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Isoflavonoids as wound healing agents from Ononidis Radix

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ABSTRACT

Ethnopharmacological relevance: Dried roots of *Ononis spinosa* L. are traditionally used for their diuretic, anti-inflammatory and wound healing effects.
Aim of the study: Isolation of the bioactive compounds of *Ononis spinosa* L. subsp. *leiosperma* (Boiss.) Sirj.
Materials and methods: Ethyl acetate extract prepared from the roots of *Ononis spinosa* L. subsp. *leiosperma* (Boiss.) Sirj. was subjected to silica gel column. The fractions were tested for their wound healing and anti-inflammatory activities. Linear incision and circular excision wound models and hydroxypyroline estimation assay were used for the wound healing activity. Carrageenan-induced hind paw edema, TPA-induced ear edema and acetic acid-induced increase in capillary permeability tests as acute inflammation; FCA-induced arthritis as chronic inflammation models were used for the assessment of anti-inflammatory activity. Antioxidant capacities of the fractions were tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) scavenging activity assay, reducing power assay and hydroxyl radical (OH^-) scavenging assay. The isolation procedure was continued with the active fraction (Fr-E5).
Results: Fr-E5 exhibited remarkable wound healing activity with the 33.4% tensile strength value on the linear incision wound model and 51.4% reduction of the wound area at the day 12 on the circular excision wound model. Hydroxyproline content of the tissue treated by Fr-E5 was found to be 30.9 ± 0.72 μg/mg. Acetic acid induced increase in capillary permeability test results revealed that Fr-E5 inhibited inflammation by the value of 40.3%. Fr-E5 showed 28.1–32.2% inhibition in carrageenan-induced hind paw edema test while did not possess activity on TPA-induced ear edema and FCA-induced arthritis models. Trifolirhizin, ononin, medicarpin-3-*O*-glucoside, onogenin-7-*O*-glucoside and sativanone-7-*O*-glucoside were isolated from Fr-E5 and tested for their wound healing activities using by measuring their inhibition of hyaluronidase, collagenase and elastase enzymes. Ononin and sativanone-7-*O*-glucoside inhibited hyaluronidase and elastase enzymes by 31.66% and 41.75%; 45.58% and 46.88% values respectively at the dose of 100 μg/mL.
Conclusion: Among five isolated compounds, ononin and sativanone-7-*O*-glucoside were found to inhibit hyaluronidase and elastase enzymes. According to the results, these compounds may majorly be responsible for the wound healing activity of the extract.

1. Introduction

The genus *Ononis* L. which belongs to Fabaceae family, is represented by more than 75 species in Europe as well as Atlantic Islands, West Asia and North Africa (Al-Khalil, 1995; Reyes-Betancort and Scholz, 2008). Dried roots of the plant is recorded in European Pharmacopoeia as “Ononidis Radix”. The roots of *O. spinosa,* are known to be used against irritations of the skin, itches, wounds and dermatitis in Central Asia and Russia (Mamedov et al., 2005). *O. spinosa* which is widely known as “kayiskiran” in Turkey, is used as a folk remedy against urinary tract diseases and kidney stones due to its anti-inflammatory and diuretic effect as well as against eczema and some other skin disorders for wound healing (Altanlar et al., 2006; Sever Yilmaz et al., 2006). The roots of *O. spinosa* are traditionally used for wound healing externally (Baytop, 1999). The decoction prepared from the roots is widely used against skin disorders, wounds and burns (Çakilcioğlu and Türkoğlu, 2010; Ecevit Genç and Özhatay, 2006). The use of Ononidis Radix due to its diuretic and anti-inflammatory effects is recorded in the monographs of Comission E (Blumenthal, 1998) and ESCOP (2003). It is also known that the roots of this species are used for anti-inflammatory and diuretic purposes (Leporatti and Ivancheva, 2003).

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Phytochemical studies on *O. spinosa* reveals that flavonoid derivatives (such as trifolirhizin, ononin, formononetin, genistein, biochanin A, daidzin, medicarpin, homopterocarpin), sterols (β-sitosterol, campesterol, stigmasterol, stigmastan-3,5-dien), terpenic substances (β-amyrin, α-onocerin) (Benedec et al., 2012; Daruhazi et al., 2008; Jimenez-Gonzales et al., 2008; Klejdus et al., 2008, 2007).

Isoflavonoid derivatives, which widely occur in Fabaceae plants, not only have a role on the defense mechanism of the plants but also are important constituents for human health. (Jasinski et al., 2009). Isoflavonoids are found to possess for several biological activities especially eustrogenic, antimicrobial, antioxidant and anti-inflammatory activities. Besides, it is known that these compounds may be used for the prevention of cardiovascular diseases and cancer (Sharma and Ramawat, 2013; Yu et al., 2016). Although there are quite a number of studies which proves the anti-inflammatory activity of isoflavones using *in vivo* and *in vitro* models, the mechanism underlying the activity have not been clarified yet. According to the investigations, antiinflammatory activity of isoflavones may arise from different mechanisms. Antioxidant activity; inhibition of proinflammatory cytokines and chemokines such as interleukins and tumor necrosis factor-α as well as inhibition of NF-κB transcriptional system and proinflammatory enzymes such as prostaglandins, leukotrienes, arachidonic acid and nitric oxide (Yu et al., 2016).

Although there are several ethnopharmacological researches that report the traditional uses of *O. spinosa*, the studies, which scientifically prove the traditional uses are insufficient. In this study, it is aimed to isolate bioactive compounds from the roots of *O. spinosa*. For this purpose; extracts were prepared from the roots of *O. spinosa* L. subsp. *leiosperma* (Boiss.) Sirj. by using *n*-hexane, ethyl acetate and methanol respectively. Wound healing and anti-inflammatory of the extracts were investigated. Linear incision and circular excision wound models and hydroxypyroline estimation assay were conducted for the investigation of wound healing activity. Acute inflammation models *i.e.* acetic acid-induced increase in capillary permeability test, carrageenan-induced hind paw edema and TPA-induced ear edema models as well as a chronic inflammation model *i.e.* FCA-induced arthritis model were used for the assessment of anti-inflammatory activity. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) scavenging activity assay, reducing power assay and hydroxyl radical (OH-) scavenging assay were conducted for the evaluation of antioxidant capacities of the fractions. Wound healing activities of isolated compounds were evaluated by the investigation of the inhibition of hyaluronidase, collagenase and elastase enzymes.

2. Material and methods

2.1. Plant material

Plant samples were collected from Konya, Central Anatolia (37° 15, 245N; 32° 08, 485 E; 1249 m) in June 2011. Taxonomic identification of the plants were confirmed by Hayri Duman from Gazi University, Department of Biological Sciences, Faculty of Art and Sciences. The voucher specimens are kept in the herbarium of Ankara University Faculty of Pharmacy (AEF26000). The plant name was checked with http://www.theplantlist.org/tpl1.1/record/ild-9255.

2.2. Extraction and isolation

2.2.1. Extraction

The roots of the plant materials were dried at room temperature. Dried and powdered materials (1200 g) were extracted with *n*-hexane (12 L × 3) and ethyl acetate (12 L × 3) respectively at room temperature by continuous stirring. 24 h of extraction period was repeated three times with fresh solvent for each extract. Following the extraction, each extract was filtered. At the end of the extraction with ethyl acetate, the solvent was removed under reduced pressure at 50 °C.

2.2.2. Fractionation of the ethyl acetate extract of O. spinosa roots

The ethyl acetate extract of *O. spinosa* roots were subjected to silica gel column (Kieselgel 60, 70–230 mesh, 0.063–0.200 mm, Merck) using dichloromethane: methanol mixture starting with pure dichloromethane and increasing concentration of methanol, subsequently dichloromethane: methanol (50:50, v/v) and pure MeOH. The fractions were combined according to the TLC analysis and six fractions (Fr-E1-6) were obtained.

2.2.3. Fractionation of Fr-E5

Fr-E5 was subjected to silica gel column initially with pure dichloromethane and then with dichloromethane: methanol (60:40, v/v). Fractions of 100 mL were collected (Sfr-1–92) and combined according to the TLC analysis yielding 16 subfractions and a pure compound (OS-3) which was crystalized from Sfr-16.

2.2.4. Isolation and purification procedure

Sfr-15–16 was subjected to preparative TLC using silica gel coated plate (Silika gel 60 F_{254}, aluminium plates, 20 × 20) and ethyl acetate: methanol:water (100:13.5:7, v/v) mixture as a mobile phase yielding five subfractions (Prep1-5) one of which (Prep2) was crystalized as a pure compound (OS-1). Subfractions Prep3 and Prep5 were subjected to sephadex column using methanol as the mobile phase. Two compounds were crystalized from the subfractions of Prep5 (OS-2 and OS-3). Two other compounds were purified from subfraction 11–12 obtained from the sephadex column of Prep3 using semipreparative HPLC. HPLC conditions were shown in Table 1.

Sfr-17-18 and Sfr-19-27 were subjected sephadex column using methanol as eluent. OS-3 is crystalized from Sfr-19-27 and OS-1, OS-3 were crystalized from sephadex column fractions of Sfr-17-18. OS-4 and OS-5 were obtained from the subfractions of Sfr-17-18 using semipreparative HPLC (Table 1).

The composition of the extract and fractions were analyzed using HPLC where acetonitrile and 0.02% *o*-phosphoric acid in water are used as gradient elution. The analysis started with 10% acetonitrile and the percentage of acetonitrile was increased to 100% in 36 min and at the end of the analysis an isocratic flow of acetonitrile was used to clean the column for 4 min. For the subfractions, the analysis were cut at 25 min. ACE 5 C18 (250 mm × 4,6 mm; 5 μm) column and Diode Array Dedector (210 nm) was used for the analysis with the flow rate of 1 mL/min and the injection volume was 10 μL.

Isolation and purification procedure is summarized at Fig. 1.

2.3. Structural analysis of isolated compounds

The structure of the compounds were established by MS, ^{1}H- and ^{13}C NMR as well as 2D NMR (COSY, TOCSY, HSQC, HMBC) techniques using Varian Mercury 400, 400 MHz High Performance Digital FTNMR Spectrometer. Waters 2695 Allia Micromass ZQ LC/MS was used for the mass spectrometric analysis.

Table 1
Semipreparative HPLC conditions.

Column	ACE 10 C18 (250 mm × 10 mm; 10 μm)	
Column Temperature	40 °C	
Detector/Wavelength	Diode Array Dedector/210 nm	
Flow Rate	5 mL/min	
Injection Volume	25 μL	
Mobile Phase		
Time	***Acetonitrile (%)***	***Water (%)***
0	10	90
15	47.5	52.5
15.01	100	0
20	100	0