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# Antinociceptive Effects of *Paeonia daurica* subsp. *macrophylla* Root Extracts in Mice

**Hamidreza Monsef-Esfahani[1], Sahand Jamshidi[1], Paria Sharafi-Badr[2], Mohammad Sharifzadeh[3], Mahdi Vazirian[1], Seyede Nargess Sadati Lamardi[4]***

*[1]Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*
*[2]Department of Pharmacognosy and Pharmaceutical Biotechnology, School of Pharmacy, Iran University of Medical Sciences, Tehran, Iran*
*[3]Department of Toxicology and Pharmacology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*
*[4]Department of Traditional Pharmacy, School of Persian Medicine, Tehran University of Medical Sciences, Tehran, Iran*

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## Abstract

This study aimed to assess the antinociceptive activity of extracts and fractions of Paeonia daurica subsp. macrophylla in BALB/c mice. Various doses of hydro-alcoholic extract (HE), hexane fraction (F-hexane), methanol (F-MeOH), and chloroform (F-$CHCl_3$), as well as aqueous extracts (AE), were evaluated by a well-known model, a formalin-induced pain test in mice. All extracts, piroxicam 0.1 mg/kg, and negative control groups were administered 30 minutes before formalin injection. Flinching, licking, and biting reflexes were measured as painful factors compared with controls at intervals of 0 to 5 minutes, 0 to 15, and 0 to 60 minutes after formalin injection. The acute oral toxicity test of total ethanolic and aqueous extracts showed no signs of toxic effect up to a dose of 5000 mg/kg. In the formalin test at a time interval of 0 to 5 minutes, there was no significant difference between the results of the study groups. In the range of 0 to 15 minutes, the effect of AE (1 g/kg), HE (2, 3 g/kg), and F-hexane (1 g/kg) was significantly higher than the positive control group ($p<0.01$). In the time interval of 0 to 60 minutes as the total time of the experiment, the effect of AE (0.25 g/kg), AE (0.5, 1 g/kg), HE (2, 3 g/kg), F-hexane (1 g/kg) were significantly different than the positive control group. It can be concluded that extract of *P. daurica* subsp. *macrophylla* might be helpful in the treatment of pain in humans.

**Keywords:** *Paeonia* roots; Antinociceptive effect; Acute toxicity; Persian medicine; Piroxicam

## Introduction

Pain is predominantly a defensive mechanism and signal of the body which occurs with damage to various tissues, and activation of the nervous system causes the patient to search for a cure [1]. Pain cascades are not static and may cause changes in the neural structure [2]. These changes may occur, especially in chronic pains. Nociceptive pain can be considered an alarm system for the presence of a potentially damaging stimulus in different organs and tissues [3]. Effective analgesics for clinical use, with lower side effects, are still required. Analgesics continue to be the natural product component, despite its well-known undesirable side effects [4].

*Paeonia* L. species (Paeoniaceae) are plants considered to be traditional ornamental and medicinal plants [5]. The plants of this genus are rich in nutrients, proteins, microelements, and vitamins. Moreover, the flowers and roots of peony have been used to make various folk foods and as anticonvulsant, anxiolytic,

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*Corresponding Authors: Seyede Nargess Sadati Lamardi
Department of Traditional Pharmacy, School of Persian Medicine, Tehran University of Medical Sciences, Tehran, Iran
Email: n_sadati@tums.ac.ir

hypoglycemic, analgesic, sedative, anti-inflammatory, anti-osteoporotic effects, and mainly its roots are used as anti-allergic and antipyretic in Chinese medicine [6-10]. Roots of the peony have been used as a traditional medicine for their antinociceptive effects [10,11]. Paeoniflorin can improve the effects of adenosine A1 receptor agonists, induce sedation and increase the threshold of nociceptive responses [12].
*Paeonia daurica* subsp. *macrophylla* (Albov) D.Y.Hong. (Paeoniaceae) are native to the South Caucasus and Iran and are also known as Whitman peony. This subspecies was formerly known as a separate species under P. *macrophylla*, but in 2002 it became a subspecies of *P. daurica* [13,14].
*Paeonia officinalis*, known as "*Oode-Saleeb*," in traditional Persian medicine, has been used to manage epilepsy, nightmare, tremor, paralysis, uterine problems, and as a stomach analgesic [17]. GC-MS analysis of hydro distilled essential oil (EO) of the roots of *P. daurica* subsp. *macrophylla* collected from the north of Iran showed that the main composition of the EO was salicylaldehyde (20.32%), beta-pinene-oxide (13.35%), and thymol acetate (61.12%) [18]. Recent studies reported antioxidant and anticonvulsant activity of the root of *Paeonia daurica* subsp. *macrophylla* without toxicity. Furthermore, three compounds, benzoic acid, veratric acid, and oleanolic acid, were isolated and identified from this plant [9].
In 2010, in a study by Prieto and colleagues on the effects of traditional Chinese medicine plants on the inflammatory properties of leukocytes and platelets, it was found that one of the species called *P. cocos* has anti-inflammatory effects by inhibiting $A_2$ phospholipase. This approach can be used as a sign in continuing this study to investigate the anti-inflammatory and antinociceptive effects [14]. In this study, the antinociceptive and acute toxicity effects of aqueous extract (AE), hydroalcoholic extract (HE), and its partitioned fractions hexane (F-hexane), methanol (F-MeOH), and chloroform (F-$CHCl_3$) from *Paeonia daurica* subsp. *macrophylla* root was investigated.

## Materials and Methods

### *Plant material*

The roots of *P. daurica* subsp. *macrophylla*, with the voucher number 6620-TEH, was identified by Prof. Gholamreza Amin and deposited at the herbarium of the faculty of pharmacy, Tehran University of Medical Sciences.

### *Extraction and isolation*

The air-dried roots (500 g) were powdered and exhaustively extracted by the maceration method (aqueous ethanol, EtOH 80%) (Merck, Germany, three-time for 48 h), and hydroalcoholic extracts were concentrated by a rotary instrument (Heidolph Laborota, Germany) at 40 ºC to yield 96 g, HE. The column chromatography (1.52×23 cm) method further partitioned the HE. The silica was used as an adsorbent (Mesh size 230-400 ASTM, Merck, Germany), and the column was eluted with pure solvent n-hexane as a mobile phase (Merck, Germany). The polarity of the solvents was increased with the gradient of 10-100% chloroform and with a 1-5% gradient of methanol consecutively. Then the residue was successively submitted to F-hexane (1.3 g), F- $CHCl_3$ (3.26 g), and F-MeOH (43 g) fractions, respectively, according to their TLC profiles. To prepare P. *macrophylla* aqueous extract (AE), the dried roots were suspended in distilled water (190 g dried roots per 2 L water), and the mixture was boiled for 60 min at 90 ºC with occasional stirring. Aqueous extracts were combined, and the obtained residue was cooled and lyophilized (34 g, AE). In addition, the final volume is stored until further evaluation. All the extracts and fractions were maintained in saline containing Tween 80 (5%, v/v).

### *Investigation of in vivo antinociceptive effects of the studied plant extracts*

Animals and treatments
120 male NMRI albino mice weighing 25±5 g (Tehran University of Medical Sciences, Iran) were housed (12-h light/dark cycle, 25 ºC). The Ethics and Animal Care Committee of Tehran University of Medical Sciences code was IR.TUMS.VCR.REC.1395.687. In addition, this study followed the National Institute of Health Guidelines for the Care and Use of Laboratory Animals [19].

Preparation of administration dose
Animals were divided into five groups of six mice each. Different doses of the extracts were dissolved in an appropriate volume of normal saline. All the mice were divided into 5 groups. The positive control group (piroxicam), at the concentration of 0.1 mg/kg, for the HE group (1, 2, and 3 g/kg), aqueous extract, F-hexane, F-$CHCl_3$, and F-MeOH (250, 500 and 1000 mg/kg) were prepared based on previous studies. Sterile isotonic saline solution was used as the negative control. All the extracts and control groups were sonicated for 10 min at room temperature and gavaged [20,21]. Thirty minutes before the experiment's start, the mice were placed under a funnel for adaptation to the environment.

Acute toxicity test
According to existing studies, acute toxicity was evaluated in rats. To control the toxicity, quantity, and quality of $LD_{50}$, animals were observed for signs of toxicity and mortality in the initial critical period of 4

h and then for 7 days. Symptoms of toxicity included paw licking, body stretch, respiratory distress, diarrhea, and death. The average lethal dose is calculated based on doses that cause 0 to 100% death. For this purpose, doses of 1000, 2500, and 5000 mg/kg of HE and AE were used by gavage in groups of 5. These doses are used because they are at least 10 to 100 times more effective than those used in animal studies [23,24].

### *Formalin test*

In this experiment, the formalin test was used to assess antinociceptive effects in mice. Half an hour before formalin injection, the desired dose of extracts and drug was gavaged in mice. Subcutaneous injection of 0.5% formalin with a volume of 20 µL was used on the left foot of male mice. Immediately after the formalin injection, the rat was placed on a flat glass surface under a glass funnel, with a 45-degree angle mirror below that surface, allowing the animal to see the sole. The control group received normal saline, and the treatment groups received different doses of the studied extracts 30 min before formalin injection. The number of flinching, licking, and biting reflexes are measured as painful factors and compared with the control group [22].

### *Statistical analysis*

A one-way analysis of variance (ANOVA) was used, followed by a Tukey test to determine the significant differences between all groups using R statistical software, version 3.5.0. Differences between experimental and control groups with a P value less than 0.01 were considered significant differences.

## Results

### *Acute toxicity test*

In the doses of 1000, 25000, and 5000 mg/kg of HE and AE (orally, time intervals of 0 to 4 h, up to 7 days), there were no signs of toxicity leading to death, but in all investigated doses, drowsiness was observed in the animal, which disappeared about 4 h. Therefore, it can be concluded that these extracts do not have acute toxic effects up to a dose of 5000 mg/kg.

### *Formalin test*

The results of the formalin test are summarized in table 1. Based on the available data, The tested samples (orally administered) in 0 to 5 min intervals (as one of the primary periods) did not show any significant difference between five plant extracts with different doses and piroxicam as positive control and normal saline, as negative control group.

At the intervals of 15 to 60 min, the effect of the AE (1 g/kg) and HE (2000, 3000 mg/kg) were significantly higher than the control group (p value ≤0.01, ≤0.001, and ≤0.001, respectively). At the time interval of 0 to 60 min as the total test time, the effects of F-hexane (1000 mg/kg) and AE (250, 500, 1000 mg/kg), and HE (2000, and 3000 mg/kg) showed a significant difference compared to the negative control group. Meanwhile, F-hexane and AE (1000 mg/kg) and HE (3000 mg/kg) exhibited a significant difference compared to the piroxicam group (Figure 1).

Based on the mentioned analyses, it seems that among the prepared extracts, the F-MeOH had an almost negligible effect on the pain mechanism in mice, indicating the purification and excretion of pharmacologically active substances in other fractions. Also, based on the available data, it can be seen that the significant differences between groups in the time intervals of 0 to 15 min and 0 to 60 min were precisely the same. Therefore, it can be said that F-hexane, AE, and F-$CHCl_3$ were more effective than other samples and compared to the control, so further studies on these extracts are suggested. Also, a dose-response relationship was observed between all the samples. Moreover, in all samples, the ratio of reducing the pain reflex to increasing the dose was linear.

## Discussion

The advantage of the formalin test to assess pain over other methods is that it differs from most pain models in that moderate to persistent pain is caused by tissue damage, and since tissue damage occurs, it is closer to clinical pain and, therefore, more useful for assessment [25,26]. Thus, in this measurement, based on the total time that the animal spends licking and biting the injected foot in different behavioral states, it is considered the time of pain. The most important feature of the formalin test is that rodents show two responses to pain, which have two different mechanisms. The first stage appears immediately after the formalin injection, which lasts for 3 to 5 min. This pain is apparently due to direct chemical stimulation of pain receptors [27]. Evidence shows that formalin increases the activity of C fibers and does not act on A-fibers [28]. After the first 5 min, at 10 to 15 min, the animal does not show any particular behavior. After 15 to 20 min, the second phase of pain initiates. Then the animal starts licking the soles of its feet again, which lasts for about 20 to 40 min. Peripheral inflammatory processes appear to be involved in this phase, as nonsteroidal anti-inflammatory drugs such as piroxicam reduce pain in the second phase. At the same time, they seem to be ineffective in the first phase. Even if small amounts of formalin are injected, taking aspirin-like does not reduce the pain of the first stage. This suggests two types of pain in these organisms with two different mechanisms. Various studies show that bradykinin plays a role in the first phase;

**Table 1.** Formalin-induced reflexes (licking, flicking, and biting) and inhibition by the tested samples.

| | Intervals of 0-5 min (Early Phase) | | Intervals of 15-60 min (Late Phase) | | Intervals of 0-60 min | |
|---|---|---|---|---|---|---|
| Groups (mg/kg) | Mean±SD | CV% | Mean± SD | CV% | Mean± SD | CV% |
| $C^-$ | 32.2±4.54 | 0.00 | 346±57.8 | 0.0 | 459±64.6 | 0.0 |
| AE (250) | 28.2±2.99 | 10.60 | 281±22 | 7.83 | 374±26.4** | 7.06 |
| AE (500) | 27.5±1.76 | 6.40 | 280±16.1 | 5.75 | 375±14.9** | 3.97 |
| AE (1000) | 27±2.83 | 10.48 | 264±18.6** | 7.05 | 354±25.6*** | 7.23 |
| $CHCl_3$ (250) | 27.3±1.63 | 5.97 | 298±16.6 | 5.57 | 399±17.9 | 4.49 |
| $CHCl_3$ (500) | 26.8±2.64 | 9.85 | 296±8.43 | 2.85 | 390±16.5 | 4.23 |
| $CHCl_3$ (1000) | 29.8±3.82 | 12.82 | 286±21.7 | 7.59 | 389±24.8 | 6.38 |
| Hexane (250) | 33.5±2.95 | 8.81 | 301±15.8 | 5.25 | 410±18.4 | 4.49 |
| Hexane (500) | 31.7±2.8 | 8.83 | 301±30 | 10.00 | 407±34.4 | 8.45 |
| Hexane (1000) | 31.2±2.32 | 7.44 | 255±43.2** | 16.94 | 352±43** | 12.19 |
| MeOH (250) | 36±3.85 | 10.69 | 380±18.8 | 4.95 | 502±17.8 | 3.55 |
| MeOH (500) | 34±2.45 | 7.21 | 345±13.2 | 3.83 | 461±16.7 | 3.62 |
| MeOH (1000) | 34.5±3.02 | 8.75 | 314±53.6 | 17.07 | 422±60 | 8.0 |
| HE (1000) | 33.3±2.73 | 8.20 | 308±14 | 4.55 | 418±13.9 | 3.33 |
| HE (2000) | 32.7±3.27 | 10.00 | 270±24.9** | 9.22 | 373±34.8** | 9.33 |
| HE (3000) | 33±3.03 | 9.18 | 236±8.04*** | 3.41 | 335±15.1*** | 4.51 |
| Piroxicam 0.1 | 32.5±3.08 | 9.48 | 255±28.8*** | 11.29 | 359±36.2*** | 10.08 |

Values are expressed as mean±SEM. Results evaluated at P<0.01 were considered significant compared to control and standard drug piroxicam (**p < 0.01, ***p < 0.001 compared with control group), N=6.
*$C^-$: Negative control; *normal* saline, AE: Aqueous extract; $CHCl_3$: Chloroform fraction; Hexane: Hexan fraction; MeOH: Methanol fraction; HE: Hydroalcoholic extract SD= Standard deviation; CV%: The coefficient of variation percentage.

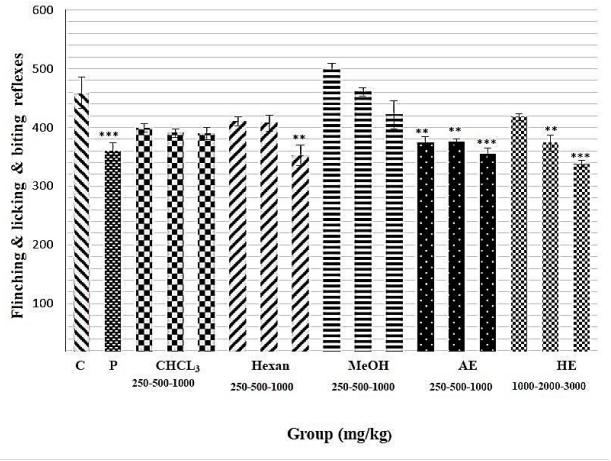


**Figure 1.** Flinching, licking, and biting reflexes pain results in 0 to 60 minutes. Error bars shown in this figure are mean±SD for n≥6 (*p<0.05, **p<0.01, ***p<0.001 compared with control group). *$C^-$: Negative control; normal saline; P: Piroxicam (0.1 mg/kg); AE: Aqueous extract; $CHCl_3$: Chloroform fraction; Hexane: Hexane fraction; MeOH: Methanol fraction; HE: Hydroalcoholic extract

while histamine, serotonin, and prostaglandin are responsible in the second phase. This can also indicate that the second stage is inflammatory. According to observations, inflammation is not enough to cause behavior similar to that seen in the second phase, but spinal processes are also involved in developing the second phase [29].

Ambient temperature is important in determining the behavioral response in mice's second phase of pain response. The importance of ambient temperature is mainly due to its effect on tissue temperature, as a decrease in tissue temperature causes the inflammatory reactions necessary for the second phase to develop more slowly and to a lesser extent [30]. Stress also affects the animal's behavior after formalin injection. Factors such as intense light, high atmospheric pressure, noise, odors, and human activity at the test site may affect the response. Therefore, the formalin test should preferably be performed in a dedicated room [31,32].

In this study antinociceptive effect of different extracts of *P. daurica* subsp. *macrophylla* root was

evaluated by formalin test. Several studies reported analgesic and anti-inflammatory efficacy of *Paeonia* species. Total glucosides of peony are used to treat rheumatoid arthritis in China. In a study by Zhang and colleagues, the anti-inflammatory effects of total glycosides of *Paeonia lactiflora* root on neuropathic pain at doses of 60, 120, and 240 mg/kg were demonstrated [13], as well as the anti-inflammatory effects of Paeoniflorin, the main active ingredient in the extract of *Paeonia* species has been studied and confirmed in rheumatoid arthritis [14]. According to the above studies, it seems that the anti-inflammatory effect of *P. daurica* subsp. *macrophylla* is responsible for its analgesic effects. Comparing the effects of different fractions of this plant with an anti-inflammatory drug demonstrated that, like piroxicam, the fractions obtained from this plant are more effective on chronic pain than acute pain. Based on this study, it seems that a species of this plant found in Iran also has analgesic and possibly anti-inflammatory effects similar to the species studied previously.

Recently, anticonvulsant effects of aqueous extract, hydroalcoholic crude extract, and its chloroform, ethyl acetate, and methanol fractions of *P. daurica* subsp. *macrophylla* root was studied employing a pentylenetetrazol (PTZ)-induced convulsion model on mice. All the plant extracts, especially aqueous extract, except ethyl acetate fraction, significantly delayed the onset and decreased the duration of PTZ-induced myoclonic convulsions and generalized tonic-clonic seizures (GTCS), considerably reducing the GTCS and mortality rate [9].

It can be concluded that knowledge of the potency of *P. daurica* subsp. *macrophylla* extract and constituents might be helpful in the discovery of new insight into pain treatment. Accordingly, further phytochemical investigation of the most active extract of *Paeonia daurica* subsp. *macrophylla* due to the antinociceptive and anticonvulsant effects of the aqueous extract is suggested.

## Conflict of Interests

We declare that there is no conflict of interest.

## Acknowledgments

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