

Each rat was placed on a 50 cm × 50 cm × 1 cm wooden table and after a 15 minutes habituation period, one drop (40 µl) of NaCl 5 M solution was topically applied on the surface of the cornea using a pipette (Transferpette® S 10-100 µl Brand Co., Germany). After topical application of NaCl 5 M solution, rats always wiped with the forepaw and sometimes rapidly scratched the eye with the hind paw. The numbers of eye wipes performed with ipsilateral forepaw were counted for a period of 30 seconds. Also, each burst of hind paw scratches were counted as one wipe [23,24]. The test was performed pre-drug and post-drug administration at the same eye of the same animal with minimum 30 minutes interval. Subcutaneous treatments (s.c.) with vehicle (Tween 80, 0.5% in saline 200 µl), EOVAC (25, 50, 100, 150 and 200 mg/kg) were given 30 minutes prior to the second eye wipe test (n=6 per group). Morphine sulfate (1.25, 2.5 and 5 mg/kg i.p.) administrated 30 minutes before the second eye wipe test. Naloxane (1 mg/kg i.p.) and atropine (1 mg/kg i.p.) L-arginine (100 and 300 mg/kg) and methylene blue (5 and 10 mg/kg) were administrated 40 minutes before the last eye wipe test. The effect of drugs and essential oil from the maximal possible effect (% MPE) was calculated for eye-wipes according to the following formula:

%MPE=100 × post drug wipe count − pre drug wipe count/(0 − pre drug wipe count)

Statistics

Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Tukey *post-hoc* test using IBM® SPSS® software version 19 (IBM Company, USA). In figures, all values are expressed as mean±standard error of mean. A value of *p<0.05, **p<0.01 was considered as statistically significant.

RESULTS

Subcutaneous administration of EOVAC at doses of 100, 150 and 200 mg/kg but not 25 and 50 mg/kg showed an inhibitory effect on eye wipe response (30.08%, 32.41% and 38.80% respectively p<0.05, Fig. 1). As shown in Fig. 2, morphine (1.25, 2.5 and 5 mg/kg i.p.) produced an analgesic effect in eye wipe response (34.80%, 41.24% and 71.86% respectively, p<0.05, p<0.05 and p<0.01). Administration of naloxane (1 mg/kg i.p.) alone had no any effect on eye wipe response (Fig. 3). Pre-treatment of animals with naloxone completely inhibited anti-nociceptive effect of EOVAC 200 mg/kg and morphine 5 mg/kg in eye wipe response (1.33% and −14.62%, respectively, p<0.01) (Fig. 3). Administration of atropine (1 mg/kg i.p.) alone had no any effect on eye wipe, but pre-treatment of animals with atropine inhibited anti-

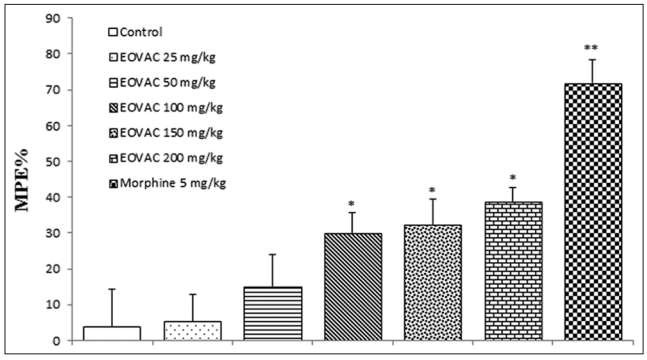

Fig. 1: Analgesic effect of essential oil extracted from *Vitex agnus-castus* and morphine on corneal pain response induced by NaCl 5M solution applied to corneal surface in rat. Maximal possible effect (%MPE) is considered as an index for comparison between results of two tests (for calculating %MPE=100 × post drug wipe count − pre drug wipe count/[0 − pre drug wipe count]). The values are expressed as mean±standard error of mean (n=6/group). The data are compared with one-way analysis of variance (ANOVA) followed by Tukey honestly significant difference *post-hoc* test; *p<0.05, **p<0.01 versus control

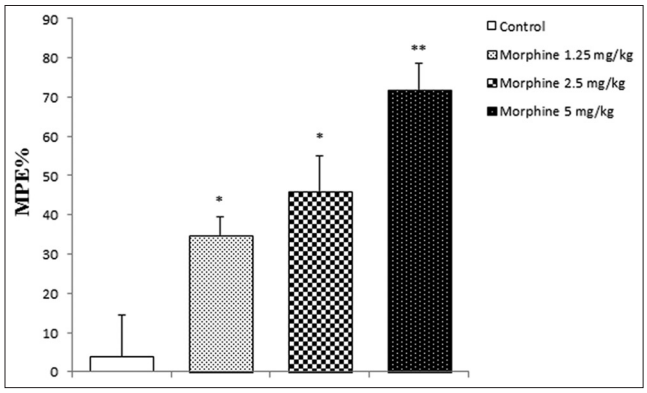

Fig. 2: Analgesia produced by intraperitoneal injection of different doses of morphine on corneal pain response induced by Nacl 5M solution applied to corneal surface in rat. Maximal possible effect (%MPE) is considered as an index for comparison between results of two tests (for calculating [%MPE=100 × post drug wipe count − pre drug wipe count/(0 − pre drug wipe count]). The values are expressed as mean±standard error of mean (n=6/group). The data are compared with one-way analysis of variance (ANOVA) followed by Tukey honestly significant difference *post-hoc* test; *p<0.05, **p<0.01

nociceptive effect of EOVAC 200 mg/kg in eye wipe response (−18.63%, p<0.01) (Fig. 4). Administration of L-arginine (100 and 300 mg/kg i.p.) alone had no any effect on eye wipe responses (Fig. 5). Pre-treatment of animals with L-arginine did not change anti-nociceptive effect of EOVAC 200 µg (38.65%, p<0.05) (Fig. 5). Methylene blue (5 and 10 mg/kg i.p.) alone had no any significant effect on the NaCl induced eye wiping behavior and also pre-treatment of animal with methylene blue (5 mg/kg i.p.) could not produce any significant effect on EOVAC (200 mg/kg s.c.) induced anti-nociception. (33.62%, p<0.05) (Fig. 6).

DISCUSSION

Topical administration of one drop NaCl 5 M solution to the corneal surface produced acute chemical pain responses in this study. It has been shown that application of NaCl, capsaicine and nicotin on the corneal surface produce vigorous response in the nociceptive neurons in trigeminal subnucleus caudalis in rat [25]. Two different classes of small-caliber primary afferents that synapse in the trigeminal

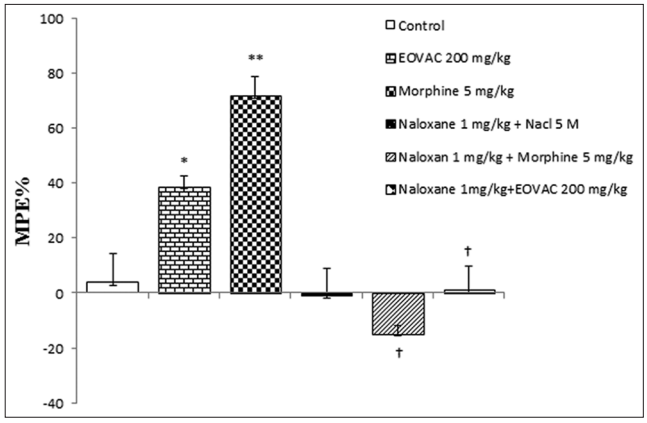

Fig. 3: Effect of pretreatment with naloxone on anti-nociceptive activity of essential oil extracted from *Vitex agnus-castus* (EOVAC) and morphine in corneal pain response induced by NaCl 5M solution applied to corneal surface in rat. Maximal possible effect (%MPE) is considered as an index for comparison between results of two tests (for calculating %MPE=100 × post drug wipe count – pre drug wipe count/[0 – pre drug wipe count]). The values are expressed as mean±standard error of mean (n=6/group). The data are compared with one-way analysis of variance (ANOVA) followed by Tukey honestly significant difference *post-hoc* test; *p<0.05, **p<0.01 versus control. †p<0.01 versus EOVAC 200 mg/kg and morphine 5 mg/kg

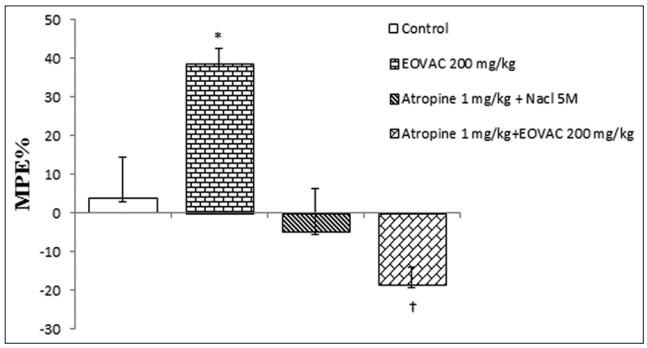

Fig. 4: Effect of pretreatment with atropine on anti-nociceptive activity of essential oil extracted from *Vitex agnus-castus* (EOVAC) in the corneal pain response induced by NaCl 5M solution applied to corneal surface in rat. Maximal possible effect (%MPE) is considered as an index for comparison between results of two tests (for calculating %MPE=100 × post drug wipe count – pre drug wipe count/[0 – pre drug wipe count]). The values are expressed as mean±standard error of mean (n=6/group). The data are compared with one-way analysis of variance (ANOVA) followed by Tukey honestly significant difference *post-hoc* test; *p<0.05 versus control, †p<0.01 versus EOVAC 200 mg/kg

brainstem nuclear complex (thin myelinated, A-delta or unmyelinated, C-type fibers) respond to chemical, mechanical and thermal noxious stimuli on the corneal surface (Belmonte *et al*., 2004). It has been reported that hyperosmotic solution like NaCl can activate TRPV1 and TRPM8 receptors on corneal nociceptors [26,27].

In the present study, systemic administration of morphine suppresses eye wipe response in the acute corneal pain and also pretreatment with naloxone prevent this suppressive effect of morphine in acute chemical corneal pain. These results showed that the analgesic effect of morphine is mediated by naloxone-sensitive mechanism in this model of pain.

Our results in the present study indicate anti-nociceptive effect of EOVAC (100, 150 and 200 mg/kg) on hypertonic saline induced corneal pain

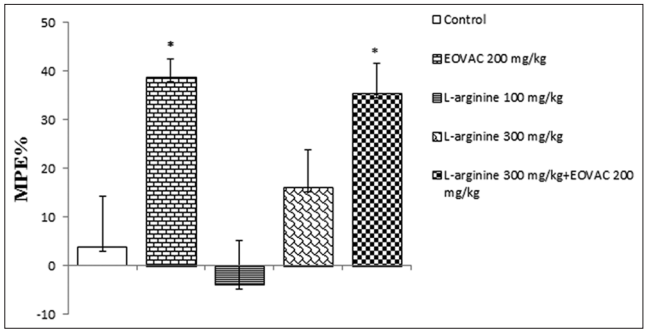

Fig. 5: Effect of pretreatment with L-arginine (300 mg/kg) on antinociceptive activity of of essential oil extracted from *Vitex agnus-castus* (EOVAC) (200 mg/kg) against NaCl 5M induced corneal pain response in rat. Maximal possible effect (%MPE) is considered as an index for comparison between results of two tests (for calculating %MPE=100 × post drug wipe count – pre drug wipe count/[0 – pre drug wipe count]). The values are expressed as mean±standard error of mean (n=6/group). The data are compared with one-way analysis of variance (ANOVA) followed by Tukey honestly significant difference *post-hoc* test; *p<0.05

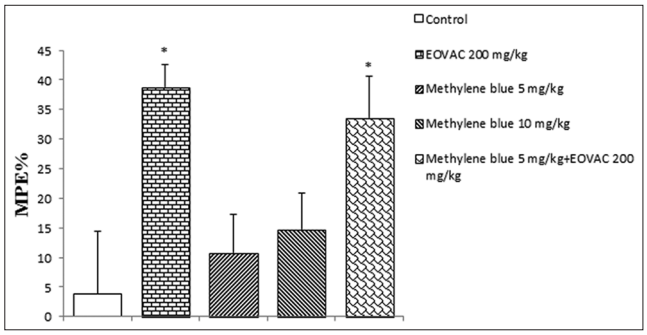

Fig. 6: Effect of pretreatment with methylene blue (5 mg/kg) on anti-nociceptive activity of essential oil extracted from *Vitex agnus-castus* (EOVAC) (200 mg/kg) against NaCl 5M induced corneal pain response in rat. Maximal possible effect (%MPE) is considered as an index for comparison between results of two tests (for calculating %MPE=100 × post drug wipe count – pre drug wipe count/[0 – pre drug wipe count]). The values are expressed as mean±standard error of mean (n=6/group). The data are compared with one-way analysis of variance (ANOVA) followed by Tukey honestly significant difference *post-hoc* test; *p<0.05

in rats and also these data provide some evidence for determination of possible mechanisms of anti-nociceptive action of EOVAC in this model of nociception. The anti-nociceptive effect induced by the EOVAC (200 mg/kg, s.c.) was significantly inhibited by naloxone and atropine. On the other hand, the administration of L-arginine and methylene blue did not alter EOVAC induced analgesia. These findings suggested that the analgesic effect of EOVAC may be mediated through opioidergic and cholinergic system but not via L-arginine/NO/cyclic GMP pathway in this model of nociception.

Cholinergic system has an important role in the pain modulation [28]. EOVAC contains some of the terpenes like (-)-Linalool and α-phellandrene that produced analgesia via activation of the cholinergic system [29,30].

Webster *et al*., (2011) reported that different fractions of VAC extract act as an agonist of μ and δ but not κ opioid receptors [31]. In addition, 4 days feeding of rats with VAC caused a significant increase in brain and blood levels of β-endorphin (endogenous opioid agonist) [32].

Opioidergic activity exhibited by VAC may be one of the important mechanisms of action of VAC in reduction of pain and treatment of PMS syndrome [31].

Therapeutic effects of VAC in PMS syndrome were already well documented [19]. In an open-labeled clinical observation on migrainous women with PMS, the VAC (40 mg/day) administrated for 3-month period and the results indicated that VAC could improve preventative management of both menstrual related and non-menstrual-related migraine headaches and it was safe and well tolerated by patients [33].

Our results in the acute trigeminal model of pain showed that EOVAC had a central analgesic activity. This effect of EOVAC may be due to its lipophilic nature. Because of this lipophilic character, the EOVAC allowed to absorb rapidly from the injection site and also rapidly penetrated the blood-brain barrier and reached the central nervous system [34].

EOVAC contains some important monoterpenes and sesquiterpenes like α-pinene, α-bisabolol, 1,8-cineol, β-caryophyllene and limonene [7]. *R*-(+)-limonene and α-pinene have analgesic effects in different models of pain [35]. In addition, sabinene (1%) has been reported act as an anti-inflammatory agent in the crystalline induced ocular inflammation in rabbit eyes [36]. β-caryophyllene is another component exist in the EOVAC, this sesquiterpene has a functional non-psychoactive CB_2 cannabinoid receptor agonistic activity [37]. Cannabinoid receptor CB_2 and its selective agonist accepted as a new pharmacological target for the treatment of pain [38].

CONCLUSION

In conclusion, our results indicate that the EOVAC produced analgesic activity in acute trigeminal model of nociception in rat. Also, our finding in the study of evolved mechanisms, suggested that this analgesic effect of EOVAC may be mediated through opioidergic and cholinergic system but not via L-arginine/NO/cyclic GMP pathway in this model of pain.

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