Original article

Potent anti-nociceptive and anti-inflammatory effects of methanol fraction of *Otostegia persica* extract and its components

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Abstract

Background and objectives: *Otostegia persica* (Labiatae) is an endemic plant of Iran and is used for its anti-inflammatory properties in folk medicine of Sistan and Baluchestan province. The aim of the present study was to investigate the anti-nociceptive and anti-inflammatory effects of *O. Persica* different fractions and identification of the natural compounds from the most active fraction. **Methods:** Total extract of *O. Persica* was fractionated with petroleum ether (PE), chloroform (CL), ethyl acetate (EA), *n*-butanol (BU) and methanol (ME). The analgesic activities of different fractions were determined by formalin test. Then, activity of effective fractions was investigated on carrageenan-induced paw edema assay. Finally, the compounds of effective fraction were isolated and their structures were elucidated. **Results:** Anti-nociceptive activity of EA and BU fractions (100 mg/kg) and ME fraction (100 and 200 mg/kg) demonstrated significant difference with normal saline during the second phase of the formalin test. ME fraction showed higher analgesic effects in comparison to indomethacin ($p<0.05$), with IC_{50} equal to 85.87 mg/kg. Among EA, BU and ME fractions which were selected for anti-inflammatory investigation, EA could not reduce rat paw edema after 6 h. The swelling inhibition percentage of ME was similar to that induced by indomethacin at the same time ($p>0.05$). Vicenin-2 and isorhamnetin-3-*O*-glucoside were elucidated from ME as the effective anti-inflammatory fraction. **Conclusion:** It was concluded that the existence of flavonoids in *O. persica* extract could play an important role for its anti-nociceptive and anti-inflammatory effects similar to various non-steroidal anti-inflammatory drugs (NSAIDS) and inhibitors of nitric oxide synthase (NOS).

Keywords: carrageenan, flavonoids, formalin test, Labiatae, *Otostegia persica*

Introduction

Inflammation is a major and complex process caused by several factors like microbial infections, immunological reactions and tissue injury [1]. Leukocytes, monocytes and macrophages release pro-inflammatory mediators such as nitric oxide (NO), prostaglandin E2 (PGE2), cytokines, tumor necrosis factor (TNF-α) and interleukin-1 (IL-1) in response to

activation signals [2,3]. Phagocytosis of foreign particles is associated with an increase in oxygen uptake by neutrophils and production of large amounts of reactive oxygen species (ROS). Also, the expression of phospholipase A2,5-lipoxygenase (5-LOX), cyclooxygenase- 2 (COX-2) and inducible nitric oxide synthase (iNOS) are increased [4]. The remission of many diseases including arthritis, atherosclerosis, and even cancer is related to the treatment of chronic inflammation [5].

There have been many plants and natural products which have shown anti-inflammatory activities [6,7]. *Otostegia persica* (Burm.) Boiss., (Labiatae), is an endemic plant of Iran [8]. It is used for treatment of headache, diabetes, stomachache, rheumatoid arthritis, toothache in folk medicine of Sistan and Baluchestan province, Iran [9]. The people of Hormozgan province (Iran) consume the aerial parts of *O. persica* for treatment of cough, headache, gastric discomfort, reduction of palpitation, regulating blood pressure, and also as laxative, antipyretic and parasite repellent agent [10]. Previous investigation has shown that the aerial parts of the plant reduced the signs of morphine withdrawal syndrome [11].

The aim of this study was the evaluation of the effective anti-nociceptive and anti-inflammatory fraction of *O. persica* extract and elucidation of its constituents.

Experimental
Plant material
Top flowered aerial parts of *O. persica,* were collected in May 2013, around the Taftan mountain of Sistan and Baluchestan province, Iran and were dried in the shade. A voucher specimen was identified by Dr. Gh. Amin and deposited at the Herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran (TEH-6684).

Extraction and fractionation
The dried aerial parts of *O. persica* (1256 g) were powdered and macerated with 80% methanol at room temperature. This procedure was continued until all compounds of plant were extracted and

controlled by Thin Layer Chromatography (TLC). After concentration under reduced pressure, the crude extract (631.5 g) was fractionated with petroleum ether (PE, 20.2 g), chloroform (CL, 115 g), ethyl acetate (EA, 8.5 g), *n*-butanol (BU, 22.6 g) and methanol (ME, 148.9 g), respectively.

Animals
Albino Wistar rats (150-200 g) of either sex were obtained from the animal house facilities of the Department of Toxicology and Pharmacology, Tehran University of Medical Sciences, Tehran, Iran. Animal had free access to animal feed and water *ad libitum,* and were housed in standard environmental conditions (12/12 h light/dark cycle and 25 ± 2 °C temperature) throughout the study. This study was carried out according to the protocol approved by the animal ethics committee of Tehran University of Medical Sciences (IR.TUMS.REC.1395.2898) and each animal was tested once only.

Anti-nociceptive activity
The analgesic activity of *O. persica* fractions was determined by the reported method for formalin test [12]. The rats were divided into seven groups each containing six. One hundred mg/kg of different fractions according to the pilot study, and indomethacin as the positive control (5 mg/kg) [13] were dissolved in normal saline and administered intraperitoneally in a volume of 0.5 mL. The rats in the negative control group received only 0.5 mL normal saline. Thirty min later, 40 μL of a freshly prepared 1% formalin solution was injected subcutaneously into the right hind paw of each rat. The rats were monitored for 1 h in a standard cage that served as an observation chamber. The time spent licking and biting responses of the injected paw were recorded as pain indicator. Anti-nociceptive effect was determined in two phases. Five min after formalin injection was recorded as the early phase and the period between 15 and 60 min as the second phase. In all stages, each animal was tested only once. The best anti-nociceptive fraction was selected for IC_{50} determination.

Anti-inflammatory activity

Anti-inflammatory activity was measured using carrageenan-induced rat paw edema assay [14]. The rats were allocated randomly to five groups of six animals each. Those fractions which passed the formalin test were selected for anti-inflammation investigation. Different fractions (100 mg/kg) and indomethacin (5 mg/kg) were administered intraperitoneally 1 h before induction of inflammation. Negative control animals received an equal volume of normal saline. In the next stage, 0.1 mL of carrageenan (Sigma-Aldrich, USA) suspension in normal saline (1% w/v) was injected into the sub plantar tissue of the left hind paw of the rats. The paw volumes of rats were measured using a vernier caliper at the end of 0, 30, 60 min and hourly interval for 6 h to determine the diameter of edema. Data were expressed as inhibition percentage according to the formula:

Inhibition percentage= [(C_t-C_0) control-(C_t-C_0) test/(C_t-C_0)control] × 100

Where, C_0 and C_t represented paw volume mean after 0 and t h after carrageenan injection.
The best anti-inflammatory fraction was selected for IC_{50} determination.

Statistical analysis

The data were expressed as Mean ± SEM or Mean ±SD. One Way Analysis of Variances (ANOVA) and post hoc of Tukey were used to analyze the results and $p < 0.05$ was considered significant.

Elucidation of compounds

According to the anti-nociceptive and anti-inflammatory results, ME fraction demonstrated the best activities and it was selected for elucidation of compounds.
ME (5 g) was submitted to column chromatography on Sephadex LH-20 (3.6×53 cm) and eluted with MeOH to give three subfractions. Subfraction 2 (2 g) was subjected to a RP-18 silicagel column (2.8×53 cm) using a step gradient of aqueous methanol (20-80% MeOH), to afford 7 subfractions (MF_{2A}-MF_{2G}). MF_{2E} (70 mg) was further separated on RP-18 silicagel column (1.0×45 cm) eluted with H_2O:

MeOH (9:1) to give 2 subfractions. Compound **1** (8.0 mg) was isolated from MF_{2E1} (30 mg) by chromatography on Sephadex LH-20 column (1.2×67 cm) and methanol as solvent. MF_{2F} (40 mg) was chromatographed on Sephadex LH-20 column (2.5×90 cm) eluted with methanol to yield 4 subtractions. Subtraction MF_{2FD} (7 mg) was pure (compound **2**).
The isolated compounds were identified using different spectroscopic methods.

Results and Discussion

Anti-nociceptive activity

Table 1 has shown the effect of different fractions of *O. persica* extract on formalin-induced pain. All fractions demonstrated analgesic effects like indomethacin (5 mg/kg, *i.p.*) as the positive control in the first phase of study ($p > 0.05$). During the second phase, there were significant difference between normal saline and EA, BU fractions (100 mg/kg) and ME fraction (50, 100 and 200 mg/kg) ($p < 0.05$). ME fraction at the dose of 100 and 200 mg/kg showed higher analgesic effects in comparison to indomethacin ($p < 0.05$ respectively). ME fraction showed the best anti-nociceptive activity, among other fractions of *O. persica* extract with IC_{50} equal to 85.87 mg/kg.

Anti-inflammatory activity

EA, BU and ME fractions which showed anti-nociceptive effects were selected for anti-inflammatory investigation (table 2). All groups markedly reduced swelling in carrageenan-induced rat paw edema model after 6 h, except the group which received EA fraction. ME fraction at the dose of 100, 200 and 400 mg/kg and BU fraction at the dose of 100 mg/kg demonstrated significant anti-inflammatory activities which were comparable with indomethacin after 6 h ($p > 0.05$).
By comparing the inhibition percentage results of different concentration of ME, it was found that there was no dose dependent manner in anti-inflammatory activity of ME fraction.

Spectral data

Vicenin-2: Apigenin 6,8-di-C-β-D-glucoside (**1**)

Table 1. Anti-nociceptive activity of different fractions of *Otostegia persica* extract in formalin test in rats

Groups	Dose (mg/kg)	Phase I (0-5 min) Licking time (min)	Phase I (0-5 min) Inhibition %	Phase II (15-60 min) Licking time (min)	Phase II (15-60 min) Inhibition %
PE fraction	100	58.83±15.50	8.08	56.83±9.16	10.8
CL fraction	100	52.50±14.37	17.97	58.87±13.20	7.6
EA fraction	100	47.00±14.41	26.56	35.33±6.05[a]	44.54
BU fraction	100	45.17±9.31	29.42	43.33±7.01[a]	31.99
ME fraction	50	62.83±6.76	1.83	40.54±5.36[a]	36.37
ME fraction	100	61.00±10.14	4.69	26.83±5.38[ab]	57.88
ME fraction	200	59.66±5.50	6.78	22.12±5.40[ab]	65.28
Indomethacin	40	55.50±3.39	13.28	33.87±5.15	46.84
Normal saline	0.5 ml	64.00±5.79	-	63.71±15.50	-

Results demonstrated as Mean ±SEM and analyzed with ANOVA and Tukey post hoc test; PE: petroleum ether, CL: chloroform, EA: Ethyl acetate, BU: butanol and ME: methanol fractions

a: There was significant difference between remarked group and normal saline ($p<0.05$)

b: There was significant difference between remarked group and indomethacin ($p<0.05$)

Table 2. Anti-inflammatory effects of different fractions of *Otostegia persica* extract in carrageenan test in rat

Groups	Dose (mg/kg)	Mean of Paw volume(mm) 0 min	30 min	60 min	120 min	180 min	240 min	300 min	360 min	Inh%*
EA fraction	100	3.11±0.1	4.40±0.4	4.30±0.2	4.10±0.3	4.51±0.1	5.07±0.5	5.31±0.1	5.32±0.2	-92.17
BU fraction	100	4.10±0.1	4.82±0.2	4.74±0.4	5.26±0.2	5.10±0.6	5.70±0.5	5.30±0.4	4.98±0.5[b]	23.48
ME fraction	100	4.32±0.2	5.06±0.3	4.75±0.1	4.79±0.3	5.11±0.2	5.21±0.5	5.33±0.2	5.08±0.2[b]	33.91
ME fraction	200	3.52±0.2	5.05±0.4	5.12±0.4	4.80±0.3	4.88±0.3	5.16±0.4	4.87±0.3[b]	5.07±0.5[b]	34.78
ME fraction	400	3.93±0.4	4.97±0.3	5.11±0.3	4.72±0.3	4.94±0.5	4.69±0.5[ab]	4.66±0.4[ab]	4.67±0.5[ab]	35.96
Indomethacin	5	3.68±0.4	4.70±0.2	4.65±0.3	4.45±0.3[a]	4.54±0.3[a]	4.34±0.5[a]	4.44±0.5[a]	4.51±0.7[a]	27.83
NS	0.5 ml	4.16±0.1	5.30±0.1	4.75±0.2	5.03±0.2	4.88±0.4	5.21±0.1	5.30±0.3	5.31±0.2	0

Results demonstrated as Mean ±SD and analyzed with ANOVA and Tukey post hoc test ; PE: petroleum ether, CL: chloroform, EA: Ethyl acetate, BU: butanol and ME: methanol fractions; NS: Normal Saline

a: There was significant difference between remarked group and normal saline($p<0.05$)

b: There was no significant difference between remarked group and indomethacin ($p>0.05$)

*: Inh%: Inhibition percentage after 360 min

Yellow amorphous powder; ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.98 (2H, *d*, J=8.5 Hz, H-2′, 6′), 6.91 (2H, *d*, J=8.5 Hz, H-3′, 5′), 6.66 (1H, *s*, H-3), 4.86 (2H, *d*, J=9.68 Hz, H-1″, 1‴), 3.5-4.5 (10H, *m*, H-2″-6″, H-2‴-6‴); EIMS, 40 eV, *m/z*: 296 [Fl+ 2 CH_2], 284 [296-CO], 255 [284- H_2O], 180 [A_1], 118 [B_1].
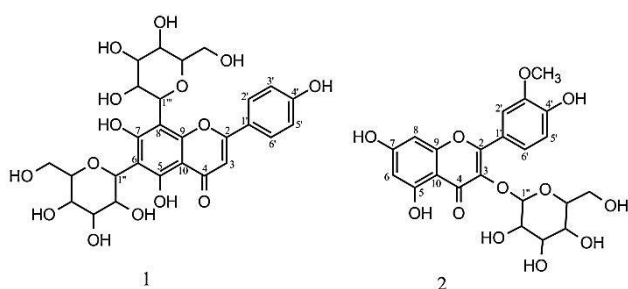
Isorhamnetin-3-*O*-glucoside (**2**)

Yellow amorphous powder; UV (MeOH) λ_{max} 255, 295*sh* and 357 nm; ^{1}H-NMR (400 MHz, DMSO-d_6): δ 8.04 (1H, *bs*, H-2′), 7.40 (1H, *bd*, J = 8 Hz, H-6′), 6.90 (1H, *d*, J = 8 Hz, H-5′), 6.27 (1H, *bs*, H-8), 6.07 (1H, *bs*, H-6), 5.47 (1H, *d*, J = 6.7 Hz, H-1″), 3.81 (3H, *s*, OMe-3′), 3.20-4.30 (5H, *m*); EIMS, 40 eV, *m/z* (%): 315 [M]⁺, 300 [M-Me], 153 [A_1], 136 [B_2], 109 [B].

Structure of compounds **1** and **2** were confirmed by comparison with published data [15-17]

(figure 1).

Formalin test is a valid model for investigation of central (spinal) sensitization after peripheral inflammatory state [18]. In this test, the initial nociceptive scores peaked at 5 min by direct effect of formalin (first phase) and then after 15–60 min (second phase), inflammation was occurred by release of serotonin, histamine, bradykinin and prostaglandins and at least sensitization of central nociceptive neurons [19,20]. The anti-nociceptive effects of different fractions of *O. persica* in the first phase, demonstrated no inhibition activity on peripheral nociception. In the second phase, only EA, BU and ME fractions of *O. persica* showed antinociceptive effects, indicating that the analgesic effects of these fractions were mediated centrally by release of neurotransmitters.

Figure 1. Structure of pure compounds; Vicenin-2 (**1**), Isorhamnetin-3-*O*-glucoside (**2**)

The carrageenan-induced inflammation is a widely used experimental model which consists of two phases. The initial of edema, is not inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, and is mediated through the release of histamine, serotonin and kinins in the first hour. The second phase of swelling is attributed to the release of prostaglandins, lysosome enzymes and slow reacting substances in 3 h and neutrophil infiltration [21-23]. On the other hand, inflammation of second phase is correlated with induction of inducible cyclooxygenase (COX-2) in the hind paw which can be blocked by the NSAIDs [24,25]. Another important mediator in acute inflammation is nitric oxide (NO) which is produced from constitutive and inducible nitric oxide synthase (cNOS and iNOS). cNOS and iNOS are responsible for development and maintenance of inflammation, respectively. The non-selective inhibitors of NOS have shown to inhibit carrageenan-induced paw edema at all-time points whereas the selective iNOS inhibitors inhibited paw edema after 5-10 h [23].

The results of anti-inflammatory investigation of different fractions of *O. persica* demonstrated that they could not reduce inflammation in the first 4 h following carrageenan administration while there was significant difference between indomethacin and normal saline after 2 h ($p<0.05$). It was concluded that the mechanism of action of *O. persica* fractions was different from indomethacin and its fractions could not inhibit cyclooxygenase-2 (COX-2) in the first 4 h of the experiment. The anti-inflammatory activity of

ME fraction at the dose of 400 mg/kg after 4 h and at the dose of 200 and 400 mg/kg after 5 h were comparable to indomethacin ($p>0.05$). BU and ME fractions showed equal responses with indomethacin after 6 h ($p>0.05$). These results indicated that they might act like selective iNOS inhibitors.

Previous studies have demonstrated that ME fraction of *O. persica* was rich in flavonoids including kaempferol, quercetin, apigenin derivatives and 3′,7-dihydroxy-4′,6,8-trimethoxy-flavone [26-28]. In the present study, other flavonoids named isorhamnetin-3-*O*-glucoside and vicenin-2 (apigenin 6,8-di-C-b-D-glucoside) were elucidated from methanol fraction of *O. persica*. There were many reports about the anti-inflammatory effects of flavonoids [29,30]. Nuclear factor-kappa B (NF-κB) and signal transducer and activator of transcription 1 (STAT-1) are two critical transcription factor for iNOS. It has been approved that kaempferol, quercetin, apigenin and isorhamnetin inhibited the activation of NF-κ B while kaempferol and quercetin also inhibited the activation of STAT-1 [31,32]. Other studies exhibited kaempferol, quercetin, apigenin and isorhamnetin could inhibit COX-2 expression [33,34]. The mentioned mechanisms for *O. persica* flavonoids confirmed our results about anti-inflammatory effects of ME fraction.

O. persica showed both anti-nociceptive and anti-inflammatory effects like various non-steroidal anti-inflammatory drugs (NSAIDS). It was concluded that the existence of flavonoids in ME fraction of *O. persica* could play an important role in inhibition of iNOS and COX-2 expression.

Declaration of interest

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

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