Original Article:

Antinociceptive and Anti-inflammatory effects of Hydroalcoholic extract of aerial parts of *Thymus carmanicus* in male mice

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ABSTRACT

Different species of *Thymus* are capable of producing various pharmacological effects. The present paper aims to investigate the analgesic and anti-inflammatory effects of hydro-alcoholic extract of aerial parts of *Thymus Carmanicus Jalas*. In this experimental research, using the formalin, writhing and tail immersion tests, anti-inflammatory effects were studied in mice by xylene-induced ear edema and mercury immersion methods. Male NMRI mice weighing 20-25g were divided into five groups: negative control, positive control and three experimental groups, being injected intraperitoneally by 250, 500 and 1000 mg/kg of hydroalcoholic extracts of *Thymus carmanicus*. The most effective dose of the extract was selected for the possible involvement of opioidergic systems. Animals were studied by pretreatment with opioid antagonist, naloxone. The data were analyzed through ANOVA and Tukey’s test. Results indicated that in the formalin test, the doses of 500 and 1000 mg/kg of the extract significantly reduced the score of analgesic in experimental groups (P<0.001). Meanwhile, pretreatment with naloxone inhibited some of the extract induced antinociceptive effects in comparison to control group. In writhing and hot tail immersion tests, all doses of the extract significantly alleviated pain in experimental groups (P<0.01). Moreover, the xylene-induced ear edema (P<0.001) as well as foot edema in mercury immersion method was considerably reduced (P<0.05). This study not only showed that the hydroalcoholic extract of aerial parts of *Thymus carmanicus* has analgesic and anti-inflammatory effects but also indicated that some of the antinociceptive properties of *Thymus carmanicus* are mediated by opioidergic mechanism, which in turn validated the traditional uses of the plant in the treatment of pain.

Key Words: Analgesic; Anti-inflammatory; Hydro-alcoholic extract; Naloxone; *Thymus carmanicus*; Male mice

INTRODUCTION

Pain is usually the result of destruction of a tissue or damage to a tissue caused by chemical, thermal, mechanical or electrical stimulus[1] and its remedy, particularly chronic pain caused by unrelieved damages, has always been an important issue. All over the world, plants are huge resources with widespread biological features, such as analgesic, anti-inflammatory, anti-diabetic, anticancer, anti-oxidant and antibacterial effects for human beings. These properties have been proved by modern analysis techniques[2] and are related to secondary metabolites such as polyphenols, flavonoid, alkaloids, terpenoids, coumarin, curcumin, etc. In addition, control and treatment of the pain have yet remained among problematic issues in the pharmaceutical industry. Most of the analgesic treatments are related to two primary groups of opioids and nonsteroidal anti-inflammatory drugs. Both groups have many side effects, including digestive problems, renal lesions, respiratory failure and drug addiction; hence, designing analgesic drugs with the least side effects is highly desirable. One of the methods to achieve the aforementioned goals is to use herbal medicine which is a rich resource of effective compounds. Plants are introduced as the main source of effective organic compounds owing to their diversity and variety[3]. Reliable evidence of traditional medicine which has been practiced for a long time and has robust scientific background
in usage of herbalism in treatment can be a valuable base for finding more effective medications[4].
Among the potential candidate plants is Thymus carmanicus. In this research, the analgesic and anti-inflammatory effects of the extract of areal parts of Thymus carmanicus was analyzed in the hope of using this plant as a proper substitute for narcotic medication.

MATERIALS AND METHODS

Collection, Recognition, and Preparation of the Extract

The plant was found in Karkas Mountain (Esfahan province, May 2012) and then was recognized in the faculty of pharmacology of Shahid Beheshti University (by Mohammad Kamalinezhad). The extract of areal part of Thymus carmanicus was prepared through maceration method and then was dried in shade in 25°C after being soaked in ethanol. The obtained solution was dried in Ben Murray[5].

Laboratory Animal

In this research, male mice from NMRI weighting among 20-25 gr. were used and kept in laboratory condition of 23 ± 2°C and 12/12 light cycle [6]. Usage of animals was in accordance with the ethical committee of the university.

METHODS

Formalin test

To run this test, five groups, each composed of eight mice were used. Fifteen minutes prior to the commencement of the test, dosages of 250, 500 and 1000 mg/kg of the extract were intraperitoneally injected in each group. In control group, normal saline and in positive control, morphine was injected. Subsequently, 0.02 ml of %2.5 formalin was injected under the skin of the right paw of the animal. It was then returned to the test box. The average of licking time during 0-5 minutes was taken as the first phase of pain [7] and the average of 15-30 minutes as the second phase or peripheral phase of formalin test [6].

Tail Immersion test

In tail immersion test, fifteen minutes after the injection of the extract or morphine, each mouse is kept in restrainer to acclimatize. Then, the tail of each mouse is immersed in 49°C water in periods of 0, 2, 4 and 6 minutes once every 2 minutes over a period of 6 minutes and then the time in which the animal subject withdrew its tail is measured [8].

Xylene test

It is used to measure edema. Following the injection of the specified dosage of extract, 0.02 ml of xylene was injected in anterior and posterior of the right ear. After two hours the animals were killed and seven mm slices of both ears were weighted [9].

Writhing test

One hour after giving a specific dosage of extract, an intraperitoneally injection of acetic acid, 0.7 percent (0.1 ml/10 g injection volume) is received, and the number of writhing twisted by these animals was counted for 30 min (a writhing is indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb) [10].

Foot edema test

15 minutes ahead of the start of the test, a specific dosage of the extract is injected: Normal saline for the control group and dexamethasone for the positive control. Subsequently, 0.02 ml formalin %2.5 is injected in the right foot sole of the animal. The animal’s foot is immersed in the mercury at 5, 10, 20 and 30 minutes after the injection to specify its volume. The inflamed foot is immersed up to a known point (marked previously) before and after inducing edema.

Using the equation \( V = \frac{M}{q} \), the changes in volume can easily be found [11].

Analysis of the Antagonist Effect of Opioid Receptors on Antinociceptive Effect Caused by Extract

To study the effect of opioid receptors on antinociceptive caused by extract, 2 mg/kg of naloxone is injected 15 minutes before the injection of the most effective dosage of the extract, and then formalin test is carried out and analyzed [12].

Statistical methods used

Data analysis was performed by SPSS version 17. Subsequent to the data validation, one-way variance analysis and then Turkey’s test were carried out. To analyze the results, the significance level of \( P < 0.05 \) was used as a borderline for statistical inference and the histogram was created by EXCEL.

RESULTS

Formalin Test Result

The test revealed that in comparison to the
control group who had received normal saline in acute phase (0-5), dosages of 500, 1000 mg/kg of hydroalcoholic extract of aerial parts of *Thymus carmanicus* have more significant effect on reducing pain.

Furthermore, all dosages of this extract have important impact upon second phase of pain (20-40min). Findings show that in the experimental groups, average pain has inverse relationship with increase in dosage. The most effective dose is 1000 mg/kg and in comparison with the control group, who had received normal saline, showed the highest score in the score of analgesic (*P* < 0.001). Moreover, in the second phase of pain, the average score of analgesic among positive control groups, the one that received a dosage of 1000 mg/kg, indicated a significant difference with the group that received a dosage of 500 mg/kg (Chart1,2).

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**Results of Antagonist Effect of Opioid Receptors on Analgesic Caused by Extract of Aerial Parts of Thymus Carmanicus Formalin Test**

In this method, one group received naloxone and the second received naloxone as, together with the most effective dosage of the extract. Results demonstrated that the naloxone significantly reduces and prevents the analgesic effects of the extract in acute phase (*P*<0.05) in both naloxone and extract groups. Statistically, there is a clear contrast between the group which solely received the extract and the group which received both the extract and the naloxone. In acute phase, there was no noteworthy difference between the group receiving the extract and naloxone with the control groups, but the difference with the group receiving the extract alone was noticeable. Moreover, it was observed that pretreatment with naloxone did not have significant impact on reduction of analgesic effect of the extract in second phase of pain (Chart1,2).

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**Figure 1. Acute phase in formalin test**

A: In comparison to the sham group (*P*<0.001)

b: In comparison to the sham group is significant (*P*<0.01)

c: In comparison to the positive control group is significant (*P*<0.001)

d: In comparison to the Naloxone group is significant (*P*<0.05)

e: In comparison to the Naloxone group + Extract 1000 is insignificant (*P*<0.05)
**Results Obtained from the Effect of Extract on Edema in Xylene Test**

Results indicate that the extract in all mentioned dosages has significant effect on the reduction of edema in comparison to control groups. Moreover, edema volume decreases with the increase of dosage. Eventually, the average of edema volume between positive control group and the group treated with different dosages of the extract did not indicate major difference (P>0.05) (Chart3).

**Figure 2. chronic phase in formalin test**

a: In comparison to the sham group is significant (P<0.001)
b: In comparison to the control group is significant (P<0.001)
c: In comparison to the Naloxone group is significant (P<0.001)
d: In comparison to the Naloxone group is significant (P<0.001)
e: In comparison to the Naloxone and Extract group is significant (P<0.001)

**Figure 3. Xylene test**

a: In comparison to the sham group is significant (P<0.001)
b: In comparison to the sham group is significant (P<0.01)
c: In comparison to the sham group is significant (P<0.05)
d: In comparison to the Receiving dexamethasone group is significant (P<0.01)

Results Obtained from the Analgesic Effects of Extract in Tail Immersion Test
Observing the obtained results, dramatic difference between 1000 mg/kg dosage group and the sham group is noticed. This group shows that the average response time to pain is increased (P < 0.01).
(Chart4).

Figure 4. Tail immersion test
a: In comparison to the sham group is significant (P<0.001)
b: In comparison to the sham group is significant (P<0.01)
c: In comparison to the sham group is significant (P<0.05)
d: In comparison to the control group is significant (P<0.001)
e: In comparison to the control is significant (P<0.01)
f: In comparison to the control is significant (P<0.05)

Result Obtained from the Effect of Hydroalcoholic Extract of Aerial Parts of Thymus Carmanicus on Pain in Acetic Acid
All of doses reduce the number of writhing during the registered time (1 hour); in this regard, there was a marked difference between doses and sham group. The most effective doses were 500 and 1000 mg/kg (P < 0.01). Furthermore, there was only a subtle difference between the groups with administered doses and the group having received morphine (Chart5).
Figure 5. Acetic acid test
a: In comparison to the sham group is significant (P<0.01)
b: In comparison to the sham group is significant (P<0.05)
c: In comparison to the control group is significant (P<0.01)

Result Obtained from the Effect of Hydroalcoholic Extract of Aerial Parts of Thymus Carmanicus on Pain in Foot Edema Test

Using plethysmometer method revealed that the injection of Thymus carmanicus in 250 and 1000 dosages has significantly reduced the edema volume in animal foot in comparison to negative control group (P < 0.05)(Chart6).

Figure 6. Foot edema test
a: In comparison to the sham group is significant (P<0.05)
DISCUSSION

Based on the findings of this research, the extract of this plant reduces pain in acute and chronic stages of formalin test. It appears that the chronic pain is due to the direct stimulation of pain receptors and activities of type C nerve fibers [13]. While in acute pain, microglia cells activate the release of Katapsyn S, and subsequently, FKN solution (sFKN) is released from neurons. SFKN produces an interactive effect by CX3CR1 receptors on microglia, and then MAPK P38 path is activated which in turn leads to the release inflammatory mediators. Eventually, the edema mediators bind with their receptors on neurons. These subsequent processes cause pain[14]. Chronic pain is eliminated by anti-inflammatory medications [15]. It should be mentioned that the first step of pain, corresponding to the neurogenic and acute pain is sensitive to medications acting through opioidergic system. The second step of pain is the inflammatory pain which is eliminated by both opioid and anti-inflammatory non-steroid drugs [16]. Drugs that have their primary mechanism of action inside the CNS (opioids) have eliminating effect on both steps of formalin test while the peripherally selective medications act on the second step[17]. The results of this research indicate that the hydroalcoholic extract of aerial parts of Thymus carmanicus not only reduces pain in acute phase, but also affects the chronic phase of pain. Therefore, the central analgesic effects can be proposed for these extracts. Among the compounds available in Lamiaceae family are flavonoids which are analgesic and anti-inflammatory compounds in plants, and their direct effects on prostaglandins synthesis are certainly specified [18]. Flavonoids inhibit nitric oxide synthase, the compound that increases after formalin injection; therefore, its reduction leads to pain relief [19]. Previous studies indicate that flavonoids limit the activity of N-methyl-D-aspartate receptors resulting in the reduction of intercellular calcium, and subsequent to this reduction, the activity of the nitric oxide synthase calcium-dependent phospholipase A reduces, and thus flavonoids show their analgesic effect by reduction of NO and prostaglandins[20,21]. Based on the available evidence, flavonoids limit cyclooxygenase enzyme as well as the production of prostaglandin E from arachidonic acid, in response to edema stimulus[22] and thus prevents the pain receptors activation produced by these molecules to become sensitive which in turn reduces pain feeling that accompanies these responses. Regarding the fact that edema is known as a peripheral process causing pain in the second phase of formalin test, the anti-inflammatory effect of flavonoids is produced by checking the production of inflammatory cytokines, such as tumor necrosis factor (TNF) from activated macrophages. These pre-inflammatory substances in inflammation increase the prostaglandin synthesis. Therefore, It is likely that some parts of analgesic effect of this extract might be the result of their anti-inflammatory effect [23]. Furthermore, flavonoids are capable of reducing the activity of complement system which in turn ends in breaking the connections of inflammatory driving cells and covering tissue of damaged area (or the area injected with formalin). These processes are subsequent to the reduction of intensity of inflammatory and eventually less pain is experienced [24]. Thymol and Carvacrol are primary components of the essential oils of the plants in Lamiaceae family[25]. Thymus carmanicus is among the plants with highest volume of Pulegone. Main constituents forming the essential oils of this extract were recognized in Thymus carmanicus as Thymol 88%, and Carvacrol 14/2[26]. Carvacrol is widely used as antiseptic and antibacterial agent in different medications[27]. It significantly decreases licking time in first and second phases of formalin test [28]. Studies have shown that monoterpene has dedicated receptors in cell membrane [29] leading to the decline of intracellular flow in rest time and increase in cell stimulation threshold[30]. According to these reports, the extract of Thymus carmanicus affects calcium channels in neurons membrane (particularly nociceptive neurons) and decreases intracellular calcium flow through which irritability and the amount of synapse transformation decrease results in pain reduction eventually. Moreover, it is reported that monoterpene affects opioid receptors in a way that it interrupts the transfer of pain signals [31]. Another component in extract of Thymus carmanicus is Pulegone[26]. It is one of the circular oxygenated Monoterpene and pleasant aroma. It has anti-inflammatory effect. Moreover, its anti-histaminic activity has been observed in hamsters. Observations revealed
that Pulegone is receptor antagonist with similar effects to Dextchloropheniramine[32].
Naloxone as the general antagonist of opioid receptors is capable of eliminating the analgesic caused by these receptors. This medication has a high tendency to compound with μ receptors and by blocking these receptors, it prevents their antagonism mechanism. A case in point is morphine[33] and probably extract of Thymus carmanicus. This conclusion is drawn regarding the harness of the analgesic effect of the extract in acute phase by naloxone. Moreover, it is anticipated that the extract of Thymus carmanicus induces its analgesic effect through opioid receptors. With regards to the chemical compounds in Thymus carmanicus plant, pulegone, 1-8 cineole, thymol, menthol and carvacrol can be mentioned. Eventually, the findings of the present study clearly indicate that the hydroalcoholic extract of Thymus carmanicus, considering its constituents including especially pulegone, has analgesic and anti-inflammatory effects.

ACKNOWLEDGMENT
The authors would like to thank Mr. Mohammam Kamalinezhad for his valuable assistance in this research.

“The authors declare no conflict of interest”

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