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Original article

Antinociceptive and Anti-Inflammatory Effects of *Geum iranicum* Khatamsaz Methanol Extract in Mice

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Abstract

Background and objectives: Traditionally, *Geum* species from Rosaceae family have been used for treating inflammatory disorders. *Geum iranicum* Khatamsaz is endemic to Iran. The aim of this study was to investigate the antinociceptive and anti-inflammatory activities of *Geum iranicum* methanol extract *G. iranicum* methanol extract using classical models. **Methods:** The methanol extract of *G. iranicum* roots was evaluated for antinociceptive activity by acetic acid-induced writhing, formalin and hot-plate tests in male Swiss albino mice. The anti-inflammatory effect was investigated by Carrageenan-induced paw edema method. **Results:** The extract significantly inhibited both the first and second phases of formalin-induced nociception in mice at the dose of 100 mg/kg compared to the control group. In acetic acid-induced writhing test and hot plate method, the extract significantly reduced pain behavior in all doses (25, 50 and 100 mg/kg). The antinociceptive activity of the extract was significantly reduced by naloxone (4 mg/kg). The anti-inflammatory activity of the extract was found to be dose dependent. The extract at the dose of 100 mg/kg exhibited significant reduction of paw edema in all surveyed times. **Conclusion:** The results showed that the methanol extract of *G. iranicum* roots possessed central analgesic activity via modulation of opioid receptors as well as anti-inflammatory activity. The observed effects could be attributed to the presence of constituents like triterpenoids, eugenol, sucrose and tannins in the extract.

Keywords: analgesics; anti-inflammatory agents; *Geum*; Rosaceae

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Introduction

Inflammation is characterized by classical signs of edema, erythema, pain, heat, and subsequently loss of function. It is the complex biological response of vascular tissues to harmful stimuli including pathogens, irritants, or damaged cells. Inflammation is a protective attempt by the organism to remove the injurious stimuli as well as initiating the healing process for the tissue [1,2]. Chronic inflammatory diseases remain one of the world's major health problems [3,4]. Pain is a global health issue which affects the quality of life and also increases the rate of mortality in elderly patients and individuals with chronic diseases [5-7]. Various nonsteroidal anti-inflammatory drugs can reduce pain and inflammation by blocking the metabolism of arachidonic acid through isoforms of cyclooxygenase enzyme, thereby reducing the

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production of prostaglandins. Unfortunately, there are many side effects associated with the administration of nonsteroidal anti-inflammatory drugs the most common being gastrointestinal ulcerations and bleeding [7,8]. In spite of the efficacy provided by analgesic opioids, these drugs have a low therapeutic index and they are usually accompanied by side effects such as respiratory depression and dependency [9,10]. Therefore, development of new therapeutic agents with improved efficacy and safety profile should be considered.

Throughout history, man has used many different forms of pain relief therapies, including medicinal herbs, which are widely used in view of their low cost and fewer side effects [11,12]. According to the World Health Organization (WHO), around 80 % of the world's population relies predominantly on plant-based drugs [13].

The survey of the effectiveness of plant-based remedies used in the folk medicine has given great reflections because they are inexpensive and have less side effects [7]. The genus *Geum* belongs to the Rosaceae family and comprises about 70 species distributed throughout the world mostly across the northern hemisphere. These plants are mainly perennial and herbaceous [14,15]. *Geum iranicum* Khatamsaz is endemic to Iran [16]. Traditionally, *Geum* species have been used for treating inflammatory disorders [15] such as rheumatism [17], gastric inflammation [18] and hemorrhoids. Based on previous studies, various biological activities have been reported from *Geum* species such as antifungal, antibacterial, antiviral, anticoagulant, antioxidant, angiogenesis and radical scavenging effects [19-25]. A literature review revealed few reports on the anti-inflammatory and antinociceptive activities of *Geum* species. In a research, the hydroalcoholic extract of *G. kokanicum*, collected from Iran, showed anti-inflammatory effect and reduced xylene-induced mice ear edema and also antinociceptive activity was observed in formalin test [26]. In Swedish traditional medicine, the root of *Geum urbanum* has been used for inflammatory disorders. The aqueous extract of *G. urbanum* root was found to have potent inhibitory activity on prostaglandin biosynthesis and platelet activating factor (PAF)-induced exocytosis [27]. In the present study, we aimed to evaluate the antinociceptive and anti-inflammatory activities of the methanol extract of *Geum iranicum* Khatamsaz root using various models in mice for the first time.

Material and Methods

Ethical consideration

All experimental protocols were in accordance with the National Institutes of Health (NIH) guidelines for care and use of laboratory animals. Animal studies were approved by Ethics Committee of Mazandaran University of Medical Sciences, Sari, Iran with ethical code of IR.MAZUMS.REC. 94.1274, 2015.

Plant material

Roots of *G. iranicum* were collected from 75 km north of Shirvan, of Khorasan-e-Shomali province, Iran, during the flowering stage. A voucher specimen (no. 6714 THE) was deposited at the Herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. One kg of the dried roots were cut into small pieces and macerated for 48 h (four times) with two liters of ethyl acetate at room temperature. The dried marc, after extraction with ethyl acetate, was extracted with methanol using the same method. The extracts were concentrated by a rotary evaporator.

Determination of total phenolics and tannin contents

Total phenolics and tannin contents of the methanol extract were determined by Folin-Ciocalteu method [28]. Calibration curve was plotted using various concentrations of gallic acid (12.5, 25, 50, 100 and 200 μg/mL). For determination of tannin content, tannins in the extract solution were precipitated with polyvinylpolypyrrolidone (PVPP). After centrifuging of the solution, the phenolics content of the supernatant was calculated and subtracted from total phenolic content. The total phenolic and tannin contents were expressed as milligrams of gallic acid equivalents per gram of dried extract [28].

Animals

Male Swiss albino mice (20-30 g) from the Animal Facility Centre of Mazandaran University of Medical Sciences were used for this study. Number of animals in each experiment group was six. They were kept in standard polypropylene cages and housed under standard condition of 12-h light: 12-h dark cycles, at room temperature of 25±2 °C and 70% relative humidity provided

with water and standard diet ad libitum. All experimental protocols of the study were in accordance with National Institutes of Health guidelines for care and use of laboratory animal [29]. Moreover, all procedures were previously approved by the Ethics Committee of Mazandaran University of Medical Sciences.

Antinociceptive activity

Formalin test

Thirty minutes after oral treatment with the vehicle (normal saline, 10 mL/kg), morphine (5 mg/kg), indomethacin (10 mg/kg) or different doses of the the methanol extract of *G. iranicum* (25, 50 and 100 mg/kg), the animals received 20 µL of formalin (2.5 % formaldehyde in saline solution), which was injected into the sub-plantar region of the right hind paw. Following the formalin injection, the animals were placed in individual cages that permitted unrestricted observation. The time (expressed in seconds) that the animals spent licking the injected paw was considered to represent nociception. The first phase of the nociceptive response normally peaks at 0-5 min and the second phase 15-30 min following the formalin injection [12].

Writhing test

Three groups of animals received different oral doses of the methanol extract of *G. iranicum* (25, 50 and 100 mg/kg), 1 h prior to i.p injection of acetic acid (0.1 mL/10 g body weight, 0.75% v/v in normal saline). Control group received vehicle (10 mL/kg of normal saline). After the acetic acid injection, the number of writhing in each mouse was counted during continuous observation for 30 min, beginning at 5 min after acetic acid injection. Morphine (5 mg/kg, p.o.) pretreated animals were used as positive control. To assess the possible participation of the opioid system in the anti-nociceptive effect of the plant extract, naloxone was injected (4 mg/kg, i.p.) 15 min before the administration of the extract (100 mg/kg, p.o.) or morphine (5 mg/kg, p.o.) in a separate group of animals [30].

Hot plate method

The hot-plate apparatus (Borj Sanat, Iran) was set to 54 ± 0.1 °C. Animals were placed on the hot surface inside the Plexiglas cylinder (20 cm in diameter) and the time (in seconds) spent to licking of their hind paws or jumping (whichever occurred first), was recorded as the pain response latency (reaction time). A 30 seconds cut-off time was set to prevent tissue damage. After recording a baseline reaction time for each animal, they immediately received their own orally administration (25, 50 and 100 mg/kg of the extract, 5 mg/kg of morphine or 10 mL/kg of normal saline) and their reaction time to hot plate was recorded after 30, 45 and 60 min. The mean reaction time in each time point after drug administration was compared to the baseline reaction time in each group for evaluating the optimum response time. To evaluate the between-group comparisons, the percentage of maximum possible effect (MPE%) against thermal stimulus in each time point for each animal was calculated by the following formula [31]:

$$\text{MPE\%}=\frac{\text{test latency-baseline latency}}{\text{cut off time -baseline latency}}\times 100$$

To assess the possible participation of the opioid system in the anti-nociceptive effect of the plant extract, naloxone was injected (4 mg/kg, i.p.) 15 min before the administration of the extract (100 mg/kg, p.o.) or morphine in a separate group of animals.

Anti-inflammatory activity

Carrageenan-induced paw edema in mice

Normal saline (10 mL/kg), the methanol extract of *G. iranicum* (25, 50 and 100 mg/kg) and indomethacin (10 mg/kg) were orally administered 45 min before injection of 1% carrageenan into the sub-plantar area of the mice' right hind paw. Paw volume was measured at base-line and 1, 3 and 5 h after carrageenan injection by using a plethysmometer (Ugo Basile, Switzerland) [32].

Motor incoordination test by rotarod

The test and standard compounds were administered 1 h before placing the animals on the mice specific rotarod apparatus (Borj Sanat, Iran). Animals were orally administrated 25, 50 and 100 mg/kg of the methanol extract of *G. iranicum*. Normal saline (10 mL/Kg) and diazepam (2 mg/kg) were used as the negative and positive controls, respectively. The animals were observed and the time (in seconds) they could remain on rotating rod (7 rpm) was recorded [33].

Statistical analysis

Data have been expressed as mean ± SEM and

analyzed statistically by one-way analysis of variance (ANOVA) followed by Dunnett's post test. P-values less than 0.05 were considered significant.

Results and Discussion

The extraction yields with ethyl acetate and methanol were 1.1% and 16%, respectively. The methanol extract was used for the animal experiments. In the formalin test, pretreatment of animals with the methanol extract of *G. iranicum* roots succeeded in significantly reducing nociceptive response in the first phase only at the dose of 100 mg/kg compared to the control group. In the second phase, significant dose dependent analgesic and anti-inflammatory effects were observed when the extract was administered at all three doses compared to the control group. Morphine demonstrated marked analgesic effect in both phases and indomethacin showed remarkable anti-nociceptive response in the second phase (table 1).

Table 1. Effect of the the methanol extract of *Geum iranicum* roots on formalin-induced pain in mice

Treatment	Dose (mg/kg)	Duration of Licking (s)	
		0-5 min (Neurogenic pain)	15-30 min (Inflammatory pain)
Control	-	62.60±7.332	228.3±44.02
Extract	25	60.00±4.785	142.4±9.190*
	50	55.40±9.304	73.20±5.054***
	100	36.40±3.076*	19.60±6.713****
Morphine	5	26.60±4.118*	36.40±5.202****
Indomethacin	10	79.40±5.006	4.40±2.205****

Results have been expressed as mean±SEM in seconds (n=6); *p<0.05 compared to the control group; ***p< 0.001 compared to the control group; ****p< 0.0001 compared to the control group

Oral administration of the extract (25, 50, 100 mg/kg) significantly (p<0.001) and dose-dependently reduced the number of writing and stretching induced by intraperitoneal injection of acetic acid in mice compared to the control group. The reference drug morphine (5 mg/kg) also produced significant protective effects (p<0.001). Prior administration of naloxone significantly increased the number of abdominal constriction in both morphine (p<0.001) and the extract (p<0.01) groups (table 2).

Table 3 shows the results of the hot plate test. Three doses of the extract increased the reaction time to the thermal stimulus as maximum possible effect (%) in a dose-dependent manner at all experimented times.

Table 2. Effect of the methanol extract of *Geum iranicum* roots on acetic acid-induced abdominal writhing test in mice

Treatment	Dose (mg/kg)	Number of writhing
Control	-	31.00±1.065
Extract	25	9.500±3.603***
	50	4.167±2.442***
	100	3.667±2.539***
Morphine	5	8.333±1.626
Morphine / Naloxone	5/ 4	33.67±1.403###
Extract/ Naloxone	100 / 4	26.43±2.603##

Results have been expressed as mean±SEM; (n=6); *** p< 0.001 compared to the control group; ###p<0.001 compared to the group receiving morphine; ##p< 0.01compared to the group receiving extract (100 mg/kg)

The 25 and 50 mg/kg doses significantly increased reaction time at 30 min and 45 min but not at 60 min. However, the 100 mg/kg dose of the extract did significantly increase the reaction time at all three times. Morphine as a typical opioid analgesic exhibited significant increase in response to thermal stimulus at all three surveyed times.

We further investigated whether naloxone could reverse the anti-nociceptive activity of the extract. Data showed that anti-nociceptive effect of both the extract (100 mg/kg) and morphine were significantly reduced by pretreatment with naloxone when compared to the same group without receiving naloxone.

Intraplantar injection of 1% carrageenan into the mice hind paw induced edema and increased paw volume in the control group. The results of anti-inflammatory activity showed that the extract could exhibit anti-inflammatory activity in a dose-dependent manner. At the dose of 100 mg/kg, the extract showed significant inhibition of paw edema at all surveyed times. The 50 mg/kg dose of the extract significantly decreased inflammation at 1 h and 3 h after carrageenan injection, but the 25 mg/kg dose of the extract could exhibit significant anti-inflammatory activity just at 1 h after carrageenan injection as compared to the control group (table 4).

Rotarod data in table 5 revealed no alteration in motor coordination of mice before and after repeated administration of different doses of the extract in comparison to the control group. Diazepam (2 mg/kg) significantly disrupted motor coordination and decreased the ability of mice to remain on rotating rod. The results demonstrated that the methanol extract obtained from the root of *G. iranicum* Khatamsaz exhibited both antinociceptive and anti-inflammatory activities in experimental animal models.

Table 3. Effect of the methanol extract of *Geum iranicum* roots on the hot-plate test in mice

Treatment	Dose (mg/kg)	MPE (%)		
		30 min	45 min	60 min
Control	-	20.67±7.205	19.17±7.106	12±3.812
Extract	25	43.75±3.326*	40.00±11.32*	26.88±3.399
	50	59.13±5.25**	61.38±7.104**	39.00±3.218
	100	70.75±8.815***	70.29±8.179***	41.63±8.679*
Morphine	5	59.40 ± 11.14*	69.20 ± 10.57**	69.40±9.913***
Morphine / Naloxone	5 / 4	16.20±3.397##	20.20±4.565##	13.60±2.926##
Extract / Naloxone	100 / 4	29.60±6.337##	26.00±7.021##	23.80±16.57#

Results have been expressed as mean±SEM in maximum possible effect (MPE) (n=6); *p< 0.05 compared to the control group; **p< 0.01 compared to the control group; ***p< 0.001 compared to the control group; #p<0.05 compared to the same group without naloxone; ##p<0.01compared to the same group without naloxone

Table 4. Effect of the methanol extract of *Geum iranicum* roots on carrageenan-induced hind paw edema in mice

Treatment	Dose (mg/kg)	Inflammation (%)		
		1 h	3 h	5 h
Control	-	33.4±4.285	32.8±6.184	34.2±4.587
Extract	25	14.60±4.534**	20.2±4.853	29.2±2.853
	50	13.2±3.720**	18.6±3.470*	28.40±1.860
	100	11.76±1.98***	14.32±2.033*	22.42±3.146*
Indomethacin	10	7.8±1.96***	6.6±2.6***	11.07±6.957**

Results have been expressed as mean±SEM; (n=6); *p< 0.05 compared to the control group; **p< 0.01 compared to the control group; ***p< 0.001 compared to the control group

Table 5. Effect of the methanol extract of *Geum iranicum* roots on skeletal muscle relaxant property in mice

Treatment	Dose (mg/kg)	Time to remain on rotating rod (s)
Control	-	100.8±14.16
Extract	25	105.8±14.25
	50	105.2±6.560
	100	103±10.22
Diazepam	2	13.6±9.125***

Results have been expressed as mean±SEM in seconds (n=6); ***p< 0.01 compared to the control group (normal saline)

The formalin test is a clinical valid chemical model which assesses the way an animal responds to moderate and continuous pain [34]. It has been shown that nonsteroidal anti-inflammatory drugs (NSAIDS) exhibit clear anti-nociceptive effects in the formalin test in mouse models [35]. Peripherally acting drugs as indomethacin can reduce nociceptive behavior during the second phase while centrally acting drugs such as morphine could inhibit both phases [36-38]. In this study the extract of *G. iranicum* significantly reduced licking and shaking in the injected paw by dose depended manner. This result supported the hypothesis that the anti-nociceptive action of the plant extract may be mediated by a peripheral mechanism and its potential in the treatment of chronic pain. Writhing test is another chemical model that is generally used for screening anti-nociceptive activity. In this test, acetic acid induces abdominal constriction by releasing endogenous mediators which stimulate the pain nerve endings [39-41]. The extract showed significant inhibition on acetic acid-induced writhing response. The hot plate test is generally used for central anti-nociceptive (supra spinal analgesia) effect in mice [42]. In this study both the extract and morphine increased pain latency, compared with the control group.

Administration of the extract and morphine with naloxone eliminated their analgesic effect, which indicates that the analgesic activity of the extract is partly dependent on endogenous opioid systems [43].

Carrageenan-induced paw edema is a common model of acute inflammation in animals. The edema occurs in different phases and several mediators have roles in each one. The initial phase (1.5 h) is mediated by the release of histamine and serotonin, the second phase from 1.5 to 2.5 h is mediated by bradykinin and the third phase may be mediated by prostaglandin from 2.5 to 6 h after carrageenan injection [40,44]. In the present study, the extract significantly suppressed the paw edema at the first and second phases in a dose dependent manner, suggesting that the possible mechanism of action of *G. iranicum* extract may involve inhibition of prostaglandin biosynthesis.

Several studies have been conducted on the anti-inflammatory and antinociceptive activities of some species of the genus *Geum*. In a research, ethyl acetate fraction of *G. japonicum* methanol

extract suppressed NO production associated with antioxidant activity and direct NO clearance. Furthermore, this fraction inhibited production of stimulated PGE_2, TNF-α and IL-1β [45]. In another study, aqueous extracts of *G. urbanum* (root) and *G. rivale* (herb) showed a potent inhibitory activity on PAF-induced exocytosis. Also, the extract of *G. urbanum* demonstrated high inhibition on prostaglandin biosynthesis. In this study, eugenol was mentioned as one of the active compounds. Several studies reported the inhibitory effect of eugenol on prostaglandin biosynthesis. In a research, the oral administration of eugenol significantly inhibited carrageenan-induced paw edema and exhibited significant antinociceptive activity against chemical stimuli in writhing test [27,46-48].

Ramezani et al. reported both antinociceptive and anti-inflammatory effects of the hydroalcoholic extract of *G. kokanicum* in mice and attributed the observed effects to presence of steroids, triterpenoids and essential oil in the extract [26]. In our previous works, phytochemical analysis of the essential oil and extracts of *G. iranicum* root were performed [49,50]. Eugenol (83.9%) was the major compound in the essential oil. The ethyl acetate extract was rich in fatty acids and steroids. Various compounds including eughenol, niga-ichigoside F1 (triterpene glycoside), catechin, gein (diglycosidic eugenol), hydrolysable tannins and sucrose were isolated from the methanol extract by column chromatography. In the literature, there are some reports referred to the anti-inflammatory and antinociceptive effects of the isolated compounds. Niga-ichigoside F1 isolated from the extracts of *Rubus* species (Rosaceae) was found to have antinociceptive and anti-inflammatory activities. In a formalin-induced pain model, both phases of pain were inhibited by this compound [51,52].

Based on our previous study, the amount of sucrose (measured by HPLC) in the root of *G. iranicum* was considerable [25]. In several animal and human studies, the ingestion of sucrose increased the hypothalamic/CSF opioid level and also the analgesic effects of sucrose was reversed by administration of naloxone, as an opioid antagonist [53]. Anti-inflammatory effects of catechin derivatives have been considered in various studies and catechin was mentioned as a cyclooxygenase 1 inhibitor [54-56]. In traditional medicine, tannin-rich plants have been used for inflammatory disorders. The roots of *Geum* species are rich in tannins. In our study, the total phenol and tannin contents of *G. iranicum* roots methanol extract were 215.42±0.28 and 184.1±0.42 mg gallic acid equivalents per gram of dried extract, respectively; while Oszmianski et al. reported the presence of both condensed and hydrolysable tannins in the root of *G. rivale*. High amounts of ellagic acid and catechin were found in the extract [57]. In another study, the root of *G. urbanum* was referred to as an ellagitannin-rich plant material [58]. Granica et al. reported the role of ellagitannins on modulation of the inflammatory response of human neutrophils [59]. Based on our previous work, the methanol extract of *G. iranicum* was rich in hydrolysable tannins. As noted above, various bioactive compounds in the methanol extract of *G. iranicum* can be responsible for the observed anti-inflammatory and antinociceptive effects.

In conclusion, the results of the present study indicated that the methanol extract of *G. iranicum* roots posessed antinociceptive and anti-inflammatory effects, suggesting this plant as a good candidate for painful conditions. Further experiments are warranted to explore its detailed mechanism of action and isolation of the active constituents.

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Author contributions

Nematollah Ahangar and Somayeh Shahani designed the study, performed data analysis, interpretation of results and manuscript editing; Maryam Feizbakhsh and Sara Pirhayati performed the experimental studies; Fatemeh Mirzaee was involved in manuscript preparation

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

CSF: cerebrospinal fluid; HPLC: high performance liquid chromatography; IL-1β: interleukin-1 beta; I.P.: intraperitoneal; MPE%: percentage of maximum possible effect; NO: nitric oxide; NSAIDS: nonsteroidal anti-inflammatory drugs; PAF: platelet activating factor; PGE2: prostaglandin E2; P.O.: per os; PVPP: polyvinylpolypyrrolidone; SEM: standard error mean; TNF-α: tumor necrosis factor alpha; WHO: world health organization