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Anti-inflammatory and Anti-nociceptive Effects of *Teucrium orientale* Ethanolic Extract in Experimental Animals

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ABSTRACT

Background: *Teucrium orientale* is commonly used as an analgesic, antibacterial, antifungal, and antioxidant, protecting the liver and digestive system, and treating type 2 diabetes.
Purpose: Evaluation of anti-nociceptive and anti-inflammatory properties of ethanolic plant extract using animal models.
Methods: The ethanolic extract was prepared using the maceration method from ground-dried aerial parts of the plant. Analgesic effects were determined by acetic acid-induced writhing and hot plate test on mice. The anti-inflammatory effect was evaluated using Carageenan and Cotton pellet tests on rats.
Result: In the writhing test, TOE (100, 200 and 400 mg/kg) caused a significant reduction in abdominal contractions. Inhibition percentages of abdominal contractions in test groups (100, 200 and 400 mg/kg), morphine and mefenamic acid were 87.40%, 91.80%, 98.29%, 94.40% and 96.89%, respectively, which indicate the visceral analgesic effects.
In hot plate tests, the pain threshold significantly increased in test groups . Extract in the doses of 100mg/kg ($P<0.001$), 200mg/kg ($P<0.0001$) and 400mg/kg ($P<0.001$) significantly reduced the paw edema in the carrageenan test at the second hour. In the cotton pellet test, the prescribed doses of the plant (100-200 mg/kg; $P<0.0001$) significantly reduced the formation of granuloma tissue and reduced the rate of edema. The percentage of inhibition of granuloma tissue by indomethacin and extract at doses of 100 and 200 mg/kg were 39.19%, 66.75% and 75.49%, respectively, and the percentage of inhibition of exudates were 26.5%, 55.57% and 67.24%, respectively.
Conclusion: These results clearly showed the anti-nociceptive and anti-inflammatory effect of *Teucrium orientale* extract in animal models.
Keyword: Analgesic, Anti-inflammation, *Teucrium orientale*, Carrageenan, Cotton pellet, Mice, Rat.

INTRODUCTION

The genus Teucrium is one of the largest genera of the Lamiaceae family which belongs to the subfamily Lamioideae and comprises nearly 340 herbaceous perennial species displaying remarkable variation (Wink and Kaufmann, 1996). They are commonly found in the Middle East and Mediterranean climates. Iran, particularly, is one of the original centers of the genus Teucrium with 12 species, is described by the Persian name of 'Maryam-Nokhodi' of which 25% are endemics (Mozaffarian 2006). For more than 2000 years Teucrium species have been used as antiseptic, diaphoretic, diuretic and antipyretic agents. Some Teucrium species are used as hypoglycemic agents in Saudi Arabia and North Africa and possess antifeedant activities. Some Teucrium extracts exhibited central nervous system depressant action (Ulubelen, Topu et al. 2000). Antinociceptive activity of the aqueous extract of *Teucrium persicum* have been well documented (Miri, Sharifi-Rad et al. 2015).
The diversity, chemical properties, variation, and species richness have led to much research on the genus Teucrium. Phytochemical investigations have reported flavonoids, aromatic compounds, rearranged neo-clerodane or abietanediterpenoids, terpenoids such as sesquiterpenoids and triterpenoids, steroids and phenolic acids and their derivatives (Ghahraman 1996) as the main compounds of these species. Teucrium species are considered a rich source of neo-clerodane diterpenoids and are regarded as chemotaxonomic markers for neo-

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clerodanes (Dehghan, Tahmasebpour *et al*. 2013). Most of Teucrium species are rich in various biologically active compounds, especially essential oils.

T. orientale is one of the native perennial species distributed in some parts of Iran, especially in the southern regions. The leaves are green and ovate. The blue flowers have a white or yellow corolla, in a globular inflorescence and appear from April to June. The fruits are light brown to dark brown nutlets with a latticed surface (Çakir, Mavi et al. 2006). This plant is extensively exploited as a medicinal plant. Significant hypoglycemic, hepatoprotective, nephroprotective(Amiri 2010) and antioxidant potentials (Küçük, Güleç et al. 2006) of *T. orientale* have also been identified. Antioxidant (Kucukbay, Yildiz et al. 2011) and antimicrobial activities (Küçük, Güleç et al. 2006, Kucukbay, Yildiz *et al*. 2011) have been reported from *T. orientale* essential oil.

T. orientale var. glabrescens is one the variety of this species and is widely used in the south Iran as a natural pain killer and anti-inflammatory agent for inflammation-based disorders via different preparations such as infusion, tincture and decoction. Since the literature survey revealed that there was no report on *T. orientale var. glabrescens* analgesic and anti-inflammatory effects, we tried to assess the anti-nociceptive and anti-inflammatory impacts of *T. orientale var. glabrescens* for the first time and assess the pharmacological basis for its folkloric utilization as a natural analgesic and anti-inflammatory agent. In this work, the analgesic and anti-inflammatory features of the studied plant were explored utilizing different standard experimental test models.

MATERIAL AND METHODS

Plant Materials

The aerial parts of *T. orientale* subsp. glabrescens were collected in May 2014 from Qotb-Abad, Hormozgan Province, Iran (27°46′12″N 56°04′27″E, 1100 m). The specimen was identified by R. Asadpour, and a voucher specimen was deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences, Islamic Azad University, Tehran, Iran, under code number 1129-AUPF.

Preparation of the Ethanolic Extract

The ethanolic extract was prepared using the maceration method from ground-dried aerial parts of the plant.

Chemicals and Drugs

Morphine sulfate, Mefenamic acid, Indomethacin, Carrageenan and acetic acid were purchased from Temad Pharmaceutical Co. (Iran) and Merck (Germany).

Experimental Animals

The present experimental study was conducted on 54 male Wistar rats weighing 200-250 g and 66 male mice weighing 20-25 g. All animals were kept under controlled conditions (22-24°C and 12 h light/dark cycles) and were provided with standard rat food and water *ad libitum*. Experiments were carried out in accordance with local guidelines for the care of laboratory animals of the Faculty of Pharmacy and Pharmaceutical Sciences, Tehran Medical Sciences, Islamic Azad University (IAUPS), Iran (ethical Approval Number: IR.IAU.PS.REC.1397.264).

Anti-nociception Activity Against Acetic Acid-induced Pain Behavior in Mice

The writhing acetic acid test was made originally as described by Siegmund *et al*.(Siegmund, Cadmus et al. 1957). This method was employed to preferentially evaluate the possible peripheral effects of *T. Orintum* extract as analgesic agent. TOE (100, 200 and 400 mg/kg), mefenamic acid (30 mg/kg), morphine (5 mg/kg) and the vehicle (saline,10 ml/kg) were intra-peritoneal injected into 5 separate groups of mice, respectively, 30 min prior to injection of acetic acid (1 %, 10 ml/kg). Mefenamic acid is a well-known peripheral analgesic drug thatwas used as a positive control in the current investigation. The mice were then located in an observation box and the number of writhes (abdominal constrictions) was counted for 30 min after acetic acid injection by direct observation. The mean number of writhes for each experimental group and the percentage decrease, compared to that of the control group, were calculated over30 min.

Anti-nociception Activity Hot-Plate Test in Mice

The hot-plate method was employed for the purpose of standard assessment of possible centrally-mediated analgesic effects of the TOE. The temperature of a metal surface was maintained at 55 ± 0.1°C and the animals' latency to licking paws or jumping was determined before and after drug administration. TOE (100, 200 and 400 mg/kg; i.p.), morphine (5 mg/kg; i.p.) and the vehicle (saline, 10 ml/kg; i.p.) were injected to animal groups separately. The latency was recorded before and after (30, 60 and 120 min) the administration of the agents. A 15-s cut-off time was used to prevent tissue damage.

Anti-inflammatory Activity Against Carrageenan-induced rat Paws Edema

Carrageenan-induced paw edema in male Wistar rats was used to evaluate the anti-inflammatory effect of *T. Orintum* extract(Morris 2003, Rangriz, Mousavi et al. 2016). To investigate the acute anti-inflammatory effect of the extract, the rats were divided into three groups of six: 1) the standard group, which received mefenamic acid (30 mg/kg; i.p.); 2) the control (saline, 10 ml/kg; i.p.) group; and 3) three test groups, which received TOE (100, 200, and 400 mg/kg; i.p.) 30 min before the injection of carrageenan (0.1 ml, 2%) into the sub-plantar region of the right hind paw. The paw volume was measured using a Plethysmometer (model PM 4500, BorjSanat Co., Iran) before and 0.5, 1, 2, 3, and 4 h after the carrageenan administration. Anti-inflammatory activity was identified as the inhibition percentage of edema, compared to the control group. The inhibition of edema was calculated by the following equation:

% inhibition of edema = 100 (1-Vt/Vc)

Where Vc is the edema volume in the control group and Vt is the edema volume in the test groups.

Anti-inflammatory Activity Against Cotton Pellet-induced Granuloma

The chronic anti-inflammatory activity of *T. Orintum* extract was assessed on the basis of cotton pellet-induced granuloma according to the method of Winter and Porter (Winter and Porter 1957). Four groups of six rats were used. Pellets weighing just about 60 mg each were made with 5 mm of dental cotton tampons. The pellets were sterilized in an autoclave for 30 min at 120 °C under 15 lb pressure. Rats were anesthetized and pellets were subcutaneously implanted in the axilla region of each rat through a single needle incision. Each group was treated daily, for 7 consecutive days with *T. Orintum* extract(100 and 200 mg/kg, i.p.), indomethacin (5 mg/kg, i.p.), and vehicle (saline, 10 ml/kg). On the eighth day, rats were anesthetized over again; the cotton pellets together with the granuloma tissues were separated surgically and made free from extraneous tissues. The wet pellets were weighed for the purpose of the wet weight, and then dried in an incubator at 60 °C for 24 hr until a constant weight was obtained; after that, the dried pellets were weighed for a second time. The exudates' quantity (mg) was calculated by subtracting the constant dry weight of the pellet from the immediate wet weight of the pellet. The dry weight of the granuloma was calculated after deducting the weight of the cotton pellet from the constant dry weight of the pellet and taken as an amount of granuloma tissue formation. The percent inhibitions of exudates and granuloma tissue formation were considered.

Statistical Analysis

The data at each time point were expressed as the mean ± standard error of the mean (SEM) and analyzed using Graph Pad Prism software, Version 6.0 (Graph Pad Software, Inc., La Jolla, USA). Comparisons between groups were made by one-way ANOVA and repeated measures analysis of variance (ANOVA) followed by the *post hoc* Tukey's test. Also, $p<0.05$ was considered a significant difference inmeans.

RESULTS AND DISCUSSION

Writhing Test in Mice

Figure 1 shows the anti-nociception effect of TOE in male mice using the acetic acid test. Compared to the control group, TOE (100, 200 and 400mg/kg), mefenamic acid (30 mg/kg) and morphine (5mg/kg) significantly ($P<0.05$) decreased the number of acetic acid-induced writhes or stretches by 87.40, 91.80, 98.29, 96.89 and 94.40%, respectively (Table 2).

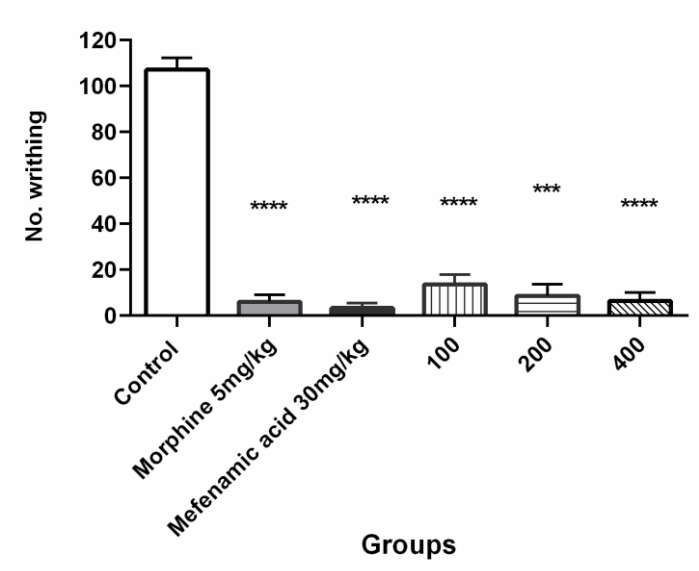

Fig. 1 Antinociception activity of *T. Orintum*extract in male mice using the acetic acid test. Each value represents the mean ± SEM of 6 mice. ***P<0.001, ****P<0.0001, Significant difference compared to the control group.

Table 1 Writhes inhibition (%) effect of i.p. *T. Orintum* Extract (TOE) injection on the acetic acid-induced writhes in mice.

Groups	Inhibition%
Morphine(5mg/kg)	94.40
Mefenamic acid(30mg/kg)	96.89
TOE(100mg/kg)	87.40
TOE(200mg/kg)	91.80
TOE(400mg/kg)	98.29

Hot-Plate Test

TOE with a lower dose of 100 and 200 mg/kg practically did not increase the reaction time against the thermal source in tested animals after 30 and 60 min treatment (Figure2). However, the higher dose of TOE (400 mg/kg) used in the present study, showed a significant (p<0.01) anti-nociceptive activity, compared to the control group. However, all doses studied of TOE after 2 hours were able to increase the reaction time in the hotplate test, which was comparable with the standard drug (morphine). Morphine induced a significant anti-nociceptive activity in the hot-plate test at all the times.

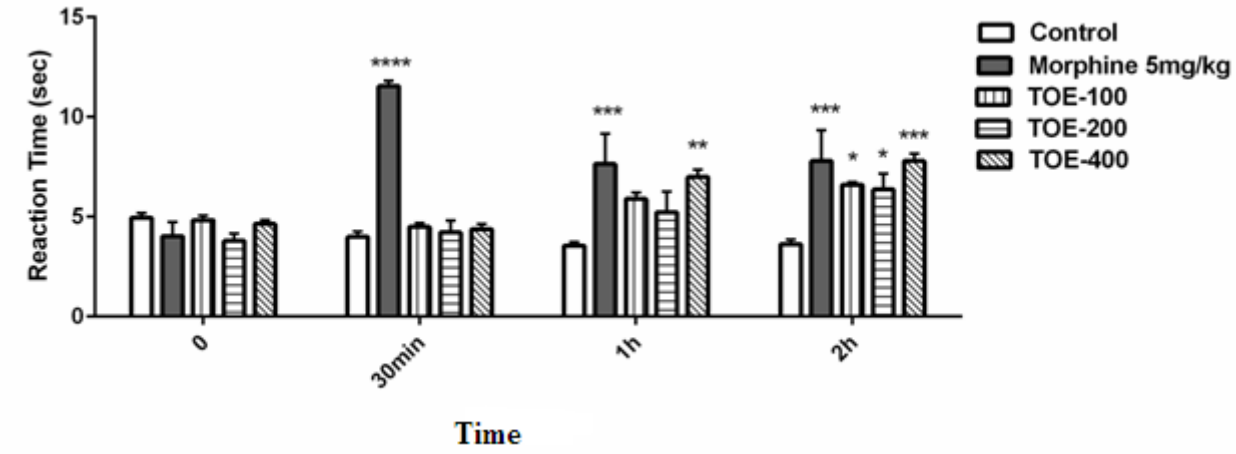

Fig. 2 Antinociception activity of *T. Orintum*extract (TOE) in male mice using the hot plate test. Each value represents the mean ± SEM of 6 mice. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 compared to the control.

Anti-Inflammatory Activity of *T. Orintum* extract

Carrageenan-Induced Inflammation Test

*T. Orintum*extract (i.p.) showed anti-inflammatory activity in the Carrageenan-induced paw edema of the rats in the second hour of testing. The anti-inflammatory activity of the extract (200 and 400 mg/kg) was comparable with that of Mefenamic acid (30mg/kg) (Fig. 3). The reduction of edema in other hours was not statistically significant. However, a dose of 100mg/kg showed an intensified inflammatory effect most of the time.

Figure 3 shows the effect of TOE in rat paw edema induced by Carrageenan. TOE (100, 200 and 400 mg/kg), as well as, mefenamic acid (30 mg/kg) significantly ($p<0.05$) inhibited the formation of Carrageenan-induced rat paw edema which was observed at the second hour of the experiment (peak of edema formation) by 79.70, 90.40, 82.06 and 65.60%, respectively (Table 2). Moreover, the inhibition percentage in the TOE-treated group was comparable to that of standard group. TOE with a dose of 100 mg/kg reduced edema only in the second hour and then induced inflammation in other hours. The percentages of rat paw edema in the carrageenan test were also compared at different times and doses (Table 2).

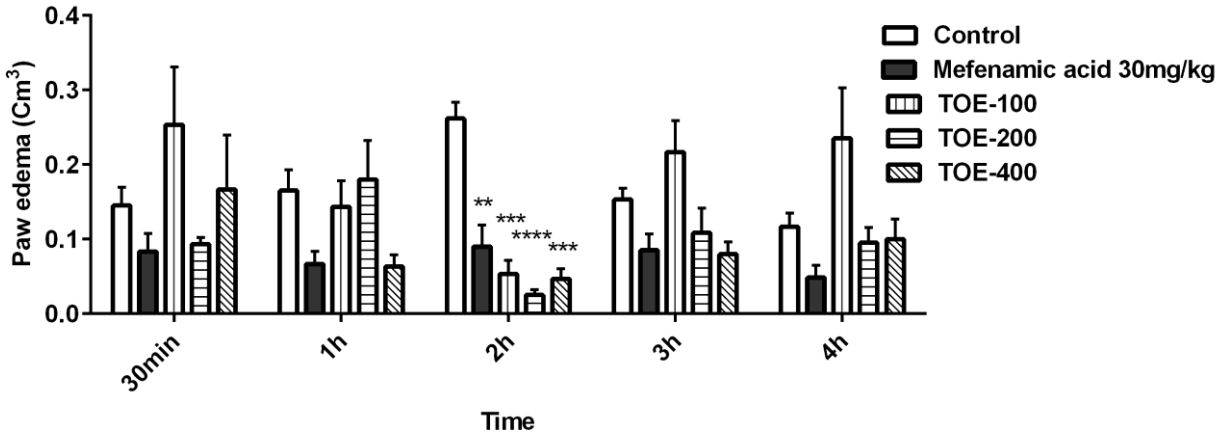

Fig. 3 Anti-inflammatory activity of *T. Orintum*extract (TOE) using the carrageenan test in male rats.

The control (Vehicle; 10 mL/kg), mefenamic acid (30 mg/kg; i.p.), and TOE (100, 200, and 400 mg/kg, i.p.) were administered 30 min before the carrageenan test. After that, the paw edema was measured at 0.5, 1, 2, 3, and 4 h after the administration of carrageenan. The data represent the mean ± SEM of 6-8 animals in each group (**$p<0.01$, ***$p<0.001$, ****$p<0.0001$) compared to the control group.

Table 2 Paw edema inhibition (%) effect of i.p. *T. Orintum* extract (TOE) injection on the inflammation induced by carrageenan in rats.

Paw edema(cm^3) at various time intervals (%inhibition)					
Time Group	30 min	1 hr	2hr	3hr	4hr
Mefenamic acid (30 mg/kg)	(42.75 %)	59.39%	65.60%	44.40%	58.90%
TOE (100mg/kg)	174% Edema induction	13.30%	79.70%	141.80% Edema induction	200% Edema induction
TOE (200mg/kg)	35.86%	109.90%	90.40%	29.40%	18.80%
TOE (400mg/kg)	115.17%	61.80%	82.06%	47.70%	14.50%

Cotton Pellet-induced Inflammation Test

In the cotton pellet-induced inflammation experiment, a significant reduction of granuloma and transude was observed just for the groups treated with T. Orintum extract (100 and 200 mg/kg, i.p.) daily for 7 days (Fig 3).

The reduction of granuloma with *T. Orintum*extracts (100 and 200 mg/kg) was 67% and 75% in comparison with the standard drug Indomethacin (40%) (Table 3).
Also, the reduction of transudingwith *T. Orintum*extract (100 and 200 mg/kg) was 55% and 67% in comparison with the standard drug Indomethacin (26%) (Table 3).

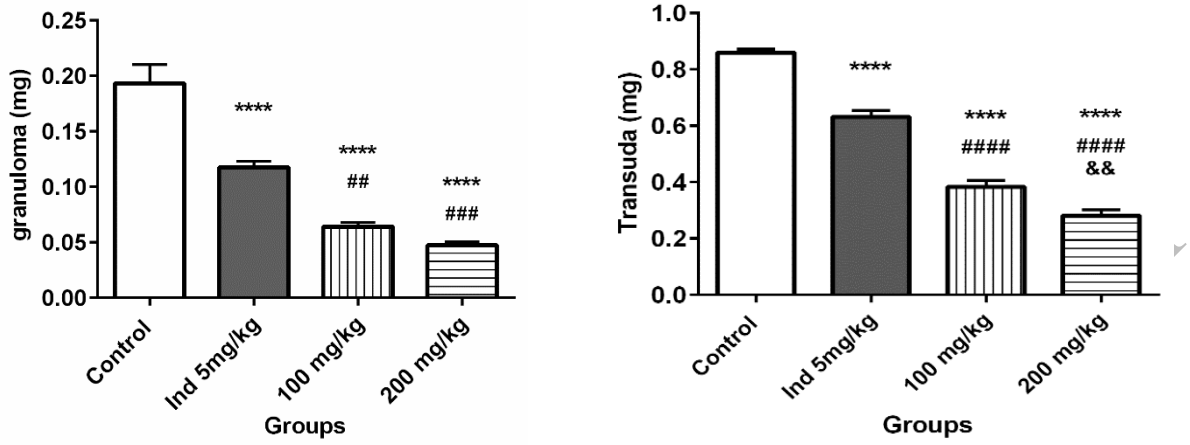

Fig. 3 Effect of *T. Orintum*extract on the cotton pellet-induced granuloma and transude in rats. Each value represents the mean ± SEM of 6 mice. *p<0.05, **p<0.01 and**** p<0.0001 compared to the control group, ## p<0.0, ###p<0.01 and#### p<0.0001 compared to Indomethacin.

Table 3 Effect of *T. Orintum* extract (TOE) on the cotton pellet-induced granuloma pouch in rats

Groups	Transudate Mean ± SEM (Inhibition %)	Granuloma Mean ± SEM (Inhibition %)
Control	0.861 ± 0.012 -	0.193 ± 0.017 -
Indo (5mg/kg)	0.632 ± 0.022 (26.5 %)	0.118 ± 0.005 (39.19 %)
TOE (100mg/kg)	0.383 ± 0.023 (55.51 %)	0.064 ± 0.004 (66.75 %)
TOE (200mg/kg)	0.282 ± 0.020 (67.24 %)	0.047 ± 0.003 (75.49 %)

DISCUSSION

Pain management is undoubtedly one of the most common and yet most difficult aspects in medicine. In spite of major scientific and technological progress in the field of synthetic drugs during recent years, drugs derived from natural products still make an enormous contribution to drug discovery today (Lahlou 2013). Therefore, a systematic method should be used to determine the efficacy of plants against pain and inflammation so as to use them as herbal anti-nociceptive and anti-inflammatory drugs.
In this study, we assessed the anti-nociceptive and anti-inflammatory activity of the extract from the aerial parts of *T. orientale subsp. glabrescens* using four pharmacological models in animals.
In the writhing test, all doses of *T. Orintum*extract caused a significant reduction in abdominal contractions, which indicates the visceral analgesic effects. Thus, the extract in our study had strong anti-nociceptive activity which was comparable with the standard drugs (morphine and mefenamic acid). In hot plate tests, the pain threshold significantly increased in test groups (100 and 200mg/kg) after 60 minutes. Indeed TOE showed an anti-nociceptive effect in a concentration and time-dependent manner.
The chemical method such as the acetic acid-induced writhing test is most commonly used for evaluating the anti-nociceptive activity of medicinal plants. Prostaglandins, initially PGE2 and then PGF2α and free arachidonic

acid are released from tissue phospholipids and consequently their levels in the peritoneal fluids increase due to intra-peritoneal administration of the irritant, acetic acid. This results in localized inflammatory response and pain feeling due to an increase in capillary permeability. In this test, the signal is transmitted to the CNS in response to pain due to irritation and the release of endogenous mediators like bradykinin and prostaglandin which contributes toincreased sensitivity to receptors. The Writhing test is sensitive to opiates as well as non-opiate analgesics. Substances that counteract this occurrence exert an anti-nociceptive effect and decrease pain sensation (Deraedt, Jouquey et al. 1980).

In this regard, some studies is available which conclude anti-nociceptive activity of essentials oils and extracts of various species of the genus Teucrium(Abdollahi, Karimpour et al. 2003, Zendehdel, Taati et al. 2011, Shah, Ullah et al. 2012, Shah, Sadiq et al. 2014). Shah et al. conducted a study in Pakistan and concluded that essential oil of *Teucrium stocksianum* had anti-nociceptive activity (Shah, Ullah et al. 2012).Abdollahi et al. in 2003 conducted a study in Iran and concluded that extract and essential oil showed the anti-nociception effect that essential oil was the main contributor in anti-nociceptive activity(Abdollahi, Karimpour et al. 2003).

In this regard, Miri et al., showed the aqueous extract of Teucrium persicum in mice with sciatic nerve ligated has induced analgesic effects (Miri, Sharifi-Rad et al. 2015).

The results of the anti-nociceptive effect of *T. Orintum* extract in our study are similar to that of essential oil extracted from *T. polium* with respect to anti-nociceptive potential. Our results revealed that administration of *T. Orintum* extract showed an anti-inflammatory effect in the carrageenan test in the second hour after treatment, although this effect was transient and did not last long enough. This transient effect can be due to an insufficient dose of the extract or the rapid inactivation of plant components in the body.

The carrageenan-induced paw edema has been usually proposed as an acute inflammation model in experimental animals that involves inflammatory mediators such as histamine, serotonin, bradykinin and prostaglandins (Winter, Risley et al. 1962, Morris 2003). The pharmacological effects of NSAIDs are related to the inhibition of the conversion of arachidonic acid and prostaglandins, which are mediators of the inflammatory process(Brunton, Chabner et al.).

In the cotton pellet test, the prescribed doses of the plant (100-200 mg/kg) significantly reduced the formation of granuloma tissue and reduced the rate of edema. The cotton-pellet granuloma is a widely used manner for the calculation of chronic anti-inflammatory substances (Spector 1969, Jahandar, Asgarpanah et al. 2018, Chitsaz, Zarezadeh et al. 2021). Thus found to be effective in chronic inflammatory conditions, which shows its efficacy in inhibiting the increase in the number of fibroblasts and synthesis of collagen and mucopolysaccharides during granuloma tissue formation (Swingle and Shideman 1972). The dry weight of the pellet correlates with the amount of granulomatous tissues, the moist weight of the pellets correlates with transudes. Chronic inflammation happens by means of the development of proliferating cells. These cells can be either spread or in granuloma form.

The results of the study of the anti-inflammatory effect of *T. Orintum* extract show that the anti-inflammatory effect of extract was more predominant in cotton pellet test than carrageenan-induced inflammation model.

The results of our study agree with the studies of many researchers who have reported anti-inflammatory effects from different species of Tucrium. However, in different studies, essential oil and or extract were used to evaluate anti-inflammatory activity; the pharmacological effects were observed in both cases. In a previous study, the ethanolic extract of *T. polium* at a dose of 500 mg/ml also produced significant inhibition of carrageenin-induced inflammation and cotton-pellet granuloma and the essential oil of the same species was found to be responsible for analgesic properties of the plant when studied by a writhing test, a visceral pain model in mice (Abdollahi et al., 2003);(Tariq, Ageel et al. 1989). Rahmouni et al reported anti-inflammatory activity of aqueous extract of *T. polium* (5 g/L) in carrageenan-induced inflammation in rats(Rahmouni, Hamdaoui et al. 2017). In another study, Menichini et al. (2009) who revealed the in vitro anti- inflammatory effect of *T.flavum*, *T.montbretii* ssp.heliotropiifolium, *T. polium* ssp. Capitatum, *T.brevifolium* essential oil by analyzing their inhibitory effects on chemical mediators released from macrophages(Menichini, Conforti et al. 2009).

A survey of literature reveals that other Teucrium spp. were previously demonstrated to have potent anti-inflammatory activity against both the acute and delayed phases of inflammation (Puntero, Peinado et al. 1997). The highest anti-inflammatory activities of *T.polium*ssp.capitatum and *T. montbretii* ssp. *Heliotropiifolium* were

documented to their major components, caryophyllene and carvacrol, which showed anti-inflammatory activity in previous research ((Passos, Fernandes et al. 2007, De Martino, Coppola et al. 2020)

Investigations about other species of Teucrium have also indicated anti-inflammatory, antiulcer inhibitory action on lipid peroxidation and anti-hyperlipidemic properties. The presence of terpenic compounds and flavonoids was proposed as a possible mechanism (Trivellini, Lucchesini et al. 2016, Uritu, Mihai et al. 2018, Zlatić and Stanković 2020).

Scientific studies are conducted globally to evaluate anti-nociceptive and anti-inflammatory efficacy of plants. The data from such studies shows that extracts and essential oils of various plants containing chemical constituents exert good anti-nociceptive and anti-inflammatory effects through various mechanisms. Although medicinal plants are often tested for their therapeutic effect as a whole plant in experiments, studies are available in which single chemical constituents have been tested. Him et al. and Ozbek et al. evaluated a positive analgesic effect of alpha pinene in their study (Him, Ozbek et al. 2008). Similarly analgesic activity of caryophyllene oxide (Chavan, Wakte et al. 2010, Fidyt, Fiedorowicz et al. 2016), myrcene(Rao, Menezes et al. 1990), limonene (do Amaral, Silva et al. 2007, de Almeida, Silva et al. 2017), and linalool (Peana, Marzocco et al. 2006) have been observed. Also it was reported anti-inflammatory effects of α-iso-cubebene(Choi, Kim et al. 2009), caryophyllene oxide(Chavan, Wakte et al. 2010), α-pinene(Kim, Lee et al. 2015), myrcene(Rufino, Ribeiro et al. 2015).

The phytochemical results indicated that essential oil major constituents of *T. Orientale subsp. glabrescens* to be β-cubebene (34.5%), α-cubebene (16.6%), α-copaene (10.1%), and trans β-caryophyllene (10.0%). The oil of the aerial parts of *T. orientale subsp. glabrescens* comprised one monoterpenoid (0.5%), 13 sesquiterpenoids (89.9%), three hydrocarbons (5.6%), and one oxygenated hydrocarbon (1.0%) (Aberumand and Asgarpanah 2017). In this way, the observed effects can be well related to the compounds in the plant, which requires further research in this area.

In conclusion, several anti-nociception and anti-inflammatory assays were utilized to evaluate the pharmacological properties of *Teucrium orientale var. glabrescens* extracts. The ethanolic extract exhibited powerful anti-visceral pain activity and chronic anti-inflammatory properties. In this regard, studying the active ingredients of this extract also is of great importance for understanding the mechanism of its pharmacological effects.

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Declaration of Interest

The authors report no declarations of interest.

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