

Evaluation of the Analgesic Effects of Teucrium Extract on Rats using the Formalin Test

Gh. Houshmand (PhD)¹, M. Goudarzi (PhD)*¹, H. Forouzandeh (PhD)¹, A. Nazari (MSc)¹,
V. Nouroollahi (DDS)²

1. Department of Pharmacology and Toxicology, Faculty of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, I.R.Iran

2. Department of Orthodontics, Faculty of Dentistry, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, I.R.Iran

Received: Dec 26th 2014, Revised: Feb 4th 2015, Accepted: May 6th 2015.

ABSTRACT

BACKGROUND AND OBJECTIVE: Pain is the most frequent symptom in different diseases. In modern medicine, there are two main categories of analgesics: opioids and non-steroidal anti-inflammatory drugs (NSAIDs). Given the importance of medicinal plants in the treatment of different diseases, this study aimed to compare the analgesic effects of the alcoholic extract of Teucrium with morphine and aspirin.

METHODS: In this experimental study, 36 Wistar rats were randomly divided into six groups of 6. The negative control group received normal saline (5 ml/kg), two positive control groups received morphine 2.5 mg/kg and aspirin 300 mg/kg, and three treatment groups received hydro-alcoholic extract of Teucrium (100, 200 and 400 mg/kg) intraperitoneally in single doses. Half an hour after the intraperitoneal injection of the extract, 50 microlitres of 2.5% formalin was injected subcutaneously into the right paw of the rats, and the analgesic effects were compared using the formalin test.

FINDINGS: In this study, the hydro-alcoholic extract of Teucrium had a dose-dependent analgesic effect, and the most effective dose of the extract was 200 mg/kg. Acute pain scores in the normal saline, aspirin, morphine and 200 mg/kg extract groups were 2.58 ± 0.09 , 1.39 ± 0.06 , 4.15 ± 0.09 and 1.61 ± 0.1 , respectively. In addition, chronic pain scores were 2.37 ± 0.09 , 0.99 ± 0.1 , 0.33 ± 0.09 and 1.18 ± 0.06 , respectively. Analgesic effects of Teucrium extract on chronic pain were lower compared to morphine, and had no significant difference with aspirin.

CONCLUSION: According to the results of this study, and regarding the presence of polyphenolic compounds in this herb, Teucrium is believed to have several analgesic properties.

KEY WORDS: Teucrium polium, Pain, Formalin test, Rat.

Please cite this article as follows:

Houshmand Gh, Goudarzi M, Forouzandeh H, Nazari A, Nouroollahi V. Evaluation of the Analgesic Effects of Teucrium Extract on Rats using the Formalin Test. J Babol Univ Med Sci. 2015;17(6):33-9.

* Corresponding Author; M. Goudarzi (PhD)

Address: Department of Pharmacology and Toxicology, Faculty of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, I.R.Iran

Tel: +98 61 33738378

E-mail: goudarzi787@gmail.com

Introduction

Pain is the most frequent symptom in diseases, and although the nature, condition and cause of pain may vary in different cases, almost half the patients referring to specialists tend to have complaints of pain. Pain is an alarming sign, which occurs under certain circumstances; for instance, due to the incidence of outside stimulation (i.e. obvious pain), or if the internal organs of the body are in unusual condition (1).

Extensive research has been conducted to discover new analgesic compounds since 1960s. In modern medicine, there are two main categories of analgesics: opioids and non-steroidal anti-inflammatory drugs (NSAIDs). Long-term use of opioids might induce dependence and tolerance, and NSAIDs may cause gastric bleeding (2, 3). Therefore, efforts continue as to search for new pain-relieving drugs with fewer complications than the existing compounds. Today, one of the important approaches to relieve pain with high performance and fewer restrictive effects is the use of medicinal plants (4, 5); these herbs have been widely applied in the treatment of diseases in traditional medicine. Moreover, disease treatment with medicinal plants, through using herbal products or whole herb extracts, is on an increasing trend as an effectual strategy across the world. Given the growing popularity of herbal therapy, comprehensive research is required as to evaluate the analgesic effects of whole herb extracts (4-8).

Plants are ample reservoirs of biologically active compounds having analgesic, anti-inflammatory, anti-diabetic, anti-cancer, antioxidant and antimicrobial properties. These features are associated with a variety of compounds which are found in these plants, such as polyphenols, flavonoids, alkaloids, carotenoids, and terpenoids. *Teucrium* is one of the most important plants used in traditional medicine, and scholars such as Hippocrates and Galen investigated the applications of this herb inclusively. *Teucrium* is a herbaceous plant, which belongs to the mint family of Labiatae. Numerous anti-diabetic, antispasmodic, analgesic, anti-inflammatory and antioxidant properties have been attributed to this herb in recent studies (8-13). The present study aimed to evaluate the analgesic effects of *Teucrium* extract on rats using the formalin test.

Methods

This experimental study was performed on 36 male Wistar rats weighing between 200 ± 20 g; the animals

were purchased from Laboratory Animals Care and Breeding Center of Jondishapour Medical University, Ahwaz, Iran. The animals were kept in cages made of polycarbonate at the temperature of $20 \pm 2^\circ\text{C}$ within a photocycle of 12 hours of light and 12 hours of darkness. Tap water and compact food were provided for the animals in sufficient amounts.

For this study, *Teucrium* leaves were collected in the spring from the region of Larestan, located in the south of Fars province, and after the identification of the plant and confirmation of the scientific term, the leaves were dried and milled. Afterwards, 200 g of the obtained powder was placed in a beaker, and ethanol 70% (30% water, 70% ethanol) was added to the powder, so that the ethanol would cover the surface of the powder. Finally, the lid of the beaker was sealed with aluminum paper, and the compound was preserved for 72 hours.

While soaking, the extract was stirred several times per day. After 72 hours of soaking, scorification was performed, and the residue was retained in a separate container. The residue was washed again with 70% alcohol and added to the previous extract; the obtained extracts were filtered through Whatman filter and concentrated using vacuum distillation technique.

The obtained concentration was kept in the oven at $30-40^\circ\text{C}$ until drying and complete loss of solvent, and was stored in a container made of dried, dark glass in cool, dry conditions. In this study, the rats were randomly divided into six groups of 6 and received intraperitoneal injections. The animal groups were as follows:

- 1) Negative control group received normal saline (5 ml/kg);
- 2) Positive control group 1 received morphine (2.5 mg/kg);
- 3) Positive control group 2 received aspirin (300 mg/kg);
- 4) Treatment group 1 received *Teucrium* extract (100 mg/kg);
- 5) Treatment group 2 received hydro-alcoholic extract of *Teucrium* (200 mg/kg);
- 6) Treatment group 3 received hydro-alcoholic extract of *Teucrium* (400 mg/kg).

In order to prepare the injectable solutions of morphine, aspirin and *Teucrium* extract in the desired concentrations, normal saline was used as solvent.

Half an hour after the intraperitoneal injection of the compounds, 50 microliter of 2.5% formalin was subcutaneously injected into the right paw of the animals. Immediately afterwards, the rats were placed

inside specific containers with transparent walls, and the pain levels were scored.

If the animal could walk without any difficulty and the injected foot was able to tolerate the whole body weight, the animal had no pain and was scored zero. In case the animal could not put the injected foot on the glass or place the weight on it, the rat had pain and was scored 1. If the animal raised the injected foot from the surface and fully kept the weight off the injected foot, the score was 2, and in case the animal licked, shook or bit the injected foot persistently, the pain score was 3 (14,15).

Quantitative data were measured in 12 five-minute blocks, and were recorded based on the pain scores at each time interval. Data recording continued until 60 minutes after the injection of formalin, and the mean of pain scores in each block was calculated based on the following formula:

$$\text{Pain score} = 0T0 + 1T1 + 2T2 + 3T3 \times 300$$

In the mean of pain scores, T0, T1, T2 and T3 are the 15-second intervals during which the rats show behaviors of zero, 1, 2, and 3 within a 5-minute period.

In all the study groups, an interval between 0-5 minutes was considered as the acute phase of pain, and 16-60 minutes was considered as the chronic phase of pain (16). The obtained results were expressed in Standard Error \pm Mean. Since data distribution was normal, parametric ANOVA was used to determine the difference between the study groups. In addition, Tukey's test was used for data analysis, and $P < 0.05$ was considered significant.

Results

In this study, a significant reduction was observed in the pain scores of the treatment groups receiving doses of 200 and 400 mg/kg of the hydro-alcoholic extract of *Teucrium*, and those receiving morphine and aspirin, in comparison with the negative control group during the first and second phases of pain in the formalin test ($p < 0.05$). However, 100 mg/kg of the extract significantly decreased the pain scores compared to the negative control group during the second phase of the formalin test ($p < 0.05$) (table 1).

Table 1. Acute and Chronic Pain Scores in the Study Groups (Mean \pm SD)

	Normal saline	Aspirin	Morphine	mg/kg 100	mg/kg 200	mg/kg 400
Acute Pain	$b. 0.58 \pm 0.2$	$a, b. 0.91 \pm 0.6$	$a, b. 0.95 \pm 0.1$	$b. 0.83 \pm 0.1$	$a. 0.61 \pm 0.1$	$a. 0.50 \pm 0.1$
Chronic Pain	$d. 0.37 \pm 0.2$	$c. 1.99 \pm 0.1$	$c, d. 0.33 \pm 0.1$	$c, d. 0.79 \pm 0.1$	$c. 0.18 \pm 0.1$	$c. 0.05 \pm 0.1$

** a: Significant difference with the saline group in acute pain scores ($p < 0.05$); b: Significant difference with the treatment group (200 mg/kg of extract) in acute pain ($p < 0.05$)

** c: Significant difference with the saline group in chronic pain scores ($p < 0.05$); d: Significant difference with the treatment group (200 mg/kg of extract) in chronic pain ($p < 0.05$)

The most significant analgesic effect was observed at doses of 200 and 400 mg/kg; since there was no significant difference between the effects of these two doses, 200 mg/kg was selected as the most effective dosage of the herbal extract of *Teucrium* (fig 1). On the other hand, evaluation of the analgesic effects of 200 mg/kg of *Teucrium* extract with morphine and aspirin indicated that the analgesic effects of the extract were lower during the first phase of pain compared to morphine and aspirin, and this difference was considered to be statistically significant ($p < 0.05$). However, there was no significant difference between the analgesic effects of the treatment group receiving 200 mg/kg of the extract with the aspirin group during the second phase of pain at any time intervals, and the analgesic effects of these compounds were observed to be identical (fig 2).

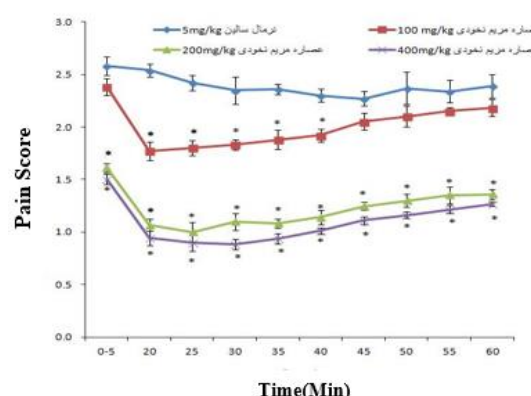


Figure 1. Comparison of analgesic effects during the first and second phases of pain in groups receiving intraperitoneal doses of extract and normal saline

** Significant difference with the normal saline group ($p < 0.05$)

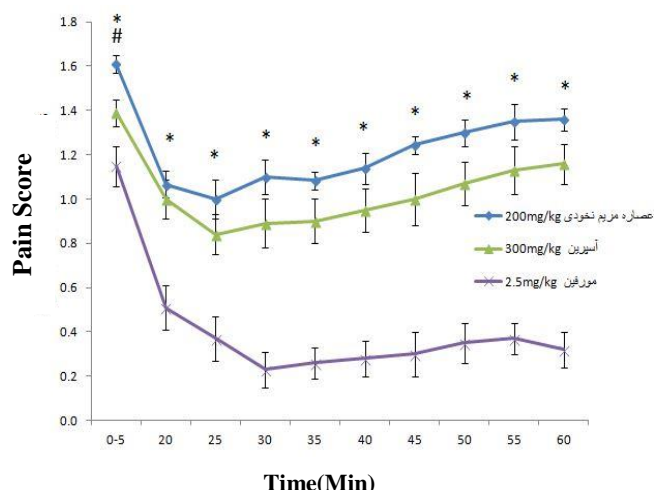


Figure 2. Comparison of analgesic effects during the first and second phases of pain in groups receiving intraperitoneal doses of *Teucrium* extract and the most effective dose of extract (200 mg/kg) with positive control groups (morphine and aspirin) using the formalin test

**Significant difference with morphine ($p < 0.05$)

**Significant difference with aspirin ($p < 0.05$)

Discussion

In this study, the hydro-alcoholic extract of *Teucrium* at doses of 200 and 400 mg/kg was observed to have analgesic effects similar to aspirin, and it seems that such pain-relieving effects could be due to the presence of phenolic compounds and flavonoids in this plant. Several tests are used to evaluate analgesic effects of different substances, and according to previous studies, the formalin test is an efficient method for the assessment of acute and chronic pain.

The acute phase of pain is normally non-inflammatory (i.e. neurogenic pain), while the chronic phase is normally associated with the subcutaneous injection of formalin causing a two-step pain, which is an important features in the process of pain assessment. Furthermore, neurotransmitters such as substance P, bradykinin, glutamate and serotonin might be involved in the incidence of formalin-induced pain (17).

The formalin test is a standard procedure to measure the tail-flick response in rats; subcutaneous injection of formalin induces two phases of pain: the first phase begins immediately after the injection, resulting in the direct stimulation of C-sensory fibers, and the second phase (i.e. delayed phase) starts approximately 15 minutes after the injection of formalin, which is caused by the inflammation process. This test is widely used for the study of the mechanism

of pain induction, as well as the analgesic properties of different compounds. Analgesic drugs that function through the central nervous system are able to inhibit both phases of pain caused by formaldehyde, while the analgesics that function through environmental factors can only inhibit the second phase of pain in the formalin test (18). Several studies in this regard have indicated that the extract of *Teucrium* contains noticeable amounts of flavonoids, which are among the main inhibitors of the enzymatic function of nitric oxide (NO) synthesis; these compounds inhibit the production of NO, which tends to increase due to formalin injection (19). Since NO could function as a mediator of pain, its reduction could result in increased analgesic activity (20). Moreover, other studies have demonstrated that flavonoids could decrease intracellular calcium through the inhibition of the activity of N-methyl D-aspartate receptor, NO synthesis and calcium-dependent phospholipase A2. Therefore, analgesic effects are produced due to the reduction of NO and prostaglandins (20, 21). Furthermore, inhibition of phospholipase A2 activity prevents the conversion of phosphatidic acid into arachidonic acid, and as a result, the synthesis of prostaglandins is inhibited (22, 23). Evidence suggests that through the inhibition of cyclooxygenase, flavonoids could prevent the production of prostaglandins from arachidonic acid in response to the inflammatory stimuli (24).

On the other hand, fats and proteins, which are the main structural component of cell membranes, could be damaged by free radicals and lose their integrity, which lead to the production of pro-inflammatory cytokines that could cause pain (25). In a study by Sharififar et al., the antioxidant properties of *Teucrium polium* were evaluated, and the obtained results indicated that the extracts and fractions of *T. polium* have revival potentials and could cause the inhibition of lipid peroxidation and collect free radicals (26).

In another study, Abdollahi et al. examined the analgesic effects of *Teucrium* extract on rats using the leg-rising test (8). In that study, doses of 150, 225 and 300 mg/kg of the extract were used, and the obtained results were remarkable. Therefore, the same dosages of the extract of *Teucrium* were used in the current study. In the study of Abdollahi et al., dosage of 225 mg/kg was observed to have the most prominent analgesic effect, while the dosage of 200 mg/kg was the most effective one in the present study. In another study in this regard, Farshchi et al. evaluated the analgesic and anti-inflammatory effects of the hydro-alcoholic extract

of *Teucrium Maryhyrcanicum*, which is another species of this herb. According to the results of that study, the anti-inflammatory and analgesic effects of this extract might be caused by central and peripheral mechanisms, and the presence of alkaloids, flavonoids and terpenoids could be another factor in the induction of such effects by this medicinal herb (27). Regarding the analgesic and anti-inflammatory properties of polyphenolic compounds, as well as the flavonoids which reduce neurogenic inflammatory pain through the activation of various nervous pathways, the function of

such analgesic components in this plant is possibly associated with the presence of phenolic compounds, and a thorough comprehension of this mechanism requires further research.

Acknowledgements

Hereby, we extend our gratitude to Ms. Zivar Amanpour, laboratory assistant of the Pharmacology Faculty of Jondishapour University of Medical Sciences, Ahwaz for her support of this research project.

References

1. Gyton AC, Hall JE. Textbook of medical physiology. 9th ed. Philadelphia: W.B. Saunders; 1996. p. 609-62.
2. Waldhoer M, Bartlett SE, Whistler JL. Opioid receptors. *Annu Rev Biochem*. 2004;73(1):953-90.
3. Macrae F, Mackenzie L, Mccoll K, Williams D. Strategies against NSAID-induced gastrointestinal side effects. Part 1. *Pharm J*. 2004;272(7286):187-9.
4. Ramezani M, Amin G, Jalili E. Antinociceptive and anti-inflammatory effects of hydroalcoholic extract of *Vitex agnus castus* fruit in mice. *J Shahrekord Univ Med Sci*. 2010;11(4):46-51. [In Persian]
5. Nasri S, Ramazani M, Yasa N. Antinociceptive and anti-inflammatory effects of hydro-alcoholic extract of *Apium graveolens*. *J Shahrekord Univ Med Sci*. 2009;10(4):25-31. [In Persian]
6. Arzi A, Hemmati AA, Mehrabi MM, Siahpush A, Nazari khorasgani Z. The study of analgesic effect of hydroalcoholic extract of *vitis vinifera* seed on rat by formalin TEST. *Jundishapur Sci Med J*. 2013;11(6):609-18. [In Persian]
7. Arzi A, Sarkaki A, Aghel N, Nazari Z, Saeidnejad S. Study of analgesic effect of hydroalcoholic extract of cinammon. *Jundishapur Sci Med J*. 2011;10(3):272-9. [In Persian]
8. Abdollahi M, Karimpour H, Monsef-Esfahani HR. Antinociceptive effects of *Teucrium polium* L. total extract and essential oil in mouse writhing test. *Pharmacological Research*. 2003;48(1):31-5.
9. Tariq M, Ageel AM, Al-Yahya MA, Mossa JS, Al-Said MS. Anti-inflammatory activity of *Teucrium polium*. *Int J Tissue React*. 1989;11(4):185-8.
10. Gharib Naseri MK, Omid Birgani F. Antispasmodic effect of *teucrium polium* leaf extract on rat ileum. *Pajoohandeh J*. 2007;12(1):59-67. [In Persian]
11. Ljubuncic P, Dakwar S, Portnaya I, Cogan U, Azaizeh H, Bomzon A. Aqueous extracts of *Teucrium polium* possess remarkable antioxidant activity in vitro. *Evid Based Complement Alternat Med*. 2006; 3(3):329-38.
12. Karimi F, Abbasi S, Bateni A. The effect of *Teucrium polium* on blood glucose in diabetes mellitus type 2; a comparison with glibenclamide. *Iran South Med J (ISMJ)*. 2002;4(2):96-103. [In Persian]
13. Gharaibeh MN, Elayan HH, Salhab AS. Hypoglycemic effects of *Teucrium polium*. *J Ethnopharmacol*. 1988;24(1):93-9.
14. Abbott FV, Franklin KB, Westbrook RF. The formalin test: scoring properties of the first and second phases of the pain response in rats. *Pain*. 1995;60(1):91-102.
15. Arzi A, Hoshmand Gh, Goudarzi M, Khadem Haghighian H, Rashidi Nooshabadi M. Comparison of the analgesic effects of royal jelly with morphine and aspirin in rats using the formalin. *J Babol Univ Med Sci*. 2015;17(2):50-6. [In Persian]
16. Dubuisson D, Dennis SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain*. 1977;4(2):161-74.
17. Sawynok J, Liu XJ. The formalin test: characteristics and usefulness of the model. *Rev Analgesia*. 2003; 7(2):145-63.
18. Shibata M, Ohkubo T, Takahashi H, Inoki R. Modified formalin test: characteristic biphasic pain response. *Pain*. 1989;38(3):347-52.
19. Toker G, Küpeli E, Memisoğlu M, Yesilada E. Flavonoids with antinociceptive and anti-inflammatory activities from the leaves of *Tilia argentea* (silver linden). *J Ethnopharmacol*. 2004;95(2):393-7.
20. Doursout MF, Liang Y, Chelly JE. NOS inhibitors exhibit antinociceptive properties in the rat formalin test. *Can J of Anaesth*. 2003;50(9):909-16.
21. Davidson EM, Coggeshall RE, Carlton SM. Peripheral NMDA and non-NMDA glutamate receptors contribute to nociceptive behaviors in the rat formalin test. *Neuroreport*. 1997;8(4):941-6.
22. Middleton E, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Reviews*. 2000;52(4):673-751.

23. Ferrandiz M, Alcaraz M. Anti-inflammatory activity and inhibition of arachidonic acid metabolism by flavonoids. *Agents Actions*. 1991;32(3-4):283-8.
24. Alcaraz M, Ferrandiz M. Modification of arachidonic metabolism by flavonoids. *J Ethnopharmacol*. 1987;21(3):209-29.
25. Cortese M, Sinclair C, Pulendran B. Translating glycolytic metabolism to innate immunity in dendritic cells. *Cell Metab*. 2014;19(5):737-9.
26. Sharififar F, Dehghn-Nudeh Gh, Mirtajaldini M. Major flavonoids with antioxidant activity from *Teucrium polium* L. *Food Chem*. 2009;112(4):885-8.
27. Farshchi A, Ghiasi G, Abdollahasl A. Antinociceptive and antiinflammatory effects of *Teucrium hyrcanicum* aqueous extract in male mice and rats. *Physiol Pharmacol*. 2010;14(1):78-84.[In Persian]