Evaluation of pharmacological mechanisms of antinociceptive effect of *Teucrium polium* on visceral pain in mice

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Summary

Teucrium polium is used for treatment of visceral pain in Iranian folkloric medicine. In this study antinociceptive mechanisms of *T. polium* hydroethanolic extract were examined by acetic acid-induced writhing test as a model of visceral pain in male NMRI mice. To reveal the antinociceptive mechanisms of the extract, we examined the effects of opioidergic, serotonergic, adrenergic and histaminergic antagonists on extract-induced antinociception. The results of this study showed that pretreatment with naloxone, chlorpheniramine and cimetidine significantly attenuate the antinociceptive effect of the extract. However, cyproheptadine and phentolamine had no effect. Our results clearly show antinociceptive effects of *T. polium* may be mediated by opioidergic and histaminegic H_1 and H_2 receptors.

Key words: *Teucrium polium*, Opioid receptor, Histamine receptors, Writhing test, Mouse

Introduction

Pain is a sensorial modality and primarily protective in nature, but often causes discomfort. It is the most important symptom that brings a patient to a physician. Analgesics relieve pain as a symptom without affecting its cause (Hasan *et al*., 2009). Currently available analgesic drugs such as opiates and nonsteroidal anti-inflammatory drugs (NSAIDs) are not useful in all cases due to their side effects. The search for new analgesic compounds has been a priority of pharmacologists and pharmaceutical industries (Mattison *et al*., 1998). Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects (Blumenthal, 2000). Thus, the study of plant species that traditionally have been used as pain killers should still be seen as a logical research strategy in the search for new analgesic drugs (Rang *et al*., 1998).

The *T. polium* is a wild-growing flowering plant belonging to the family labiatae and is found abundantly in south western Asia, Europe and North Africa (Abdollahi *et al*., 2003). This plant is used as a visceral pain killer in Iranian folkloric medicine. Previous studies have demonstrated some of the pharmacological effects of *T. polium* such as its anti-inflammatory (Capasso *et al*., 1983), anti-nociceptive (Abdollahi *et al*., 2003), anti-diabetes (Gharaibeh *et al*., 1988) and anti-hypertensive effects (Suleiman *et al*., 1988). In this study, to reveal the antinociceptive mechanisms of *T. polium* we examined the effects of opioidergic, serotonergic, noradrenergic and histaminergic receptor antagonists on *T. polium*-induced antinociception.

Materials and Methods

Preparation of crude extract

Teucrium polium aerial parts were

collected during the flowering season from Zabol (Sistan and Baloochestan province, Southeastern Iran). Samples of the plant were identified by a botanist from the division of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Iran. The plant materials were cleaned, shade dried and coarsely ground. The powdered material was soaked in hydroethanolic (70%) solvent for three days with occasional shaking. First, it was filtered through a muslin cloth and then through a filter paper. This process was repeated twice more and the combined filtrated material was evaporated on a rotary evaporator under reduced pressure to a thick semi-solid mass of dark brown color (Subhan *et al*., 2007). The percentage yields based on the dried starting material was 15.6% for dried hydroalcoholic extract (W/W).

Animals

Male albino NMRI mice weighing 25-30 g from the Pasteur Institute of Tehran were used for all the experiments. Animals were housed in a temperature (22 ± 2°C) and light-controlled room under a 12-h light/12-h dark cycle (light on at 7:00 a.m.). Food and water were available *ad libitum*. The animals were allowed to adapt to the laboratory for at least 2 h before testing and were used only once. To reduce the experimental variation, all experiments were performed during the light phase of the cycle (10:00-17:00). All experimental procedures followed the Guidelines on Ethical Standards for Investigations of Experimental Pain in Animals (Zimmermann, 1983) and were carried out according to a protocol approved by the local Animal Ethics Committee.

Writhing test

Nociception was induced by an intraperitoneal (i.p.) injection of 0.6% acetic acid in a volume of 10 ml/kg in mice. The induced nociceptive behavior is characterized by abdominal contractions known as writhing, described as an exaggerated extension of the abdomen combined with the outstretching of the hind limbs (Koster *et al*., 1959). The total number of writhing was recorded in the following periods: 0-10, 10-20 and 20-30 min immediately after acetic acid administration.

Evaluation of antinociceptive activity of *T. polium* and pretreatment with antagonists

The *T. polium* hydroethanolic extract was dissolved in saline and administered intraperitoneally at doses of 50, 100 and 200 mg/kg. Indomethacin (5 mg/kg) was dissolved in saline and administered i.p. as the reference drug for comparison (Kozak *et al*., 1998). Control group received normal saline. Antinociceptive activity was expressed as the percentage of inhibition of abdominal constrictions using the ratio:

$$\frac{(\text{control mean} - \text{treated mean}) \times 100}{\text{control mean}} = \%\ \text{writhing pain score}$$

Animals were pretreated i.p. with either saline, opioidergic receptor antagonist (naloxone, 2 mg/kg), serotonergic receptor antagonist (cyproheptadine, 4 mg/kg), α-adrenergic receptor antagonist (phento-lamine, 20 mg/kg), histamine H_1-receptor antagonist (chlorpheniramine, 10 mg/kg) and histamine H_2-receptor antagonist (cimetidine, 10 mg/kg) 15 min before i.p. administration of vehicle or the most effective dose of *T. polium* (200 mg/kg). The writhing test response was tested 30 min after the treatment with either vehicle or extract. Additionally, onset of the first abdominal writhing was recorded as latency time. The time and dose of antagonists used were chosen on the basis of preliminary studies and previous publications (Schmitt *et al*., 1974; Leza *et al*., 1990; Zendehdel and Babapour, 2010). All drugs were dissolved in 5% dimethyl sulfoxide (DMSO). Control group received vehicle.

Acute toxicity

Mice were divided into control and test groups (n = 8). The first group served as normal control. *Teucrium polium* extract was administered i.p. to different groups at increasing doses of 400, 800, 1600, 3200 and 6400 mg/kg. After injections of extracts, mice were allowed food and water *ad libitum* and all animals were observed for possible mortality cases and behavioral

changes for 72 h (Lorke, 1983).

Statistical analysis

The data were presented as the mean ± SEM. Statistical analysis was carried out by one-way analysis of variance (ANOVA) with Tukey's post-hoc test. P-values less than 0.05 were considered to indicate statistical significance.

Results

Evaluation of antinociceptive effects of *T. polium* in writhing test

The results of this study showed that hydroethanolic extract of *T. polium* at doses of 50, 100 and 200 mg/kg induced significant reduction in pain response in a dose dependent manner when compared to control group (P<0.001). Further, indomethacin significantly decreased the number of writhing as a reference drug (P<0.001). The percentage of the inhibition of writhing response induced by extract at doses of 50, 100 and 200 mg/kg were 43.33, 64.42 and 70.5%, respectively, despite the fact that indomethacin inhibited the writhing response by 80%. Furthermore, indomethacin significantly delayed the onset of first abdominal writhing (latency time) when compared to control group (P<0.001). But other groups had no effect on the onset of first abdominal writhing in comparison with control group (Table 1). The ED_{50} for antinociceptive effects of hydroethanolic extract was 62.7 mg/kg.

Effects of cyproheptadine and phentolamine on the antinociceptive action of *T. polium*

The results showed that the *T. polium* (200 mg/kg) induced significant reduction in pain response when compared to control group (P<0.001). Pretreatment with cyproheptadine and phentolamine had no effect on the antinociceptive properties induced by the extract. Moreover, none of the drugs had any effect on the onset of first abdominal writhing in comparison with control group (Tables 2 and 3).

Effects of naloxone, chlorpheniramine and cimetidine on the antinociceptive action of *T. polium*

Intraperitoneal injection of *T. polium*

Table 1: Effect of the hydroethanolic extract of *T. polium* on acetic acid-induced writhing in mice

Treatment	Dose (mg/kg, i.p.)	Latency time (sec)	Writhing count (Mean±SEM)	Inhibition (%)	P-value
Control	10 (ml/kg, ip)	189 ± 25	74.50 ± 2.68	—	—
T. polium	50	178 ± 23	43.17 ± 5.19	43.33	<0.001 vs. contol
T. polium	100	206 ± 32	26.50 ± 1.22	64.42	<0.001 vs. contol
T. polium	200	202 ± 30	21.00 ± 1.84	70.50	<0.001 vs. contol
Indomethacin	5	783 ± 67*	14.50 ± 2.15	80	<0.001 vs. contol

*P<0.001 vs. control group, Saline: Control, and n = 8 for each group

Table 2: Effect of cyproheptadine on *T. polium*-induced antinociception writhing with acetic acid in mice

Treatment	Dose (mg/kg, i.p.)	Latency time (sec)	Writhing count (Mean±SEM)	Inhibition (%)	P-value
Control	10 (ml/kg)	179 ± 18	75.17 ± 3.17	—	—
Vehicle + *T. polium*	200	200 ± 27	23.13 ± 2.97	69.2	<0.001 vs. control
Cyproheptadine	4	188 ± 31	81.5 ± 2.66	—	—
Cyproheptadine + *T. polium*	4 + 200	191 ± 29	28.83 ± 2.23	61.64	<0.001 vs. control

Vehicle is 5% DMSO, Vehicle + Saline: Control, and n = 8 for each group

Table 3: Effect of phentolamine on *T. polium*-induced antinociception writhing with acetic acid in mice

Treatment	Dose (mg/kg, i.p.)	Latency time (sec)	Writhing count (Mean±SEM)	Inhibition (%)	P-value
Control	10 (ml/kg)	181 ± 27	78.66 ± 1.81	—	—
Vehicle + *T. polium*	200	190 ± 32	24.03 ± 1.63	69.08	<0.001 vs. control
Phentolamine	20	199 ± 36	85.33 ± 1.78	—	—
Phentolamine + *T. polium*	20 + 200	177 ± 19	32.16 ± 2.53	59.11	<0.001 vs. control

Vehicle is 5% DMSO, Vehicle + Saline: Control, and n = 8 for each group

Table 4: Effect of naloxone on *T. polium*-induced antinociception writhing with acetic acid in mice

Treatment	Dose (mg/kg, i.p.)	Latency time (sec)	Writhing count (Mean±SEM)	Inhibition (%)	P-value
Control	10 (ml/kg)	185 ± 24	76.17 ± 2.33	—	—
Vehicle + *T. polium*	200	210 ± 35	27.83 ± 1.22	70.02	<0.001 vs. control
Naloxone	2	193 ± 20	84.83 ± 2.54	—	—
Naloxone + *T. polium*	2 + 200	201 ± 26	45.33 ± 4.50	40.5	<0.001 vs. control <0.01 vs. *T. polium*

Vehicle is 5% DMSO, Vehicle + Saline: Control, and n = 8 for each group

Table 5: Effect of chlorpheniramine on *T. polium*-induced antinociception writhing with acetic acid in mice

Treatment	Dose (mg/kg, i.p.)	Latency time (sec)	Writhing count (Mean±SEM)	Inhibition (%)	P-value
Control	10 (ml/kg)	186 ± 20	76.50 ± 1.87	—	—
Vehicle + *T. polium*	200	201 ± 27	24.83 ± 1.07	67.5	<0.001 vs. control
Chlorpheniramine	10	197 ± 25	75.67 ± 1.94	—	—
Chlorpheniramine + *T. polium*	10 + 200	215 ± 36	38.33 ± 2.24	49.89	<0.001 vs. control <0.05 vs. *T. polium*

Vehicle is 5% DMSO, Vehicle + Saline: Control, and n = 8 for each group

Table 6: Effect of cimetidine on *T. polium*-induced antinociception writhing with acetic acid in mice

Treatment	Dose (mg/kg, i.p.)	Latency time (sec)	Writhing count (Mean±SEM)	Inhibition (%)	P-value
Control	10 (ml/kg)	191 ± 23	78.50 ± 2.76	—	—
Vehicle + *T. polium*	200	183 ± 18	30.33 ± 1.23	66.4	<0.001 vs. control
Cimetidine	10	189 ± 27	71.33 ± 3.20	—	—
Cimetidine + *T. polium*	10 + 200	211 ± 33	50.50 ± 3.06	35.66	<0.001 vs. control <0.001 vs. *T. polium*

Vehicle is 5% DMSO, Vehicle + Saline: Control, and n = 8 for each group

extract (200 mg/kg) induced significant reduction in pain response when compared to control group (P<0.001). Pretreatment with naloxone, chlorpheniramine and cimetidine significantly attenuated the antinociceptive effects of extract (from 70.02% to 40.5%, 67.5% to 49.89% and 66.4% to 35.66%, respectively) (P<0.01, P<0.05 and P<0.001, respectively). Furthermore, none of the drugs had any effect on the onset of first abdominal writhing in comparison with control group (Tables 4, 5 and 6).

Acute toxicity

Teucrium polium extract, at doses of 400-6400 mg/kg i.p. given to mice, had no effect on their behavioral responses and no mortality during the observation period of 72 h after administration. Therefore, it can be indicated that *T. polium* extract has no toxicity profile.

Discussion

In the present study, no mortality case up to the dose of 6.4 g/kg of *T. polium* extract (i.p.) was observed. Therefore, we may suggest that the extract has no lethal toxicity in mice.

In this study, i.p. injection of *T. polium* extract showed a dose-dependent antinociceptive effect in writhing test. Previous studies reported that this effect of the plant is due to flavonoids, and the main flavonoids of this plant are quercetine and apigenine (Yazdanparast and Ardestani, 2009). Furthermore, to reveal the anti-nociceptive mechanisms of *T. polium*, the possible involvement of opioidergic, serotonergic, noradrenergic and histamine receptor antagonists on *T. polium*-induced antinociception were examined. Our findings showed that pretreatment with naloxone significantly attenuates the antinociceptive effects of the extract (from 70.02 to 40.5%), thus it reverses analgesic activity of *T. polium* to some extent. In this regard, Anjaneyulu and Chopra (2003) reported that quercetin probably acts through modulation of opioidergic mechanisms. Furthermore, Opioid agents exert their

analgesic effects via supraspinal (m_1, k_3, d_1, s_2) and spinal (m_2, k_1, d_2) receptors (Reisine and Pasternack, 1996). As the anti-nociceptive activity of the extract was inhibited by naloxone, the extract likely acts on spinal opioid receptors such as m_2, k_1 and d_2 receptors, although other mechanisms of action such as inhibition of cyclooxygenases are also possible.

In the present study, pretreatment with chlorpheniramine, H_1 receptor antagonist, and cimetidine, H_2 receptor antagonist, attenuated the extract induced-antinociception (from 67.5% to 49.89% and 66.4% to 35.66%, respectively) but cyproheptadine and phentolamine had no effect. Thus, our results suggest that the part of *T. polium*-induced antinociceptive effect is mediated by H_1 and H_2 receptors, whereas serotonergic and adrenergic receptors had no effect. However, previous studies reported that serotonin and norepinephrine play an important role in the modulation of pain response (Taherianfard and Khazaee, 2006; Zendehdel and Babapour, 2010).

The involvement of histamine in the inflammatory pain of chemicals (e.g. formalin-induced) is well-documented. Peripheral histamine specifically activates and sensitizes itch-specific nociceptive C fibers (Schmelz *et al*., 1997), while it has emerged that central histamine plays an important role in antinociception (Robertson *et al*., 1988). Central injection of histamine shows an analgesic effect in several experiments including the tail-flick and hot-plate tests (Thoburn *et al*., 1994). Previously, several lines of evidence have demonstrated that systemic or central injection of histamine or histamine agonist produces antinociception, which suggest an important role in the regulation of antinociception (Chung *et al*., 1984). Furthermore, it has been reported that blockade of the H_1 and H_2 receptors attenuate the antinociception induced by nefopam, decursinol and restraint (Girard *et al*., 2004). Both H_1 and H_2 receptor antagonists when applied intracerebro-ventricularly or into the periaqueductal gray have been shown to block histamine-induced antinociception (Thoburn *et al*., 1994). Broad functional overlap, but also a striking anatomical and molecular specificity characterizes these distinct sensations (Ikoma *et al*., 2006). Most convincing seems to be the evidence implicating histamine H_2 receptors in the periaqueductal gray in the histamine mediated antinoception (Thoburn *et al*., 1994), although H_1 receptors may be important in other areas such as the spinal cord (Suh *et al*., 1996). Taken together, our results suggest that *T. polium* may be effective on the inflammatory pain even at the central nervous system level. Since opioid, H_1 and H_2 receptors antagonist could not completely reverse *T. polium* analgesic activity, it is possible that other mechanisms influence its antinociceptive effects. According to these findings, there is evidence to suggest that the central effect and the peripheral one may result in different extract compounds, polar compound(s) which act centrally through the opioid and histaminergic system, and the other class of compounds which act peripherally (Bittar *et al*., 2000).

In conclusion, the present study proposes that *T. polium* possesses a strong antinociceptive property, acting on, at least, the central nervous system level and opioidergic, histamine H_1 and H_2 receptors appear to be involved in the production of *T. polium*-induced antinociception.

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