Original Article

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Effects of the Hydro-alcoholic Extract of Clove (Dianthus deltoides) on Inflammation and Pain Response using the Xylene Test and Hot Plate Test in Mice

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ABSTRACT

BACKGROUND AND OBJECTIVE: Clove contains large proportion of a phenolic compound called eugenol, which has antioxidant, anti-inflammatory and analgesic properties. This study aimed to evaluate the effects of hydro-alcoholic clove extract on inflammation and pain response in mice.

METHODS: In this experimental study, 48 male mice were classified into six groups of control (saline), positive control (dexamethasone) and treatment (receiving 42, 85, 170 and 340 mg/kg of clove extract) to investigate the effects of clove extract on inflammation. To evaluate pain perception, 160 mice were categorized into 4 groups, divided into 5 subgroups including the control (saline), positive control (morphine), recipients of 200 mg/kg clove extract, recipients of 500 mg/kg of extract, and recipients of 200 mg/kg of combined clove extract and naloxone (4 mg/kg). To assess pain response, the hot plate test was performed on each subgroup at 5, 15, 30 and 60 minutes after the injection of the herbal extract. In addition, two hours after the injection of 0.03 ml xylene into the back of the right ears of the mice, the sections obtained from both ears (7 mm) were compared in terms of weight.

FINDINGS: In the inflammation test, the most significant difference in the ear sections was observed between the control group and recipients of 42 mg/kg of extract (36±5.1 and 33±5.3 µg, respectively), while the least significant difference was observed at doses of 180 and 340 mg/kg (21±2.1 and 17 ±1.5 µg, respectively). In the hot plate test on the treatment groups, dose of 500 mg/kg caused the highest pain delay 15 minutes before the test, while the lowest delay was observed in the recipients of combined naloxone and clove extract (200 mg/kg) 5 minutes before the test (18.91±1.53 and 8.71±1.04 seconds, respectively).

CONCLUSION: According to the results of this study, hydro-alcoholic clove extract could have significant anti-inflammatory and analgesic effects on mice.

KEY WORDS: Clove, Hot plate, Naloxone, Morphine, Dexamethasone, Xylene.

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Introduction

Clove is a well-known medicinal plant with a long history of use in the treatment of different diseases. Clove is composed of 21-14% essence, containing 70-80% of a flavonoid called eugenol (1). Flavonoids are polyphenolic compounds, which are naturally present in plants, and have several analgesic and anti-inflammatory properties (2). In one study conducted on rats, intraperitoneal and local injection of eugenol to the paw of the animals was observed to cause a significant reduction in pain during the formalin test (3). In another research in this regard, it was reported that use of clove extract in the treatment of lactating female rats had analgesic effects on the male offspring of these animals, and the analgesia occurring during the chronic phase of pain was considered to be significant (4).

Inflammation is defined as a reaction induced by the capillary system of the body, which involves the movement of fluids and white blood cells into the extravascular tissues, manifesting through symptoms such as swelling, redness, warmth and pain. This condition is an attempt by the host to localize foreign bodies, microorganisms, antigens or other involving cells (5). Inflammation has been a major concern among researchers since this defense mechanism could cause noticeable damage to the body, giving rise to various diseases. Given the notable effects of this phenomenon on the body, searching for effective drugs as to control and manage inflammation has been of paramount importance in the field of medical research (6).

Available chemical medications, such as glucocorticoids, could remarkably relieve inflammation; however, the use of these compounds may lead to severe side effects in the patient (7). With regard to pain, due to the existence of multiple stimuli, as well as the complexity of the mechanisms involved in the direction and perception of pain as the most common clinical symptom, research for discovering more efficient pain-relieving agents remains essential (8). Frequent production and distribution of synthetic, chemical drugs among patients have led to a better understanding of the wide range of adverse effects caused by these medications (9). Regarding the high consumption of analgesics and anti-inflammatory drugs and their side effects in the modern society, comprehensive research is required on alternative, herbal and synthetic medicines. This study aimed to evaluate the analgesic and anti-inflammatory effects of clove extract on BALB/C mice.

Methods

Drugs and Medications: The purchased drugs for this study included morphine sulfate (Daroupakhsh, Iran), naloxone (Tolid-darou, Iran) and xylene (Romil, England). This experimental study was performed on 208 adult, male BALB/C mice with the mean weight of 25±3 g (160 mice to investigate analgesic effects and 48 mice to evaluate anti-inflammatory effects of the drugs).

For environmental adaptation, the animals (purchased from Tehran Pasteur Institute, Iran) were kept at the animal house of Shahrekord University of Medical Sciences, Iran, at the temperature of 21-25°C for one week. During this time, the animals had free access to sufficient food (pellets) and water. After one week, the weight of the animals was measured and recorded (10).

Extraction Method: After purchasing clove (Dianthus deltoides), the plant was evaluated and approved by a panel of experts at the Botanical Research Institute of Shahrekord University of Medical Sciences. Afterwards, the flowers were dried in shades with the appropriate temperature, and 150 grams of dried clove powder was placed in an ecological jar with 1000 ml of 80% ethanol covering the powder. After 3 days, the solution was filtered twice using a funnel and filter paper, and the obtained extract was transferred to a vacuum distillation unit in order to concentrate at a low temperature. Following that, the extract was dissolved in certain amounts of distilled water in order to obtain the desired concentrations for the study (11).

Evaluation of the Anti-inflammatory Effects of Clove Extract: In this study, the xylene test was used to evaluate the anti-inflammatory effects of the clove extract. For this test, the male mice (N=48) were randomly divided into the following groups of 8:

1) Control group (receiving intraperitoneal saline at dose of 10 ml/kg of body weight);

2) Positive control group (receiving 15 mg/kg of dexamethasone dissolved in saline intraperitoneally);

3) Experimental groups (receiving 42, 85, 170 and 340 mg/kg clove extract via intraperitoneal injection in single doses). About 15 minutes after the

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intraperitoneal injections, 0.03 ml of xylene was injected into the anterior and posterior lobe of the right ear of the animals in order to cause ear inflammation. After two hours, the animals were killed, and both ears were removed to prepare 7-mm sections from the left and right ears.

The obtained sections were weighed, and the differences between the right and left ears were calculated. Weight differences were indicative of the level of inflammation; in case there was a significant difference between the weights of two ears, the inflammation was considered as severe (2).

Evaluation of the Analgesic Effects of Clove Extract: To evaluate the analgesic effects of clove extract, the hot plate test was performed on 160 mice. The animals were randomly divided into 4 categories of 40 mice, and each category was divided into 5 subgroups of 8. Each of the five subgroups in each category consisted of one control group (receiving 10 ml/kg normal saline), one positive control group (receiving 2.5 mg/kg of morphine) and two treatment groups (receiving 200 and 500 mg/kg of clove extract).

In order to monitor the possible pain-relieving mechanism of the herbal extract in the fifth subgroup, 4 mg/kg of naloxone (an antagonist and selective blocker of opioid receptors) was injected subcutaneously into the back of the animals, and after 5 minutes, the rats received the clove extract at dose of 200 mg/kg via intraperitoneal injections (12). The delay in the pain response of the animals was measured in each category at 5, 15, 30 and 60 minutes after the injections (12).

For the hot plate test, the temperature of the hot plate was set at 52.5°C. The point when the animal was placed on the plate was marked as the starting point of the test, and the end of the test was the point when the animal began to lick its hands (i.e. pain evaluation criterion in this test). In case of lack of pain response, the experiment would be terminated after 25 seconds and the animal was removed from the hot plate (13).

In this study, the animals were tested at minimum time intervals, and at the end of each part of the experiments, the mice were excluded from the study in compliance with ethical considerations. Data analysis was performed using post-hoc ANOVA and Tukey's test, and P□0.05 was considered as significant.

Results

In the evaluation of inflammation, comparison of the xylene test results between the control subjects and other groups indicated that except for the recipients of 42 mg/kg clove extract, other groups had a significant difference with the control subjects in the weight of the right and left ear sections ($p<0.05$). In addition, comparison of the positive control subjects with other groups showed that except for the recipients of 170 and 340 mg/kg of the extract, other groups had a significant different with the positive control subjects ($p<0.05$).

On the other hand, comparison of the treatment group receiving 42 mg/kg of clove extract with other subjects was indicative of no significant difference with the control group, while a significant difference was observed between the recipients of 42 mg/kg of the extract with the other groups ($p<0.05$).

Furthermore, no significant difference was observed between the recipients of 85 mg/kg and 170 mg/kg of the clove extract. However, there was a statistically significant difference between the groups receiving extract doses of 85 mg/kg with the other groups ($p<0.05$).

These comparisons suggested that with the exception of the positive control subject and recipients of the extract at doses of 85 and 340 mg/kg, the results of other study groups had a significant difference with the treatment group receiving 170 mg/kg of the clove extract ($p<0.05$).

In addition, no statistically significant difference was observed between the results of the treatment group receiving 340 mg/kg of the extract with the positive control subjects, as well as the recipients of 170 mg/kg of the clove extract. However, there was a significant difference between the recipients of 340 mg/kg of the extract with the other study groups ($p<0.05$) (table & fig 1).

Table 1. Differences in the Weight of Right and Left Ear Sections of the Mice (Micrograms) based on the Results of the Xylene Test (N=48)

Group	Mean±SD
Control	36±5.1
Positive control	18±2.8
Extract(42mg/kg)	33±5.3
Extract(85mg/kg)	26±2.2
Extract(180mg/kg)	21±2.1
Extract(340mg/kg)	17±1.5

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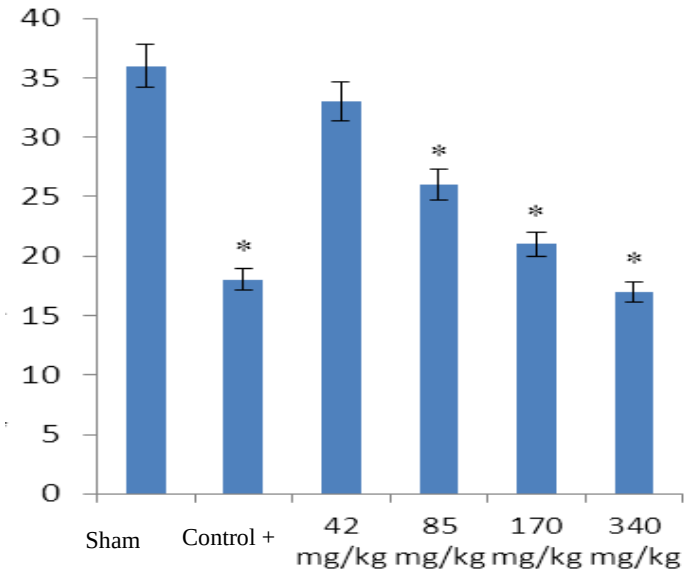


Figure 1. Evaluation of Differences in the Weight of Right and Left Ear Sections in the Study Groups (Micrograms)

*Significance difference with the control group (p<0.05)

The evaluation of pain response using the hot plate test in the first (test time: 5 minutes after intervention), second (test time: 15 minutes after injection) and third categories (test time: 30 minutes after injection) was indicative of a significant increase in the time of unresponsiveness to pain in the recipients of 200 and 500 mg/kg extracts and the positive control group (morphine), compared to the control group and subjects receiving 200 mg/kg of combined clove extract and naloxone (P<0.05).

Moreover, a significant increase was observed in the pain threshold of the recipients of morphine (positive control) compared to the groups receiving doses of 200 and 500 mg/kg of the clove extract (P<0.05). According to the comparison between the groups receiving 200 and 500 mg/kg of clove extract, the duration of unresponsiveness was higher in the animals receiving 500 mg/kg of the herbal extract (P<0.05).

On the other hand, no significant difference was observed between the control group and recipients of combined naloxone and 200 mg/kg extract in terms of the pain threshold and pain tolerance.

In addition, no significant increase was observed in the pain threshold between the fourth category (test time: 60 minutes after injection), recipients of 500 mg/kg of clove extract, and the group receiving 200 mg/kg of the clove extract.

According to the further results of this study, no significant increase was observed in the group receiving 200 mg/kg of the clove extract compared to the recipients of combined naloxone and 200 mg/kg extract. The results of the statistical analysis obtained from the groups in the fourth category were similar to those of the first three categories of animals (table & fig 2).

Table 2. Distribution of Pain Thresholds at 5, 15, 30 and 60 Minutes after the Intervention in the Four Categories (Seconds) using the Hot Plate Test (n=160)

Group	First Category (test time: 5 minutes after intervention) Mean±SD	Second Category (test time: 15 minutes after intervention) Mean±SD	Third Category (test time: 30 minutes after intervention) Mean±SD	Fourth Category (test time: 60 minutes after intervention) Mean±SD
Control	20.51±1.8	26.48±1.8	06.60±1.8	32.70±1.9
Positive control	74.22±1.21	50.75±1.23	65.82±1.19	79.73±1.18
Extract mg/kg200	79.81±0.12	61.25±0.15	65.80±0.12	81.20±0.12
Extract mg/kg500	44.95±1.17	53.91±1.18	35.61±1.15	87.88±0.13
Combined noloxone mg/kg200 and extract	04.71±1.8	38.61±1.9	13.76±1.9	68.52±0.11

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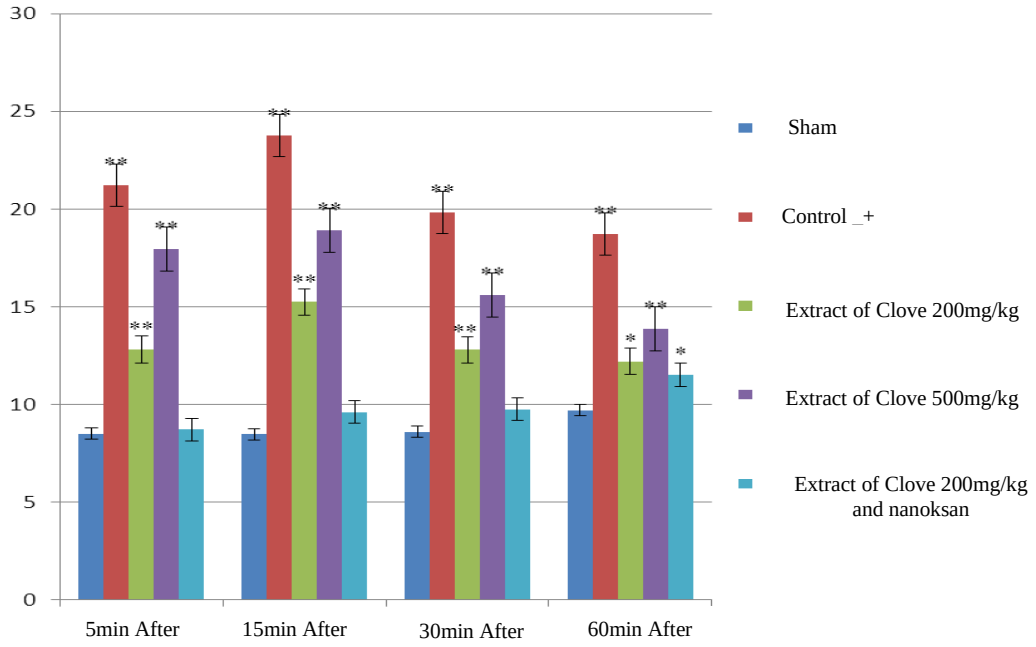


Figure 2. Comparison of Pain Thresholds in Five Subgroups of Different Categories (Seconds) using the Hot Plate Test

*Significance level at p<0.05 with the control group

**Significance level at p<0.01 with the control group

Discussion

Evaluation of the anti-inflammatory effects of clove extract using the xylene test indicated that treatment with this herbal extract (except at dose of 42 mg/kg) could cause a significant reduction in the weight of the ear sections, in a dose-dependent manner, compared to the control group. This is suggestive of the effectiveness of hydro-alcoholic clove extract in the prevention of the inflammation induced by xylene.

According to the results of the hot plate test, hydro-alcoholic clove extract cloud cause a statistically significant increase in the pain threshold in a dose-dependent manner; however, no significant increase was observed in the pain thresholds of the subjects receiving combined naloxone and 200 mg/kg extract (except for the fourth category tested 60 minutes after injection).

The findings of the present study could be explained through the assessment of the main components found in clove. This herb contains 14-21% essence, containing 70-80% flavonoids (e.g. eugenol), which are proven to have numerous antioxidant properties. Neutralization of free radicals is considered as the main function of antioxidants.

Other compounds inherent to the essential oil of clove include caryophyllene, alcohol, benzylic carbon, dimethyl-benzoate, forfovoul and ethylene (1). Flavonoids are natural compounds found in plants with remarkable analgesic and anti-inflammatory properties. Moreover, flavonoids could inhibit nitric oxide (NO) synthesis and by reducing the production of this molecule, they perform analgesic activities. On the other hand, flavonoids could decrease the levels of intracellular calcium through the inhibition of N-methyl-D-aspartate receptors. This process is followed by reduced enzymatic activity of NO synthesizers, as well as the calcium-dependent phospholipase A2; consequently, analgesic functions occur following the reduction in the levels of NO and prostaglandins.

By the inhibition of cyclooxygenase enzyme activity, flavonoids are able to suppress the production of prostaglandin E from arachidonic acid in response to inflammatory stimuli (2).

In one study conducted on inflammation and the effects of medicinal plants on this condition, flaxseed extract was shown to have significant anti-inflammatory functions at different doses (100, 200 and 400 mg/kg). Such effects are associated with the presence of active components in the extract of

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flaxseed, including phenylpropanoids, mucilage and flavonoids (14). In another study in this regard, the analgesic and anti-inflammatory functions of this herbal extract were observed to be significantly higher in the treatment groups compared to the control group; these effects were more noticeable at the higher doses of the flaxseed extract.

On the other hand, significant anti-inflammatory and analgesic properties have been reported in the treatment with the herbal extract of hypericum perforatum, which are mainly associated with the presence of antioxidant agents, such as flavonoids and iridoids, in this medicinal herb (15). Therefore, it could be concluded that flavonoids play a pivotal role in the analgesic performance of the aforementioned medicinal plants, which is a finding confirmed by the results of the current study.

Several studies have been conducted on the evaluation of the analgesic properties of different medicinal plants, some of which are in line with the results of the present study on the significant analgesic functions of clove extract.

In a study by Shirani et al., the analgesic effects of clove extract were observed to be dose-dependent, and the increase in the pain threshold was reported to be more significant in the treatment groups receiving higher doses of the extract; this finding is compatible with the results of the current study. Moreover, to investigate the mechanism of the analgesic function of this herbal extract, one of the treatment groups was administered with combined naloxone and clove extract, which was observed to have more significant pain-relieving effects on the subjects of this group compared to those receiving the extract alone (10). Given the key role of naloxone in preventing the activation of opioid receptors, it seems that the reduction in the pain threshold could be due to the presence of various active components influencing opioid receptors.

In another study, the analgesic effects of eugenol were evaluated using the formalin test in rats, and the results were indicative of the dose-dependent, anti-pain function of this compound (3). Accordingly, the does-dependent analgesic function of clove extract could be due to the significant proportion of eugenol in this plant (1, 16-19).

Pain and inflammation are considered as the major causes of oxidative stress, and flavonoids could prevent these conditions through implementing antioxidant activities (20-22). Therefore, the analgesic and anti-inflammatory effects observed in the extract of flaxseed might also be associated with antioxidant agents found in this herb. Assuming that phenolic and antioxidant compounds are the main proxies of analgesic activity, several other plants that contain these compounds may also perform such functions (23-25); thus, further research is required as to obtain more knowledge on the pain-relieving effects of different plants.

In conclusion, the results of the present study indicated that the hydro-alcoholic extract of clove could have significant analgesic and anti-inflammatory effects and may be an appropriate alternative for synthetic, anti-pain drugs. Furthermore, it seems that flavonoids are the main herbal components to perform antioxidant functions relieving pain and inflammation. Therefore, it is recommended that future studies be conducted specifically on the effective constituents of clove in the management of different medical conditions.

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