Original Article

J Babol Univ Med Sci
Vol 17, Issu 5; May 2015; P:59-65

The Evaluation of the Analgesic Effects and Acute Toxicity of Methanol Extract of Pimpinella anisum.L in Male Wistar Rats

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Received: Aug 6th 2014, Revised: Sep 24th 2014, Accepted: Feb 4th 2015.

ABSTRACT

BACKGROUND AND OBJECTIVE: Pimpinella anisum is a medicinal herb widely used in the traditional Iranian medicine to cure digestive, inflammatory, and spastic diseases. This study aimed to evaluate the analgesic effects of methanol extract of Pimpinella anisum.L in mature male Wistar rats.

METHODS: In this study, 48 male Wistar rats were divided into 8 groups of control, treated with the Pimpinella anisum.L extract (50, 100, 200 mg/kg), treated with morphine, aspirin, extract with naloxone and extract with aspirin. Extraction was performed via maceration, rating and tail flick tests. In addition, formalin tests were conducted to evaluate the analgesic effects of the extract, and all the injections were performed intraperitoneally.

FINDINGS: Co-administration of 200 mg/kg of Pimpinella anisum.L extract combined with aspirin was observed to produce significant analgesic effects ($p<0.05$) compared to aspirin alone, with a one-second reduction of time delay in the tail flick test. In the rating test, 200 mg/kg of the extract resulted in more significant analgesic effects compared to aspirin alone ($p<0.01$), reducing the number of abdominal contractions to 10. The acute toxicity of the extract was determined as 2125 mg/kg.

CONCLUSION: According to the results of this study, the methanol extract of Pimpinella anisum.L could exert analgesic effects in both the acute and chronic phases of pain.

KEY WORDS: *Analgesic, Methanol Extract, Pimpinella anisum.L, Pain.*

Please cite this article as follows:

Asgari Nematian M, Mohammadi S. The Evaluation of the Analgesic Effects and Acute Toxicity of Methanol Extract of Pimpinella anisum.L in Male Wistar Rats. J Babol Univ Med Sci. 2015;17(5):59-65.

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[DOR: 20.1001.1.15614107.1394.17.5.9.4]
[DOI: 10.22088/jbums.17.5.9]

Introduction

Pain is normally caused by the destruction or damage of a tissue due to several factors such as heat, impact, tear, strain, and electrical or chemical factors (1). Opiates and non-steroidal, anti-inflammatory drugs (e.g. aspirin) are the two main types of analgesic compounds, which, despite their widespread use, are known to yield undesirable side effects such as digestive system dysfunctions, and renal and central nervous system damages (2). Currently, approximately 25% of the available medications in the world are of herbal origins. According to the World Health Organization (WHO), about 80% of the population of the world lives in developing and underdeveloped countries, and they mostly use medicinal plants for their medicinal needs since synthetic drugs are expensive, unavailable, and lead to several side effects (3, 4). Pimpinella anisum.L is a member of the Apiaceae family. This plant is indigenous to the East Mediterranean region and contains volatile oils. It is a herbaceous plant with a straight root, cylindrical stems with no fluff between 30-50 centimeters, and it can be found in the northwestern and southwestern regions of Iran as well as in India, Turkey, and other tropical areas in the world (4).

In the traditional Iranian medicine, the extract of this herb has been used for the treatment of inflammatory, gastrointestinal and anticonvulsants disorders, as well as an anti-asthma drug in order to cure shortness of breath (5). Furthermore, aqueous and ethanol extracts of this plant are known to have antioxidant and antibacterial properties (6), and several studies have reported of antibacterial and antifungal agents in this herb (7). Recently, the extract of Pimpinella anisum.L has been shown to have anti-injury and cell-protective effects against chemically induced gastric injuries in rats (8). Moreover, the fruit extract of this plant has been demonstrated to have antiepileptic properties (9). According to several studies, the extracted oil of Pimpinella anisum.L is able to increase glucose absorption and decrease urine output in rats (10). On the other hand, chemical tests indicate that the extract of Pimpinella anisum.L is composed of compounds such as anethole (90%), estragole, ognole, methyl chavicol, anisaldehyde, and polyethylene (11). Many popular medicinal compounds such as aspirin and morphine have been extracted from plants with analgesic effects. Since focused studies on the analgesic effects of Pimpinella anisum.L are scarce, this study aimed to evaluate the analgesic effects and acute toxicity of methanol extract of Pimpinella anisum.L through scientific tests such as formalin, tail flick, and rating tests on male rats.

Methods

In this experimental study, 48 male Wister rats weighing between 220-250 grams were purchased from Pasteur Institute of Iran and were preserved in standard animal room conditions under a photoperiod of 12 hours of light and 12 hours of darkness (starting point of light phase: 7 a.m). The temperature was kept at 22 centigrade degrees, and the relative humidity was between 50-55%. The animals had free access to food and water in their metal cages and became accustomed to laboratory conditions at least 2 hours before the beginning of the tests. The time period of test performance was between 8-12 a.m. The experiments were approved by the Research Council of Islamic Azad University of Hamedan, Iran and were conducted following the moral principles of the International Association for the Study of Pain in laboratory animals (12). The rats were divided into 8 groups of 6, which were as follows:

1) Control group (under the influence of Normal Saline); 2) Receiving morphine (1 milligrams/kilogram); 3) Receiving aspirin (10 mg/kg); 4) Treatment group receiving low, medium, and high doses of Pimpinella anisum.L (50, 100, 200 mg/kg, respectively); 5) Treatment group receiving aspirin (10 mg/kg) accompanied with high doses of the extract; 6) Treatment group receiving naloxone (1 mg/kg) with high doses of the extract

In this experiment, all the injections of the extract and medications were done intraperitoneally.

Drugs used in the experiment: Morphine sulfate and aspirin were purchased from Darou Pakhsh (Iran), naloxone from Tolid Darou (Iran), and formalin and acetic acid were provided from the Merck Company of Germany.

Extraction method: Approximately one kilogram of fresh Pimpinella anisum.L leaves was collected in August 2013 from regions around Alvand mountain of Hamedan, Iran. The samples were approved by the botanical experts of Islamic Azad University of Hamedan, and the herbarium of Bu-Ali Sina University (identification and maintenance number: 179). Extraction was performed by maceration method, and following the separation of petioles, Pimpinella anisum.L leaves were dried under shades at a

[DOR: 20.1001.1.15614107.1394.17.5.9.4]
[DOI: 10.22088/jbums.17.5.9]

temperature of 25 degrees. Afterwards, the dried leaves were grinded into a powder by a mechanical grinder, and 100 grams of the powder was preserved in one liter of 80% methanol for 72 hours in order to have the required effective compounds derived out. After filtering, the obtained compound was placed into a rotary device, and the solvent was removed and preserved in a petri dish under the hood for a week to completely dry. After a week, the remainder of the extract was dissolved in different doses in a proper amount of saline (Chloroethyl sodium 0.9%) as to be used for the treatment of the male rats (13).

Pain tests:

The Rating test: In order for the animals to get accustomed to the testing environment, they were placed in the mentioned standard glass box about 30 minutes before the beginning of the test. Afterwards, the methanol extract of Pimpinella anisum.L was dissolved in certain proportions of sterile saline and was injected intraperitoneally in dosages of 50, 100, and 200 milligram per each kilogram of the animal's weight. Following that, about 80 milligrams of acetic acid per each kilogram of the body weight was injected after 15 minutes with a 0.6% density, and immediately after the injection, the number of abdominal cramps, as well as the number of back-leg stretching, were counted for 30 minutes in the animals. Each rat was used only once during the current experiment (14).

The Tail Flick test: This test was performed using a tail-flick test device (model 5500-F, manufactured in Industry Tower Company of Iran). The test was performed according to the previously proposed model (15), and a reference time of 10 seconds was considered as the definite lighting time; in other words, if the rat did not pull his tail after 10 seconds of burning heat, the stimulus would stop in order to prevent tissue damage. Following that, the animals were horizontally placed in the Plexiglas, and one third of the tail was exposed to heat. The delay time of pulling the tail was calculated 3 times, with two-minute intervals in between the tests, before and 20 minutes after the injection of the drug or extract, and the mean of these variables were estimated and recorded as the delay time before and after the injection.

The Formalin test: In this experiment, we used the Dubuisson and Dennis model (1977) in order to evaluate chronic pain. The animals were placed in the special box of formalin test one hour before the start of the test in order to become accustomed to the testing condition. The special box was Plexiglas, which was manufactured in dimensions of 30×30×30, and in order to have a clear view of the animal's movements, a mirror was set under the rat and in front of the observer at an angle of 45°. About 30 minutes after the intraperitoneal injection of the drugs, 50 micro liters of 2.5% formaldehyde was subcutaneously injected into the animals' right leg and they would be returned to the special box. Following that, the behavior of the rat was evaluated for 60 minutes, in a way that once every 15 seconds the pain motor response was recorded as 0, 1, 2, or 3. Zero applied to the cases where the animal had full balance during movement and the body weight was distributed on both legs, and score one was applied to the situation where the animal could not carry his weight on the injected leg or would try to protect that leg. Score 2 applied to the situation where the animal would raise the painful paw avoiding contact to the container's floor, and score 3 was when the animal would lick the painful paw, chew it or persistently shake the painful paw. The mean of the first 5 minutes of each test was considered as the first phase of formalin test (acute phase), and the mean of 15-60 minutes after the onset of the tests was regarded as the second phase of the formalin test (chronic phase) (16).

Drugs used in the tests: Morphine sulfate, naloxone and indomethacin were purchased from Darou Pakhsh (Iran), and acetic acid and formalin were purchased from Merck Company of Germany.

Determination of acute toxicity (Median Lethal Dose: LD_{50}): The acute toxicity was determined according to the previous laboratory model (17). In addition, different doses of the extract were injected intraperitoneally and separately to the male rats. The amount of death in the studied animals was calculated for the next 72 hours and LD_{50} of the extract was determined as well.

Statistical Analysis: The collected data were presented in the form of mean and standard error (mean ± SE). Data analysis was performed using one-way analysis of variance (ANOVA) and Tukey's HSD (Honestly Significant Difference) test, and $P<0.05$ was considered as significant.

Result

Acute toxicity of the extract: The amount of acute toxicity [LD_{50}] was intraperitoneally calculated to be 2125 mg/kg.

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The Rating test: The results of this test indicated that an injection of 100 mg/kg dose of the extract with a total rating of 14, as well as the injection of 200 mg/kg dose with a total rating of 9, could significantly decrease the ratings compared to the control group ($p<0.05$, $p<0.01$). Moreover, simultaneous injection of 200 mg/kg of the extract and aspirin with a total rating of 7 could cause more impassibility compared to other doses of the extract in the control group ($p<0.001$). Similarly, simultaneous injection of 200 mg/kg of the extract and aspirin resulted in more impassibility compared to the aspirin-only group ($p<0.05$). Moreover, simultaneous injection of 200 mg/kg of the extract with naloxone with a total rating of 35 was observed to reverse the analgesic effects compared to the group receiving 200 mg/kg of the extract with aspirin (fig 1).

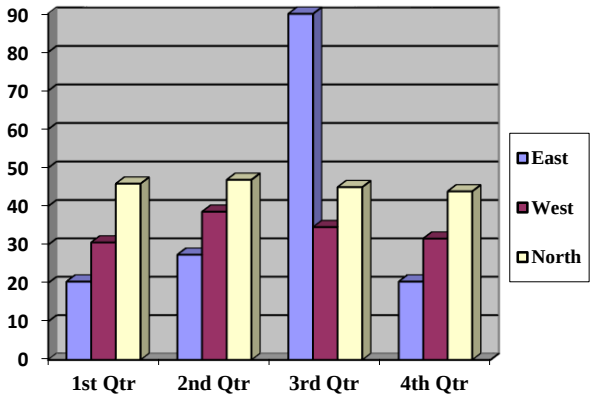


Figure 1. Comparison of Average Number of Rating in male rats with different doses of Pimpinella anisum.L in Acetic Acid test

*p<0.05, **p<0.01, ***p<0.001 (significant difference with the control group). *p<0.05,**p<0.01,***p<0.001 (significant difference with Pimpinella anisum.L group with dosage of 200 mg/kg+aspirin

The Tail flick test: In this test, injection of 100 and 200 mg/kg of the extract with the average time delay of 2.5 and 6 seconds was observed to have significant analgesic effects compared to the control group ($p<0.05$, $p<0.01$). Furthermore, the test results indicated that injection of 200 mg/kg of the extract with aspirin with the average time delay of 2.5 seconds ($p<0.05$) could increase the average time delay in the tail flick test more than the aspirin-only group(fig 2).

The Formalin test: According to the obtained results in figure 3, injection of 200 mg/kg of the extract during the acute phase of the formalin test with the pain rate of 1.7 was able to inhibit pain more efficiently compared to the control group ($p<0.05$). Moreover, injection of 200 mg/kg of the extract with aspirin was observed to inhibit pain more efficiently than the aspirin-only group with the pain rate of 1.3 ($p<0.05$). However, during the chronic phase of the formalin test, we observed an increase in the analgesic effects of the extract in doses of 100 mg/kg (pain rate: 1.5) and 200 mg/kg (pain rate: 1.4) compared to the control group ($p<0.05$, $p<0.01$). In addition, injection of 200 mg/kg of the extract with aspirin (pain rate: 1) during the chronic phase could significantly decrease pain compared to the group receiving aspirin ($p<0.05$).

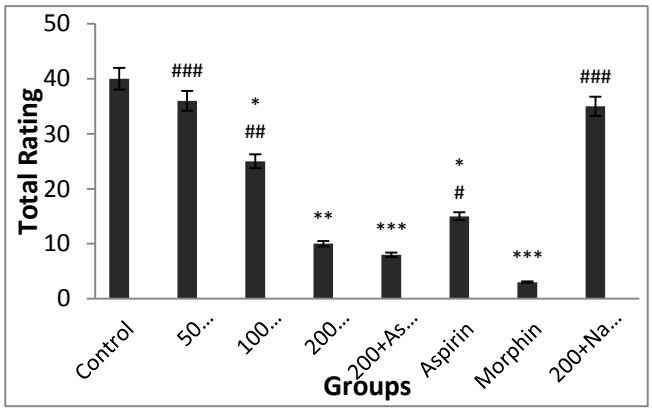


Figure 2. Comparison of Delay time in Tail flick test between the tested groups before and after treatment

*p<0.05, **p<0.01, ***p<0.001 (compared to the control group). #p<0.05, ##p<0.01, ###p<0.001 (significant difference with Pimpinella anisum.L with dosage of 200 mg/kg + aspirin

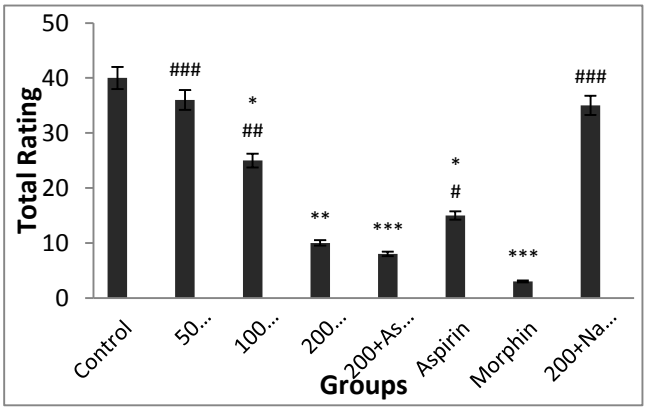


Figure 3. Comparison of average pain scores in male rats with different density of Pimpinella anisum.L extract during acute phase of Formalin test

*p<0.05, **p<0.01, ***p<0.001 (significant difference with the control group).#p<0.05, ##p<0.01, ###p<0.001 (significant difference with 200 mg/kg dosage of Pimpinella anisum.L + aspirin

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Discussion

According to the results of the present study, Pimpinella anisum.L extract has remarkable analgesic properties. In the past, medicinal herbs and plants were commonly used as a rich source against disease causing agents around the world (18). Given the fact that the acute toxicity of Pimpinella anisum.L extract has been reported to be higher than 2125 mg/kg, using doses of 50, 100, and 200 mg/kg of this extract was considered to be safe in this study. One of the most important tests used for screening the potential analgesic compounds is the Rating test, where acetic acid is used and it is also a chemical stimulation widely used in order to evaluate environmental analgesic activities (19). On the other hand, intraperitoneal injection of acetic acid is likely to cause acute inflammation of the peritoneum (20). Acetic acid can indirectly stimulate internal mediators such as Bradykinin, Serotonin, Histamine, Substance P, and Prostaglandins (21), which are also able to stimulate nociceptors, which have a high sensitivity to non-steroidal anti-inflammatory drugs such as aspirin, and opiates such as morphin (22). The results of the rating test in this study indicated that the injection of 100 and 200 mg/kg of Pimpinella anisum.L extract could noticeably inhibit pain. Furthermore, simultaneous injection of 200 mg/kg of the extract and aspirin was observed to have more analgesic effects compared to aspirin alone, which was, on the other hand, comparatively less than the pain relieving effects of morphine. The results of the current study also demonstrated that the injection of medium and high doses of Pimpinella anisum.L extract could reduce pain due to the thermal stimulus in the tail flick test. The tail flick test is normally used to evaluate spinal reflexes and identify central nociceptive pathways (22, 23). In this test, the extract of Pimpinella anisum.L was observed to have proper analgesic effects used independently and in the form of injection alongside aspirin since it was able to exert more efficient pain inhibitory effects compared to aspirin alone. Therefore, it could be suggested that Pimpinella anisum.L extract exerts its analgesic effects through the central nervous system, and the advantage of using the evaluation model of formalin pain is the ability of this model to distinguish between the compounds taking effect through the central nervous system and peripheral pain (24). Moreover, the subcutaneous injection of formalin leads to two different phases that could trigger pain; the first is referred to as the “neurogenic” (acute) phase, which is generated in the proximity of active flicking neurons directly under the influence of formalin. The second phase is known as the “inflammatory” (chronic) phase, which is caused by the activation of ventral horn neurons in the spinal cord (25). The results of the current study indicated that a dosage of 200 mg/kg of Pimpinella anisum.L extract was able to inhibit pain and the simultaneous injection of this extract with aspirin also showed more inhibitory effects than aspirin. Pain inhibition was also observed during the phase of chronic pain after administrating doses of 100 and 200 mg/kg. Consequently, this analgesic agent will ultimately result in more pain reduction during the chronic phase than the acute phase. The chronic phase of the formalin test might be triggered due to the inflammations leading to the release of compounds such as prostaglandins E_2 and F_{2a}, which at least in some cases, could render the central flicking neurons more sensitive (26). In the current study, naloxone, which is an opioid antagonist, was used in order to evaluate the interference of opioid system under the analgesic effects of Pimpinella anisum.L extract, which also prevents the activation of opioid receptors (27, 28). The results of this study also suggested that the injection of naloxone with 200 mg/kg of Pimpinella anisum.L extract could decrease the analgesic effects of the extract; therefore, it seems that the analgesic effects of this extract are correlated with the activity of opioid receptors. Several reports have confirmed the existence of compounds such as Saponin, Sapogenin, and flavonoids, such as quercetin, in Pimpinella anisum.L (29), and the analgesic agents of saponins have been mentioned in some studies in this regard (30). Furthermore, the inhibition of inducible nitric oxide and cyclooxygenase-2 synthesis has been reported to be associated with Terpenoides, which are able to independently cause pain inhibition (31, 32). Flavonoids also have numerous biological effects on protein synthesis, cell differentiation, and making of the arteries in humans (33). According to the results of the present study, Pimpinella anisum.L extract is known to have central and peripheral analgesic properties and could be a suitable alternative for chemical analgesic drugs. It seems that the existing flavonoids and tannins in the extract can reduce pain through activating several nervous paths, which is a subject in need of further investigation. The study of the mechanisms of Pimpinella anisum.L extract effects on the receptors and the interaction with neurotransmitters or their agonists and antagonists could determine the precise nervous paths under the influence of this extract.

Acknowledgements

Hereby, we extend our deepest gratitude to Mr. Hadi Golmohammadi for his sincere cooperation in this study. We would also like to thank all the colleagues who assisted us with this research project.

[DOR: 20.1001.1.15614107.1394.17.5.9.4]
[DOI: 10.22088/jbums.17.5.9]

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