Original Article

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An Investigation of the Antinociceptive and Anti-inflammatory Effects of Hydroalcoholic Extract of Inula Helenium on Male Rats

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ABSTRACT

BACKGROUND AND OBJECTIVE: Inula helenium is a medicinal plant with proven anti-cancer, anti-microbial and anti-fungal effects. The aim of this study is to investigate the antinociceptive and anti-inflammatory effects of hydroalcoholic extract of Inula helenium leaf on male rats.

METHODS: 66 male rats were used in this experimental study. The animals were divided into 6 groups (each group consisted of 6 rats) for pain assessment tests: control group, groups treated with the extract (50, 100 and 300 mg/kg), morphine and naloxone in combination with 300 mg/kg extract. Furthermore, they were divided into 5 groups (each group consisted of 6 rats) for anti-inflammatory tests: control group, groups treated with the extract (10, 50 and 100 mg/kg) and dexamethasone. Tail-flick, rating and formalin tests were used to assess pain and xylene test was used to assess inflammation.

FINDINGS: According to the results of rating (28.21±1.34) and tail-flick (5.11±1.34) tests, using 300 mg/kg extract had significant antinociceptive effect ($p<0.01$) compared with control group (41.22±4.12). According to the formalin test, using 100 mg/kg extract decreased pain rating from 2.17±0.21 in control group to 0.53±0.24, in the chronic phase ($p<0.05$). According to the xylene test, using 50 and 100 mg/kg extract decreased the inflammation of the ear in rats (4.1±2 and 3.3±1, respectively) compared with control group (0.4±7.6) ($p<0.001$ and $p<0.01$).

CONCLUSION: Results of this study showed that hydroalcoholic extract of Inula helenium leaf may benefit from antinociceptive and anti-inflammatory effects.

KEY WORDS: *Inflammation, Pain, Inula helenium, Medicinal plant.*

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Introduction

Pain is one of the fundamental issues is modern societies and although pain is an alert for tissue damages, it is an unpleasant feeling that obliges human to search for solutions to stop it through various treatment methods (1). Nowadays, nonsteroidal anti-inflammatory drugs or opioid drugs are mostly used to control pain. However, these drugs have several side effects and are accompanied by disorders in digestive system, kidney impairment or dependency, forcing human to look for alternative newer drugs that are inexpensive and accessible with fewer side effects (2). Inflammation is one of the common complications of different diseases which weakens the body's immune system, causes infection and delays recovery from diseases. Inflammatory processes are heavily dependent on pain and the chemical materials released during the process of inflammation can stimulate pain receptors and lead to inflammatory pain (3).

Medicinal plants are important sources of new chemical materials with very powerful therapeutic effects (4). Using medicinal plants to treat pain and inflammation is some sort of custom or habit in Persian medicine, though the source and principle of these plants' activity are yet unknown. However, assessment of these plants can be a logical research strategy to find new medications (5,6). The genus Inula is a perennial plant that spreads from Asia and Europe to Africa and particularly Mediterranean and South Asia. This genus is a member of Asteraceae family (7). Many plants belonging to this genus benefit from therapeutic characteristics, among which Inula helenium is one of the most important plants and the most widely used medicinal plant (8).

Inula helenium is a beautiful perennial plant with an average height of 2 meters and large glandular rhizome. It has thick hairy stem that branches in the upper part. The leaves of this plant are oval-shaped and have tiny teeth on the edge. In Iran, this plant mainly grows in surrounding areas of Tehran, Mazandaran beaches, Arak, Heydarieh and Hamedan. It is called "Rasen" in Arabic. A study by Dorn et al. demonstrated that Inula helenium extract has high selective toxicity against cell lines (HT-29, MCF-7, Capan-2 and G1) (9). Some of the unique pharmacological effects of this plant have been mentioned in Persian medicine, among them we can mention: Anti-bacterial, expectorant, tussive, sweat-stimulating, appetizing and anti-inflammatory properties (10, 11). In recent years, the antinociceptive and anti-inflammatory effects of various medicinal plants such as Tribulus terrestris (12), Pimpinella anisum (13) and Bryonia dioica (14) have been proven using tail flick, rating and formalin standard tests. Moreover, a study by Arumugam et al. demonstrated that the aqueous extract of Inula racemose, which is a member of Asteraceae family, has antinociceptive and anti-inflammatory effects (15). Considering the fact that common analgesic compounds such as morphine are derived from medicinal plants (16) and considering the claim of Persian medicine regarding anti-inflammatory effects of this plant and the close relationship between inflammatory mechanisms and pain, the antinociceptive and anti-inflammatory effects of hydroalcoholic extract of Inula helenium leaf on male rats are investigated in this study using authentic tests of xylene, rating, formalin and tail flick.

Methods

Preparation of extracts: In this experimental study, 4 kg fresh leaves of Inula helenium were gathered in July 2016 and were confirmed by a botanist in Bu-Ali Sina University in Hamedan. After isolating petioles, Inula helenium leaves were dried at room temperature (25ºC) and in the shadow. They were then transformed into dry powder using a mechanical mill. 200 g dried leaf powder was immersed in l liter methanol 80% solution for 72 hours to extract the required active ingredients. The resulting mixture was placed in rotary device after smoothening and in order to dry it, the solvent was separated and was kept under the hood in a petri dish for another week. After one week, what was left at the bottom of the container (the extract) was dissolved in appropriate amount of physiological saline (sodium chloride 9%) at various dosages and was used for treatment of male rats.

Animals: 66 male Wistar rats (220-250 g) were prepared from Pasteur Institute of Iran and were kept under standard conditions with 12/12 light cycle (the light cycle started from 7 A.M.) at 22±1 ºC. The animals were kept in metal cages with free access to special food and water. The experiments were conducted according to the ethical guidelines of International Association for the Study of Pain for animals (17). The animals were divided into 6 groups (each group consisted of 6 rats) for pain assessment tests: control group (affected by normal saline), the group treated with morphine (1 mg/kg), groups treated with the extract at low, medium and high dosages of

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Inula helenium (50, 100 and 300 mg/kg) and the group treated with naloxone (1 mg/kg) combined with high dosage of the extract (300 mg/kg). In anti-inflammatory test of xylene, rats were divided into 5 groups of 6: control group, groups treated with the extract (10, 50 and 100 mg/kg) and dexamethasone.

Inflammation test: In this test, the animals were divided into 5 groups of 6. The animals in control group received normal saline. The positive control group received 15 mg/kg body weight dexamethasone. The extract-receiving groups received any dosage of extract (10, 50 and 100 mg/kg) intraperitoneally 15 minutes before test. After inflammation test, they were administered with xylene in their ears. If animals died two hours after xylene administration, both ears were removed and 7 mm slices of left and right ear were prepared using a cork borer. Slices were then weighed and the difference between the weight of left and right ear was specified. This difference in weight shows the level of inflammation and the more different the weights are, the more inflammation is expected (18).

Pain tests

Rating test: To help animals adapt to the environment on the day of experiment, each of them was placed in the standard glass box before the experiment for 30 minutes. First, 50, 100 and 300 mg/kg hydroalcoholic extract of Inula helenium leaf dissolved in sterile physiological serum was injected intraperitoneally. After 15 minutes, 0.1 ml/kg body weight acetic acid 1% was injected and 5 minutes after intraperitoneal injection of acetic acid, the number of abdominal contractions was counted (19). In control group, the test was run after intraperitoneal injection of normal saline.

Tail flick test: This experiment was conducted using a tail-flick device (TF-5380, Borj Sanat Co., Iran). The test was carried out based on the previously proposed model (20). The applied light intensity was determined to be seven and the cut-off time was estimated at 10 sec. That is, in case the animal did not flick its tail after 10 seconds of exposure to burning light, the light was cut to prevent stimulating tissue damage. The animal was placed horizontally in the special animal cage, while the tail was free. The latency time in flicking the tail was measured three times with 2-minute intervals before injection of drug or extract and the average value was calculated and recorded as latency time. Then, 20 minutes after drug injection, another series of test were carried out 3 times and the average value was recorded as the latency time after drug injection. Morphine was injected intraperitoneally to animal and the tail flick time was recorded.

Formalin test: A special box made of Plexiglas (30×30×30 dimension) was used for this test and a mirror a 45-degree angle was placed beneath it, in front of the observer, to observe animal movements. 30 minutes after intraperitoneal injection of drugs, 50 µl formaldehyde 2.5% was injected to the animal's right paw subcutaneously. The animals were returned to the special boxes and their behaviors were studied and scored for 60 minutes. The motor response to pain was recorded every 15 seconds as numbers 0, 1, 2, and 3: Number 0: the animal moved with complete balance and its weight was equally distributed on both feet; Number 1: the animal could not tolerate its body weight on the injected foot or tried to take care of that foot; Number 2: the animal raised the painful paw and had no contact with the box floor; and Number 3: the animal licked, chewed or severely shook the painful paw (21).

Drugs: Morphine sulfate and naloxone were prepared from Darou Pakhsh Co. (Iran) and acetic acid, formalin and xylene were prepared from Merck Co. (Germany).

Statistical analysis: The analyzed data were presented as mean±SEM and one-way analysis of variance.

Results

Xylene test: Results of the study demonstrated that 50 and 100 mg/kg Inula helenium extract significantly decreased inflammation ($p<0.01$, $p<0.001$) by 4.1±2 and 3.3±1, respectively, compared with control group (0.4±7.6). 100 mg/kg extract with inhibition percentage of 49.7% could decrease inflammation almost like dexamethasone compared with control group ($p<0.01$) (table 1).

Rating test: Results of the study based on rating test demonstrated that injection of 100 mg/kg extract (32.41±1.51) decreased the number of rating (abdominal contractions of rat) compared with control group (41.22±4.12) ($p<0.05$). Moreover, use of 300 mg/kg extract (28.21±1.34) decreased the number of rating compared with control group (41.22±4.12) ($p<0.01$).

This laboratory model specified that using naloxone along with high dosage of extract reverses the antinociceptive effects of the extract when used alone. Moreover, using morphine decreased the number of abdominal contractions compared with control group ($p<0.001$). On the other hand, using 300

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mg/kg extract demonstrated a significant difference ($p<0.01$) compared with morphine group (Fig 1).

Table 1. The effect of intraperitoneal injection of hydroalcoholic extract of Inula helenium on xylene-induced inflammation in rats

Groups	Inflammation of rat ear	Inhibition percentage
Control (10 mg/kg)	7.6±0.4	
Low dosage of extract (10 mg/kg)	6.4±0.1	15.9
Medium dosage of extract (50 mg/kg)	* 4.1±0.8	34.2
High dosage of extract (100 mg/kg)	** 3.3±1	49.7
Dexamethasone (15 mg/kg)	*** 3.2±0.3	56.2

p<0.05,**p<0.01,p<0.001 significant difference with control group.**

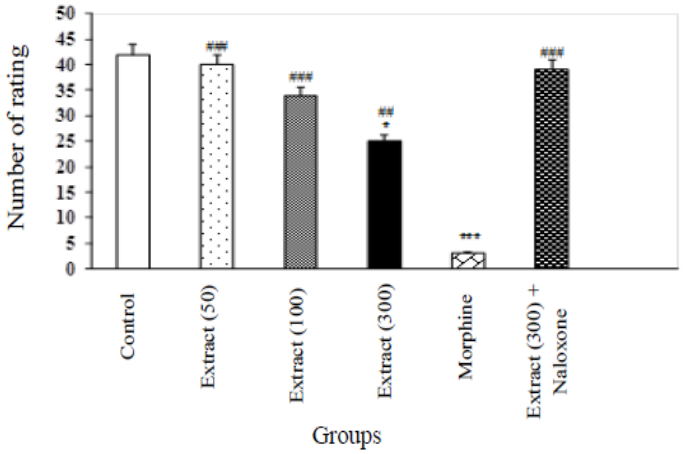

Figure 1. A comparison of mean number of rating in rats at different concentrations of Inula helenium extract in acetic acid test

***p<0.05: significant difference with control group. ## p<0.01, ### p<0.05: significant difference with morphine group.**

Tail flick test: According to tail flick test, using 300 mg/kg extract revealed a significant antinociceptive effect compared with control group ($p<0.05$) and increased the latency time of tail flick from 2.21±1.18 seconds to 5.11±1.34 seconds. However, using 50 and 100 mg/kg in this test did not show a significant antinociceptive effect compared with control group. This test also demonstrated that co-administration of naloxone and high dosage of extract reverses the antinociceptive effects of the extract. Using morphine increased the latency time of tail flick from 2.81±1.13 seconds to 8.1±4.34 seconds ($p<0.01$) (Fig 2). **Formalin test:** Results of this test demonstrated that injection of 100 mg/kg extract in the chronic phase of pain showed a significant antinociceptive effect compared with control group($p<0.05$) and the pain score changed from 2.17±0.21 units in control group to 0.9±4.71 units. Using this dosage in the acute phase of pain did not show a significant antinociceptive effect compared with control group. On the other hand, injection of 300 mg/kg extract in both chronic and acute phases showed a significant antinociceptive effect compared with control group($p<0.01$) and decreased the pain score to 0.7±0.21 compared with control group. This test also demonstrated that co-administration of naloxone and high dosage of extract reverses the antinociceptive effects of the extract (Fig3).

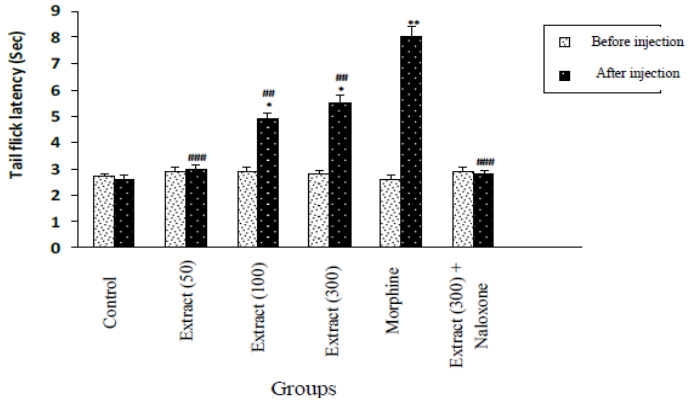

Figure 2. A comparison of different concentrations of Inula helenium extract in tail flick test

***p<0.05, **p<0.01:significant difference with control group. ##p<0.01, ###p<0.001:significant difference with morphine group.**

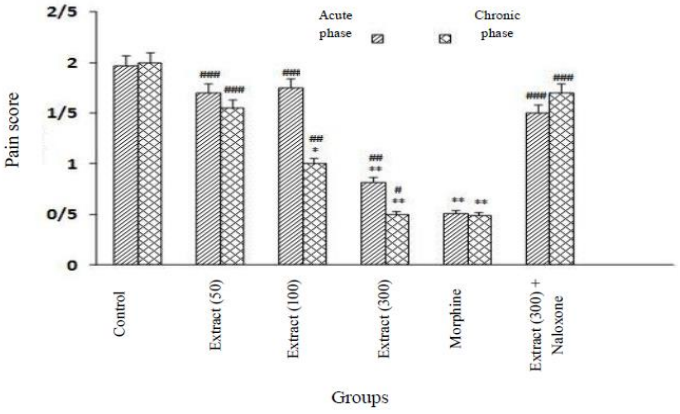

Figure 3. A comparison of mean pain score in rats with different concentrations of Inula helenium extract in formalin test

***p<0.05, **p<0.01: significant difference with control group. ##p<0.05, ##p<0.05: significant difference with morphine group.**

Discussion

Results of this study indicated that Inula helenium benefits from central and peripheral antinociceptive and anti-inflammatory effects. A study by Sayyah et al. on Cuminum cyminum (a member of Umbelliferae family) demonstrated that this plant has peripheral

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antinociceptive effects (22). Moreover, a study conducted by Barros et al. proved the antinociceptive effect of Pluchea quitoc plant using acetic acid (23).

Similar to the previous studies, the present study demonstrated that hydroalcoholic extract of Inula helenium prevents stomach cramps induced by acetic acid. Therefore, the antinociceptive effects of this plant are probably supported by environmental mechanisms. Intraperitoneal injection of acetic acid can cause acute inflammation of the peritoneum. Based on this model, it seems that the peripheral antinociceptive effects Inula helenium are indirectly created by internal mediators such as bradykinin, serotonin, histamine, substance p and prostaglandins, because all these mediators are related to stimulation of peripheral nociceptors (24). A study by Arambewela et al. demonstrated that medium dosage of Alpinia calcarata extract decreases pain (25). The results of the present study show that injection of medium and high dosages of extract decreases the pain caused by thermal stimuli in the tail flick test. Since tail flick test is used to assess spinal reflexes and identify central antinociceptive path (20), one may suggest that the extract of Inula helenium has central antinociceptive effects. Subcutaneous injection of formalin causes two different phases of pain. The first phase is the neurologic (acute) phase, which is created around active nociceptive neurons and is directly affected by formalin. The second phases in the inflammatory (chronic) phase, which is caused by activation of neurons located in the ventral horn of the spinal cord (26). In a study by Khan et al., the antinociceptive effect of Polygonatum verticillatum plant was proved using formalin test and it was demonstrated that the extract of this plant decreases pain in the chronic phase of formalin test and these effects are caused by flavonoids and alkaloids, which are present in the extract (27). According to the results of the present study, the extract of Inula helenium has inhibitory effects on pain. However, it reduces chronic phase more than acute phase. In formalin test, inhibition of chronic phase by extract may be due to inflammations that release compounds such as prostaglandins $F_2\alpha$ and E_2, which sensitize central nociceptive neurons at certain dosages (28). In order to assess the interference of opioid system in antinociceptive effect of Inula helenium extract, naloxone was used (one of the antagonist drugs of opioid system), which prevents the activation of opioid receptors (29). Results of this study show that naloxone decreases the antinociceptive effect of Inula helenium extract. Therefore, it seems that the antinociceptive effect of Inula helenium extract is due to opioid receptors. The major chemical compounds of this plant include flavonoids, monoterpenes, sesquiterpenes and diterpenes (30).

A study by Zhang et al. demonstrated that sesquiterpene composition in this plant decreases the level of Nitric oxide (31). According to the results of the present study, the hydroalcoholic extract of Inula helenium decreased inflammation and one of the anti-inflammatory mechanisms was probably due to inhibition of nitric oxide inflammatory mediator. Considering the results of this study, the extract was able to inhibit the inflammation and this anti-inflammatory effect may be due to inhibition of cyclooxygenases, particularly cyclooxygenases type-2 and prostaglandin E_2 in central nervous system (32).

As mentioned before, Inula helenium contains important photochemical compounds such as flavonoids. Flavonoids have many antinociceptive and anti-inflammatory effects (33). Flavonoids reduce intracellular calcium by inhibiting the activity of the N-methyl-D-aspartate receptor and then reduce the activity of nitric oxide synthases and calcium-dependent phospholipase A_2. Therefore, they show their antinociceptive effects by reducing nitric oxide and prostaglandins, particularly prostaglandins $F_2\alpha$ and E_2 (34). A general conclusion of the present experiment reveals that hydroalcoholic extract of Inula helenium leaf inhibits inflammation and acute and chronic pain in male rats. Although this plant's mechanism of action is not clearly known and given the results of researches on various plants with antinociceptive and anti-inflammatory effects and the presence of flavonoid in most of these plants and considering the effect of these plants on central and peripheral nervous system, the extract of this plant probably has both central and peripheral moderating effects on pain, increases resistance to pain and decreases response to acute and chronic pain. Therefore, it is suggested that more antinociceptive mechanisms and other types of extract be assessed in future studies.

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