Anti-inflammatory and anti-nociceptive effects of hydroalcoholic extract of Nepeta menthoides on pain in aerial parts in male mice

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Abstract

Introduction:
The application of herbal plants instead of synthetic drugs has been increasing in recent years because of their lower side-effects and high varieties of efficient components. An investigation on anti-nociceptive and anti-inflammatory effects of hydroalcoholic extract of Nepeta menthoides seems to be necessary due to the existence of its components.

Materials and Methods:
This study was done on 224 NMRI male mice weighing 20-25 g. Xylene-induced ear edema and Formalin test were used for demonstrating its anti-inflammatory and anti-nociceptive effects.

Results:
The hydroalcoholic extract had no toxic effect. This study showed that Nepeta menthoides aerial parts have anti-inflammatory effect at all doses, particularly at dose of 2800 mg/kg and significantly decreased nociception in chronic phases. The dose of 2800 mg/kg had the most anti-nociceptive effect in chronic phases. Meanwhile, in the immersion test, nociception decreased at a dose of 1400 mg/kg significantly.

Conclusion:
Hydroalcoholic extract of Nepeta menthoides has anti-nociceptive and anti-inflammatory effects. These effects might be due to its Nepetalactone and 1,8 cineole components.

Keywords: Analgesics, Plant Extracts, Mice

Introduction
Pain is an unpleasant sensory experience which is common in all organisms, but individuals’ sensitivity to painful stimuli is different. Pain as a protective sensory experience has a wide range (1). In fact, any kind of sensory created by damaging or destroying tissue stimulators is considered as pain (2). Opioids are among compounds that have antinociceptive properties and are divided into two categories:

1. Endogenous opioids that are found naturally in the body and are effective in suppressing or inhibiting pain.
2. Exogenous opioids such as morphine are compounds that exist outside the body and are used as painkillers.

Plants were the first pharmaceutical materials that were used for treatment. Based on experience, those plants that effectively treat various diseases have been known as healing plants or medicinal plants (4). By development of
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antinociceptive chemical drugs and their advent to the pharmaceutical market and their widespread use for pain relief, a wide range of their side effects has emerged (5). The use of these drugs creates problems such as drug resistance, dependence, euphoria etc. (6). Hence, the trend to investigate the effects of medicinal plants has been formed. Because most countries have carefully examined medicinal plants and Iran has rich resources of medicinal plants– in terms of climatic variety and widespread vegetation – it is necessary to study plants which have been suggested in traditional or modern medicine as antinociceptive drugs (7).

Nepeta genus (Lamiaceae), known as “Poone-sa” in the Persian language, has 250 species that spread from North Africa to Europe and Asia. So far, 67 species of the genus have been identified in Iran and 39 species are native to Iran (8). Since ancient times, different species of the genus have been used in traditional medicine because of anticonvulsant, anti-cough, anti-asthma, disinfectant and diuretic effects (9). In Iranian traditional medicine, species of N. bracteata and N. ispahanica as asthma reliever and anti-flatulence, the species of N. racemosa as abdominal pain reliever and disinfectant and anti-flatulence and the species of N. menthoides as sedative, anti-fever and abdominal pain reliever are consumed (10). N. cataria has been introduced as an official drug with therapeutic effects. Leaves and aerial parts of this species are used as sedative tea to relieve insomnia and headache, and they are anti-flatulence and anti-spasm as well (11).

N. menthoides Boiss. & Buhse or Nepeta menthoides is one of the exclusive species of Lamiaceae in Iran which is distributed in the northwest of the country, Azerbaijan. Sabalan Mountains and the areas around the Sufian road to Marand are mentioned as the distribution areas for Iranica flora. However, the new surveys suggest the widespread distribution of this species in the northwest of Iran. Nepeta menthoides despite being aromatic is less used by indigenous people of the region and in Ardabil province is known as – Yarpuz – but it appears to have significant medicinal properties because of chemicals similar to other Nepeta species. Nepeta menthoides is a gramineous, perennial, rising and sturdy plant reaching 15 to 40 cm height with violet flowers (12). Earlier studies have been conducted to investigate the antinociceptive effects of some species of Nepeta species such as Nepeta Caesarea and N. Italica L., but hydroalcoholic extract from N. menthoides was used for the first time to evaluate the anti-inflammatory and antinociceptive effects in this study (13-14).

Materials and Methods

In this experimental study, 224 young male mice, weighing approximately 20 to 25 grams (procured from Pasteur Institute of Tehran) were used after two days of adjustment with environment of animal house in Payame-Noor University of Tehran, at 12 hr day/night cycle and free access to food (Karaj Pars Animal Feed Co.) and water. All animal experiments were conducted according to the rules of Payame-Noor University’s Bioethics Committee. To make the extract, plant samples were purchased from a botanica in Tehran bazaar and then the genus and species of plants were approved in herbarium of Pharmacognosy Department, Faculty of Pharmacy of Tehran University of Medical Sciences. Extraction was performed by Soxhlet method. Then, 50 g of powdered aerial parts of Nepeta menthoides was placed on filter paper with small pores. Then the solvent (ethanol 70°) was poured into the flask and the upper part of the device was placed on it and the temperature was adjusted according to the boiling point of the solvent. After the solvent vapor was cooled and liquefied by condenser, it was dripped on crude powder. When the solvent surface reached siphon, it was discharged automatically and was accumulated in the flask. This
process continued regularly up to 4 hours till the extraction was completed upon the raw powder (15-16). **Method of determining the lethal dose of the extract:** Fifty-six male Syrian mice were used to determine the lethal dose of hydroalcoholic extract from *Nepeta menthoides*. The mice were divided into groups of eight animals each. Then, different doses of the plant extract were injected to them intraperitoneally. The lowest injected doses given to other species of *Nepeta* was 500 mg per kg of animal weight and the next numbers were selected by exponential equation (1000, 2000, 4000, 10000, 20000, 40000). The number of dead mice was counted at 24 and 48 hours after each injection. **Formalin test:** In this experimental study, formalin test was used developed by Dubuisson and Dennis and it is a model for assessing pain (17-18). To perform this experiment, seven randomly divided groups of eight mice were used. The first group was the negative control group to which normal saline was injected intraperitoneally. The second group was the positive control group to which 10 mg per kg morphine (dissolved in distilled water) was injected intraperitoneally. Next groups as experimental groups (each containing 8 mice) received different doses of the extract (1400, 700, 350, 70, and 2800 mg per kg) as a single dose for each animal intraperitoneally (all injections were 30 minutes before formalin administration). Intraperitoneal injections were made 15 minutes after the animal was placed under a glass funnel on the pain box with dimensions 30×30×30. In order to better observe the mouse movements, a mirror was placed at an angle of 45 degrees under it and in front of the observer (19). Immediately after injection of the extract, animal was placed under the funnel for 30 minutes for compatibility with the test chamber. Then intraplantar injection of 0.02 mL of formalin (2.5%) (Romil, UK) was given to the right hind paw of the animal subcutaneously and then the animal was returned into the special test box again. At this phase, animal’s movements were studied at two phases. When the animal licked, bit or shook its right paw violently, it was considered as licking time. The mean time of licking in the interval time between 0-5 minutes was considered as the first phase of the pain and the mean time of licking between 15-30 minutes was considered as the second or peripheral phase of the formalin test (the volume of injection of drugs and extracts was to 0.1 ml) (20). **Xylene test:** To perform this test, mice were randomly divided into seven groups of eight. The first group as the negative control group was injected with normal saline intraperitoneally. The second group that was the positive control group was injected with 15 mg per kg dexamethasone (dissolved in normal saline) intraperitoneally. Different doses of the extract (1400, 700, 350, 70, and 2800 mg per kg) were injected intraperitoneally to five experimental groups as a single dose for each animal (all injections were made 30 minutes before Xylene administration). Then, xylene (Romil Ltd., UK) was used to create inflammation in the ear of mice, so that 15 minutes after injection, 0.03 ml xylene was injected in the anterior and posterior surface of the right ear of the animal and two hours after injection the animals were sacrificed (21). Then seven-millimeter circular sections taken from animal’s left and right ear were weighed and the weight difference was determined. The weight difference indicates the amount of inflammation. In other words, greater weight difference between the two ears shows greater degree of inflammation (3). **Hot water tail-immersion test for mice:** The test was conducted by using the method described by Curtis et al. (22). To perform this test, mice were randomly divided into six groups of eight. The first group as the negative control group was injected with normal saline, and the second
group as the positive control group was injected with 10 mg per kg morphine (dissolved in distilled water) intraperitoneally. Four other groups as experimental groups received different doses of the herbal extract (1400, 700, 350, 70, and 2800 mg per kg) intraperitoneally as a single dose for each animal. All injections were made 15 minutes before the animal was placed in individual restraining cage. To perform this test, the animal was put inside the mouse restraining cage for 15 minutes under standard laboratory conditions and then the animal's tail was immersed in hot water at 49 C°, and tail withdrawal latency was measured by a stopwatch. The experiment on each animal was repeated 4 times with an interval of 5 minutes and finally the data mean was recorded for each mouse. A cut off time of 30 seconds was established if the mouse did not withdraw its tail.

**Data analysis:** In each group of experiments, the effect of different doses was recorded as mean±SD for eight mice. Data were selected completely randomly. To determine if there was a significant difference between the groups that received different concentrations of the extract with control groups, one-way ANOVA and Tukey test were used (p<0.001). After completion of the tests, the mice were slaughtered by cervical dislocation method (23).

**Results**

After injection of different doses of the extract and the elapse of 48 hours, no dead mouse was found. Given that the dose of 40,000 mg per kg was very close to the initial concentration of the extract, the next injection was avoided.

**Anti-inflammatory effects:** The review of inflammation test results showed that the extract reduces inflammation compared to the control group. There was no significant difference between the group that received dexamethasone and the group that received the extract at doses of 2800 and 1400 mg per kg, and its anti-inflammatory properties became more by increasing the dose of the extract. Therefore, the dose of 1400 mg per kg has a close effect to dexamethasone effect on reducing inflammation induced by xylene (with mean value and standard deviation of 0.0077±2.086×10⁻⁵ for this dose versus 0.0077±1.07×10⁻⁵ for dexamethasone, p<0.01) and anti-inflammatory effect of dose 2800 mg per kg is greater than dexamethasone (with mean value and standard deviation of 0.0046±7.05×10⁻⁶ for this dose versus 0.0071±1.07×10⁻⁵ for dexamethasone, p<0.001).

**Antinociceptive effect:** In the first phase of formalin test (acute phase of the pain) just the dose of 2800 mg per kg showed a significant reduction of the pain compared to the control group (with mean value and standard deviation of 41.7±8.04 for this dose compared to the control group with 10.5±22.41, p<0.001) (Figure 1). In the second phase of formalin test (chronic phase of the pain) all doses except 70 and 350 doses showed a significant reduction of the pain compared to the control group (with mean value and standard deviation of 28.86±18.31 at 700 mg per kg dose, 26.32±15.48 at 1400 mg per kg dose and 1.69±0.46 at 2800 mg per kg dose compared to the control group 108.6±25.16) (Figure 2). According to Tukey test at this phase, there was no significant difference between 1400 and 700 mg per kg doses and the group receiving morphine (with mean value and standard deviations of 26.86±18.31 at 700 mg per kg dose, p<0.01 and 26.32±15.48 at 1400 mg per kg dose compared to the group receiving morphine with 29.09±11.41, p<0.001), meaning that they showed similar antinociceptive effect in this phase (Figure 2). In the second phase of the pain, antinociceptive effect of the extract increased by increasing injection doses, so the 2800 mg per kg dose is the most effective dose with antinociceptive effect (with mean value and standard deviation of 1.69±0.46 at 2800 mg per kg
dose compared to the group receiving morphine with 29.09±15.48, p<0.001).

**Hot water tail-immersion test:** In hot water tail immersion test, which is used to measure the thermal pain threshold, there was a significant difference between all doses except 70 mg per kg and control group (with mean value and standard deviation of 4.84±0.12 at a dose of 350 mg per kg and 4.47±0.7 at a dose of 700 mg per kg and 4.52±0.2 at a dose of 2800 mg per kg compared to the control group with 2.82±0.8, p<0.01) and dose of 1400 mg per kg had the highest tail withdrawal latency (with mean value and standard deviation of 5.76±0.76 at 1400 mg per kg dose compared to the control group with 2.82±0.8, p<0.001).

All figures are plotted based on the mean and standard deviation. According to Figure 1, the mean of weight difference between the two ears for the extract group at a dose of 2800 mg per kg is the lowest value and for the negative control group is the highest value. According to Tukey test, a dose of 2800 mg per kg showed the highest anti-inflammatory effect.

![Figure 1](image1.png)

*** p <0.001, ** p <0.01 difference with the control group

Figure 1: Results of the effect of intraperitoneal injection of the total extract of *Nepeta menthoides* (Nm.) on inflammation by using xylene test compared to negative control group.

![Figure 2](image2.png)

*** p <0.001 difference with the control group

Figure 2: Results of the effect of intraperitoneal injection of the total extract of *Nepeta menthoides* (Nm.) on the acute pain (0-5 min.) by using formalin test compared to negative control group.
As seen in Figure 2, the mean time of licking for the positive control group (morphine) is the lowest value and for the negative control group is the highest value. According to Tukey test, the highest antinociceptive effect of the extract in the acute phase of formalin test is related to a dose of 2800 mg per kg.

As seen in Figure 3, the lowest time of licking at this phase is related 2800 mg per kg dose of the experimental group and the highest time is related to the negative control group.

![Graph showing mean time of licking per second](image)

**Figure 3:** Results of the effect of intraperitoneal injection of the total extract of *Nepeta menthoides* (Nm.) on chronic pain (15-30 min.) by using formalin test compared to negative control group.

![Graph showing delay of withdrawal of the tail (sec)](image)

**Figure 4:** Results of the effect of intraperitoneal injection of the total extract of *Nepeta menthoides* (Nm.) on thermal pain by using hot water tail-immersion test compared to the negative control group.

Based on Figure 4, it can be said that all doses except 70 mg per kg have more significant latency than the control group. At all doses, the tail withdrawal latency is less than the group receiving morphine.
Discussion
The results of the current applied research confirm for the first time the anti-inflammatory and antinociceptive effect of the total extract of aerial parts of *Nepeta menthoides* (Lavender). The major part of the antinociceptive effects of this plant may be due to inhibition of inflammation (the second phase of the pain). In addition, the hydroalcoholic extract of this plant has more effective antinociceptive properties than morphine in the second phase of the formalin test. In the formalin test, pain expression occurs in two phases (24). The first phase results from direct stimulation of pain receptors, while the second phase reflects the transmission of the pain message from peripheral inflammatory processes that cause pain (25). In the first phase of the pain (neurogenic pain) substance P and bradykinin, and in the second phase of the pain (peripheral inflammatory pain) histamine and prostaglandins are involved, which suggests that the second phase can be inflammatory (26). The central analgesic drugs, such as opioids inhibit both phases equally. The effect produced in the early phase may result in their immediate and direct effects on sensory receptors, bradykinin receptors or glutamatergic pathway (26). The second phase is dependent on inflammatory responses induced by arachidonic acid cascade. The second phase may be a response to inflammatory pain that can be inhibited by anti-inflammatory drugs (18).

Chemical compounds in many plants have been identified with antinociceptive properties, among which the following can be mentioned: phenanthrene, salicin, cannabinoids, salicylic acid and a large number of alkaloids, terpenoids, capsaicinoids, steroids, flavonoids, xanthines, tannins, xanthones, ligans, saponins, lactones and glycosides (27). The results of this study showed that administration of the hydroalcoholic extract from *Nepeta menthoides* further reduces the symptoms of pain due to formalin administration to the animal's paw in the second phase of the formalin test. According to the anti-inflammatory effects of hydroalcoholic extract of plants of this family, the extract of this plant contains compounds with peripheral anti-inflammatory effects. Researchers have previously shown that the extracts prepared from different species of *Nepeta* are rich in flavonoids and tannins. Previously, it has been found that flavonoids inhibit synthesis of prostaglandins and the compounds are the most important inflammatory factors activating peripheral pain receptors (28-29). This can be considered as the most important peripheral mechanism of the pain control by metabolites present in the extract. It is interesting that most drugs that stop or reduce the animal's response in the chronic phase of this experiment and are used for the treatment of chronic and neuropathic pains have no effect on acute and normal pains or have minimal effect clinically (30-31).

Comparing the results of the earlier studies conducted on *Nepeta menthoides* shows that habitat can change the essence composition of this plant. The results of these studies indicate that the most important constituent of the sample collected from Sahand Mountain slopes is Nepetalactone, while it is 1, 8-cineole in the sample collected from Sabalan Mountain which comprises approximately 59% of the total ingredients of this essence and there is no Nepetalactone (32-34).

In a study in 2000, it was determined that the use of 1, 8-cineole could reduce the pain and inflammation (35). Nepetalactone is a compound with bicyclic terpenoid structure that was isolated from *N.cataria* plant for the first time in 1941 and it is called as an antinociceptive compound of opioid in studies (13,36). According to the current study, it seems that the results are attributed to 1,8-cineole in the extract that is used to inhibit the inflammatory pain. By using formalin test model in the chronic phase, it was determined that
hydroalcoholic extract from aerial parts of *Nepeta menthoides* acts dose-dependent in creating antinociceptive effects. Although the mechanism of the herbal extract is unknown, the results of the current study confirm the antinociceptive effects of the plant.

**Conclusion**

Based on the findings of this research, whole hydroalcoholic extract of aerial parts of *Nepeta menthoides* caused a significant reduction of pain in the formalin test (acute and chronic phases) compared to the control group. Also, the extract of the aerial parts of this plant has an anti-inflammatory effect that is equal to that of dexamethasone and even surpasses it. Anti-nociceptive and anti-inflammatory effects of this plant can be attributed to its components. Further studies are needed to determine the antinociceptive and anti-inflammatory mechanisms and the effect of components in the extract on pain and inflammation.

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**Conflict of interests**

Authors had no conflict of interests in this study.

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