



Pharmacognostic and Anti-Inflammatory Properties of *Securigera securidaca* Seeds and Seed Oil

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Abstract

Background and objectives: Although weed plants are considered undesirable in a particular situation, some weed seeds can be a valuable and cheap source of therapeutic natural compounds. *Securigera securidaca* (L.) Degen & Dorfl (Fabaceae) is widely distributed in Europe, Australia and Asia as a weed plant. This study investigated the bioactive compounds of *S. securidaca* seeds as well as its potential anti-inflammatory properties. **Methods:** The fatty acid and sterol content were investigated with gas chromatography–mass spectrometry (GC-MS) and phenolic compounds were detected using high performance thin layer chromatography (HPTLC). The thermostability of the oil was studied using differential scanning calorimetry (DSC). Formalin-induced paw licking test and myeloperoxidase activity were investigated. The study was conducted by creating six groups of rats including a control group (vehicle-treated rats, 250 μ L/kg, i.p.), formalin group (50 μ L of 2.5% formalin), positive control (paracetamol, 100 mg/kg, i.p), and groups of *S. securidaca* seed oil (250, 500, 1000, 2000, and 4000 μ L/kg, i.p). **Results:** *S. securidaca* seeds contained a high level of polyunsaturated fatty acid content including linoleic acid ($64.602 \pm 0.793\%$) and oleic acid ($15.353 \pm 0.461\%$). Stigmasterol and campesterol were not detected in the oil but it contained esterified β -sitosterol (6.621 ± 0.08 mg/g). The seed oil couldn't create a significant reduction in the MPO activity. It showed a slight but not significant effect on formalin-induced pain reduction. **Conclusion:** The seed is a rich source of linoleic acid which makes it a good candidate to be used in the pharmaceutical industry.

Keywords: Hatchet Vetch; PUFA; *Securigera securidaca*; seed oil; Weed seed

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Introduction

Fabaceae is known as the second-largest family of medicinal plants, most of which have been used as traditional therapeutic remedies for different inflammatory diseases in various regions of the world, e.g., China, Japan, Iran, and India. In addition to the traditional use in folk medicine, many species of this family possess important phytochemical properties that have caused them to be widely considered as major components of pharmaceutical products. *S. securidaca* (L.) Degen & Dorfl. (Syn.: *Coronilla*

securidaca L., *Securigera coronilla* DC.) with the common names: goat pea, hatchet vetch, scorpion vetch, ax, or weed seed is an herbaceous plant belonging to the family Fabaceae [1]. The herb is widely distributed in Europe, Australia, and Asia and can be found in Iran too [2]. There are numerous records regarding the traditional application of the herb and seeds. Local practitioners and traditional healers have recommended the use of this seed which is called "Gandeh talkheh" in Persian, for the management

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of high blood pressure, hypercholesterolemia, wound healing, gastric reflux, malaria, hyperglycemia, and some inflammatory diseases [3,4].

There is considerable documentation concerning the evaluation of different pharmacological effects of *S. securidaca* seeds. These seeds have been investigated for anti-parasite [5], anti-seizure [6], anti-hypertensive [7], anti-ulcerogenic [8], anti-nociceptive [9], antioxidant [7], antiviral [4], antitumor [10], and hypoglycemic effects [11-13].

Despite the numerous pharmacological studies on the *S. securidaca*, there is not much scientific data available regarding the phytochemical and pharmacognostical assessments of the seed's oil. A previous investigation revealed that the seed extracts was very rich in flavonoids. Moreover, hydroxycoumarins, flavones, tannins, saponins, alkaloids, and cardenolides were reported from the ethanol or aqueous extracts of *S. securidaca* seeds [14-16]. Recently, amino acids such as alanine, arginine, asparagine, aspartic acid, glutamic acid, tyrosine, and valine have been identified in the composition of these seeds [17]. However, a more comprehensive assessment focusing on the pharmacognostic properties of the seed's oil was unavailable. In this regard, the current work was aimed to investigate the phytochemical constituents as well as the anti-inflammatory and analgesic activities of *S. securidaca*.

Material and Methods

Ethical considerations

Animal studies were approved by Local Research Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran with the ethical code of 2018-362.

Plant material

The *S. securidaca* seeds were collected in July 2015 from Fars province, Iran, and authenticated by the Department of Pharmacognosy Herbarium, School of Pharmacy, Shiraz University of Medical Sciences. Its voucher specimen was preserved with the code PM-51 for further references. The seed powder was passed through a number 100 sieve and kept in a dark sealed glass container.

Physicochemical analysis

Based on the standard WHO guidelines analysis methods for quality control of herbal medicines,

total ash, water soluble ash, and acid insoluble ash were determined [18]. The extraction of 100 g of seed powder with *n*-hexane was carried out by Soxhlet apparatus (6h). The dried residue was macerated for 48 h with dichloromethane and then with ethanol. The hexane, dichloromethane, and ethanolic fractions were concentrated and dried by a speed vacuum rotary evaporator at 40 °C. The solvent-free *n*-hexane fraction was considered to contain the fixed oil of the seeds. The dried fractions were weighed out and kept in Teflon capped tubes at -20°C.

Measurement of the seed oil's refractive index was performed with an automatic ATAGO Rx7000- α digital refractometer (ATAGO, Japan) at 40 °C. The seed oil's thermal behavior was studied using differential scanning calorimetry (DSC) in a Bahr DSC 310 calorimeter (Germany). The temperature was correlated with indium. The oil was cooled from 25 °C to -20 °C at 5 °C/min, maintained at -20 °C for 5 min and heated back to 300 °C. An empty DSC pan was used as an inert reference to balance the heat capacity of the sample pan.

High performance thin layer chromatography (HPTLC)

HPTLC was used for screening of the seeds' primary and secondary metabolites. A volume of 10 μ L dichloromethane and ethanol fractions (5 mg/mL) and 2 μ L of the fixed oil were applied with HPTLC (CAMAG) to the silica gel plate 60F₂₅₄ (10-20 cm, Merck). Four different solvent mixture with various polarity were used as the mobile phases including non-polar (toluene-acetone, 80:20, MP1), semi-polar (toluene-chloroform-acetone, 40:25:35, MP2), polar (*n*-butanol- glacial acetic acid-water, 50:10:40, MP3) and chloroform-acetone- water (98:1.99:0.01, MP4). Ultraviolet light at 254 and 365 nm were first used for chromatographic spots visualizing and then different reagents including phosphomolybdic acid reagent (vis.), dragendorff, 5% potassium hydroxide (vis. & UV365 nm), orcinol, NP reagent (ethanolamine diphenylborate/PEG, UV365 nm), Liebermann-Burchard (UV365 nm or vis.), vanillin-sulfuric acid, 3% iron trichloride and anisaldehyde-sulfuric acid (vis.) were sprayed onto the plates [19,20]. The chemicals and solvents used for HPTLC of the fractions were of analytical grade and purchased from Merck (Germany) or Sigma Aldrich (United States).

Fatty acids derivatization

Fatty acid methyl esters were prepared based on the procedure explained by AOAC [21]. Oil or standard fatty acids (0.2 g) were added to a Teflon capped test tube. Then to the samples, 1 mL of toluene and 2 mL methanolic sulfuric acid (1%) were added, followed by 0.1 mL of hexadecanoic acid (2 g/L), as an internal standard. After overnight incubation at 50 °C, a 5% NaCl solution was added and the prepared esters were extracted with *n*-hexane (2×5 mL). This extract was washed with 2% sodium bicarbonate solution. Anhydrous Na₂SO₄ was used to remove the remaining water. The sample tubes were centrifuged for 10 min at 3000 rpm. The supernatant was collected in a test tube and the solvent (*n*-hexane) was removed under a stream of nitrogen and kept at -20 °C. Later, 500 µL of HPLC grade *n*-hexane was used to dissolve the samples, followed by the gas chromatography-mass spectrometry (GC-MS) analysis.

Isolation and TMS derivatization of sterols

After adding internal standards (0.2 mg of free cholesterol and cholesteryl heptadecanoate), 300 µL of the oil was applied to packed silica gel in a pipette pasture and fractionated sequentially with A: 10 mL diethyl ether-hexane- (1:200, v/v, fraction 1), B: 10 mL diethyl ether- hexane (4:96, v/v, fraction 2), and C: 10 mL diethyl ether-acetic acid (100:0.2, v/v, fraction 3). Fraction 1 eluted wax esters and sterol esters while fraction 2 and fraction 3 extracted triacylglycerols and free sterols, respectively. Solvent-free fractions were obtained after drying under a stream of nitrogen. Saponification of sterol esters were carried out in a tube containing 1 mL of potassium hydroxide (33%) and 4 mL of ethanol (96%). This mixture was refluxed at 80 °C for 1 h and then cooled to room temperature. After washing this mixture with 2.5 mL distilled water, the unsaponifiable matter were extracted with 3 mL *n*-hexane (×3). The collected *n*-hexane fraction was dried under a stream of nitrogen. For preparing the TMS-derivatives of the sterols, the mentioned *n*-hexane fractions (unsaponifiable matter) was incubated in 200 µL of N,O-bis (trimethylsilyl) trifluoroacetamide at 60 °C for 2 h [22-24].

GC-MS analysis of fatty acids and sterols

After derivatization, fatty acid and sterols were

analyzed by a Hewlett-Packard 6890 GC-MS instrument. The gas chromatography was performed on an HP-5MS capillary column (phenylmethylsiloxane, 25 m, 0.25 mm i.d.).

The temperature of injector was fixed at 250 °C and the positive ion electron impact mass spectra were obtained at ionization energy of 70 eV. The carrier gas was helium with a flow rate of 1 mL/min. For fatty acid methyl esters, the oven temperature was increased from 160 °C (2 min) to 230 °C with a rate of 8 °C/min. It was held at 230 °C for 20 min.

For TMS derivatives of sterols, the column temperature was fixed at 230 °C for 1 min, and then increased to 275 °C at a rate of 1 °C/min. Finally, the oven was held at 275 °C for 30 min. For sterols, the injection temperature was 275 °C [22,25].

All of the reference compounds including standard fatty acids, stigmasterol, β-sitosterol and campesterol were purchased from Sigma (USA). Phytosterols and fatty acids in the seed oil were identified by comparing their mass spectra and retention times with those of the reference compounds as well as the mass spectra in the literature [25]. For those molecules for which neither the standard compounds nor reference spectra were available, chemical structures were determined according to the general patterns of mass spectrometric fragmentation [26].

Experimental animals

For this investigation, Sprague-Dawley female rats (180-220 g) were used. The animals were supplied from the animal center of Shiraz University of Medical Sciences, Shiraz, Iran. All of them were maintained for one week under light/ dark cycle at 25–30 °C. We adhered to all procedures as per the Shiraz University of Medical Sciences animal care and treatment guidelines. After overnight (18 h) fasting with free access to water, the animals were randomly divided into eight groups (n = 6) including (I) control group (vehicle-treated rat, DMSO 250 µL/kg, i.p.), (II) formalin group (50 µL of 2.5% formalin (v/v in distilled water), (III) positive control that received the paracetamol (APAP, 100 mg/kg, i.p.), and groups (IV-VIII), *S. securidaca* seed oil at doses of 250, 500, 1000, 2000, and 4000 µL/kg, i.p., respectively. For groups III-VIII, formalin was injected 60 min after treatments. The anti-nociceptive and anti-inflammatory properties of these doses were assessed in all groups according to flowing methods.

Formalin-induced paw licking in the rat

Sixty minutes after administration of vehicle, APAP or oil, formalin was subcutaneously injected into the plantar surface of the animals' right hind paw. Immediately after the sub-plantar formalin injection, the animals were placed in a chamber with a mirror positioned under, with a 45° angle underneath the floor to allow an unobstructed view of the paw. The nociception measurement was based on the following observational score: zero, no response in injected paw; one, limping or resting the paw lightly on the floor; two, elevation of the injected paw; three, licking or biting of the injected paw [27,28]. All responses were recorded for 45 min after the formalin injection. There were two reaction phases considered in the animal behavioral responses, first phase after 5 min and second phase between 15 and 45 min after formalin injection.

Myeloperoxidase activity

The Myeloperoxidase (MPO) activity measurement is widely used as a biochemical marker of many inflammatory diseases that are mostly accompanied by granulocyte infiltration [29]. Intraperitoneal injection of DMSO (250 μ L/kg, control) or the oil (250, 500, 1000, 2000, and 4000 μ L/kg) were done 60 min before the intra-plantar injection of formalin to the animal's paw. Six animals were considered for each group. After 4 h, pentobarbital was injected (50 mg/kg, i.p.) for anesthesia, and then paw tissues with the maximum inflammation were isolated and homogenized (IKA Homogenizer, Germany) in a solution with pH 6 containing 0.5% hexadecyltrimethyl-ammonium bromide (HTAB). The tissues were dissolved in 50 mM potassium phosphate buffer. After 20 min at 4 °C centrifugation at 3000 rpm, 0.1 mL of the aliquot supernatant was allowed to react with a 2.9 mL solution of 50 mM potassium phosphate buffer (pH 6) containing 0.0005% hydrogen peroxide and 0.167 mg/mL of O-dianisidine hydrochloride. To stop the reaction (after 5 min), 0.1 mL of 1.2 M hydrochloric acid was added to the previous solution. The absorbance change rate was measured by a spectrophotometric method at 400 nm (Cecil 9000, UK) and then the MPO activity was expressed in milliunits (mU) per 100 mg weight of wet tissue [30].

Results and Discussion

The total ash including physiological and non-physiological ashes was determined as 67.50 ± 9.35 (mg/g) from seed. The acid insoluble and water soluble ash values were determined as 16.65 ± 2.68 and 35.03 ± 9.88 (mg/g), respectively. The *n*-hexane, dichloromethane, and ethanol fraction values of *S. securidaca* seed oil were determined as 4.21%, 0.11%, and 0.53% (w/w), respectively. The primary and secondary metabolites of the seed were screened using high performance thin layer chromatography (HPTLC) with variety of chemical reagents and UV detectors (figure 1). The MP4 system was chosen as the ideal mobile phase for the *n*-hexane fraction (fixed oil). This fraction visualized by the Liebermann–Burchard indicator showed the presence of different fatty acids and steroidal triterpenes. Other secondary metabolites such as tannins, flavonoids, glycosides as well as an alkaloid spot were detected in the ethanol fraction (HPTLCs are not shown here).

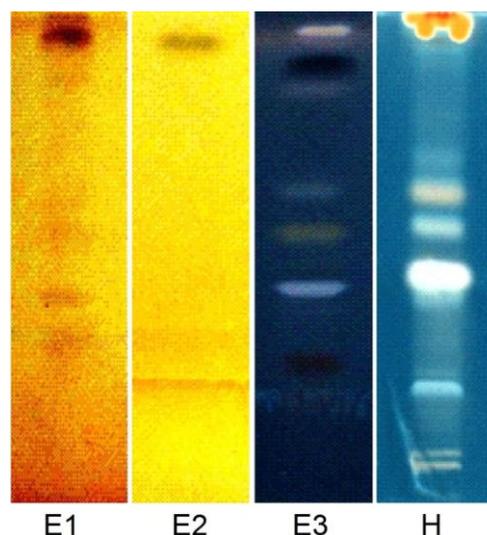


Figure 1. HPTLC fingerprints of *S. securidaca* seeds oil fractions run in different mobile phases (MP) and treated with, orcinol (E1), FeCl_3 (E2) and Liebermann-Burchard (E3, H). E1, E2 and E3: ethanol fraction run in MP3; H: fixed oil run in MP4

The total unsaturated fatty acid content of this seed oil was determined to be about 82.90%. The results of the present investigation showed that the main fatty acid in the seed oil was linoleic acid ($\text{C}_{18}:2$, $64.602 \pm 0.793\%$). The second major fatty acid was oleic acid ($\text{C}_{18}:1$, 15.353 ± 0.461), other fatty acids (1-8%) were stearic acid ($\text{C}_{18}:0$), behenic acid ($\text{C}_{22}:0$), and gondoic acid ($\text{C}_{20}:1$) (table 1). In addition to the fatty acid components, some normal alkanes and two aromatic

constituents were detected in the oil too (table 2). In addition to the noted compounds, the presence of principal phytosterols including β -sitosterol, stigmasterol, and campesterol were investigated in the seed oil. Stigmasterol and campesterol were not detected in the oil but the presence of the esterified β -sitosterol (6.621 ± 0.08 mg/g) was detectable.

Table 1. Fatty acids detected in *Securigera securidaca* seed oil

Retention time (min)	Fatty acid name	Formula	Percentage (mean \pm SD)
5.628	Tetradecanoic acid	C ₁₄ :0	0.820 \pm 0.003
6.788	Pentadecanoic acid	C ₁₅ :0	0.418 \pm 0.010
7.737	9-Hexadecenoic acid	C ₁₆ :1	0.515 \pm 0.001
8.023	Hexadecanoic acid	C ₁₆ :0	0.328 \pm 0.004
9.143	Heptadecanoic acid	C ₁₇ :1	0.474 \pm 0.026
10.086	9,12-Octadecadienoic acid	C ₁₈ :2	64.602 \pm 0.793
10.126	9-Octadecenoic acid	C ₁₈ :1	15.353 \pm 0.461
10.326	Octadecanoic acid	C ₁₈ :0	8.554 \pm 0.207
12.441	11-Eicosenoic acid	C ₂₀ :1	1.548 \pm 0.072
12.818	Eicosanoic acid	C ₂₀ :0	0.166 \pm 0.001
14.567	Heneicosanoic acid	C ₂₁ :0	0.120 \pm 0.047
16.87	Docosanoic acid	C ₂₂ :0	2.480 \pm 0.197
19.865	Tricosanoic acid	C ₂₃ :0	0.319 \pm 0.155

Table 2. Aromatic and alkane constituents of *Securigera securidaca* seed oil.

Retention time (min)	Aromatic or alkane component name	Formula	Percentage (mean \pm SD)
3.519	Phenol,2,4,bis(1,1,dimet hylethyl)	C ₁₄ H ₂₂ O	0.997 \pm 0.120
4.239	Hexadecane	C ₁₆ H ₃₄	0.506 \pm 0.014
6.457	Octadecane	C ₁₈ H ₃₈	0.495 \pm 0.032
8.263	Methyl-3(3,5-di-tert-butyl-4-hydroxyphenyl) propionate	C ₁₈ H ₂₈ O ₃	1.051 \pm 0.028
8.806	Eicosane	C ₂₀ H ₄₂	0.382 \pm 0.065
11.069	Docosane	C ₂₂ H ₄₆	0.467 \pm 0.041

Accordingly, based on the unique DSC profile for different oils which can provide useful information about the fatty acid and triacylglycerol profiles of these oils, we investigated the thermal behavior of *S. securidaca* oil with a DSC apparatus (figure 2) [31,32]. The endothermic peak at 1.938°C exhibited the melting point of this oil. Several small transition peaks could be observed at temperatures higher than 50 °C, which might be due to the presence of unsaturated components in the oil. But overall, the oil could be considered thermostable up to 300 °C.

The high content of polyunsaturated fatty acid (PUFA) in *S. securidaca* and its applications in traditional medicine encouraged us to investigate its anti-inflammatory potential as well. Maximum thickness and inflammation resulted 4 h after the formalin intra-plantar injection to the animals' paw. At this time point,

neutrophil infiltration was observed in the paw tissue.

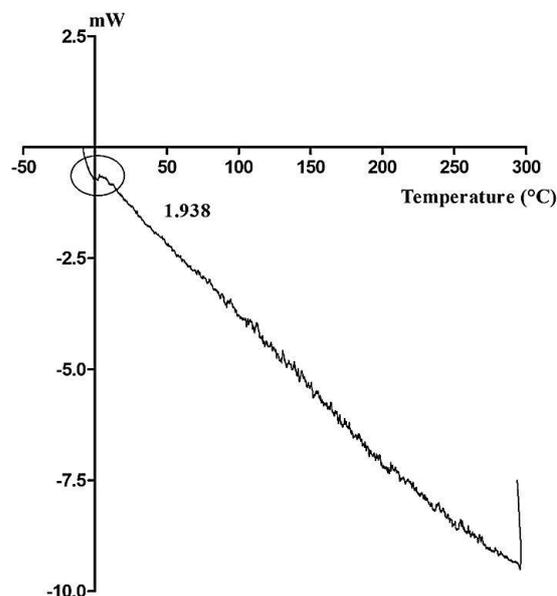


Figure 2. DSC profile of *Securigera securidaca* seed oil.

There was a clear increase in the myeloperoxidase (MPO) activity in the paw tissue (1 ± 0.02 U/mg of wet tissue in formalin-injected rats compared to 0.5 ± 0.03 U/mg of wet tissue in the control rats).

The effect of the seed oil in the primary (0-5 min) and secondary (20-60 min) phases of the paw edema test have been shown in figure 3A. In both cases, 100 mg/kg of APAP showed a significant nociceptive effect compared to the formalin group. The seed oil of *S. securidaca* in different doses (250, 500, 1000, 2000, and 4000 μ L/kg, i.p.) showed a slight effect on reduction of the formalin-induced pain. Unfortunately, none of the *S. securidaca* seed oil doses could create a significant reduction in MPO activity (figure 3B). Although, this study indicated that the *S. securidaca* seed oil contained a high percentage of unsaturated fatty acids that could exhibit suitable anti-inflammatory activity, it didn't show a significant effect on the acute inflammation model.

There are many available reports on the anti-inflammatory potential of the Fabaceae family, for instance, the African traditional plant, *Tamarindus indica* used for wound healing purposes has an effect on inflammations [33,34]. The various species of *Glycyrrhiza* genus such as *G. glabra*, *G. inflata*, and *G. uralensis* have been used in traditional medicines as anti-inflammatory therapeutics [35].

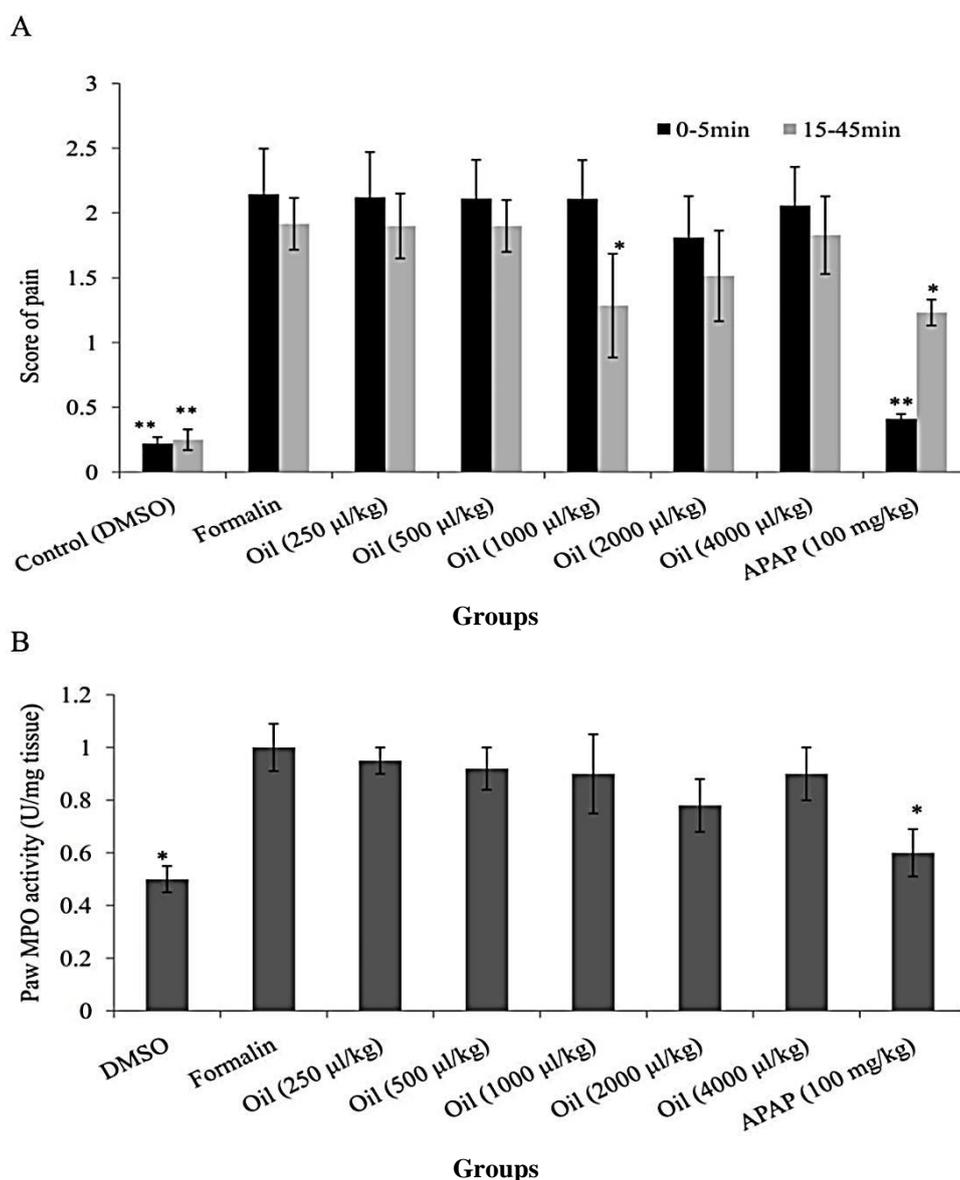


Figure 3. The effect of *S. securidaca* seed oil on inflammatory pain induced by formalin (A) and myeloperoxidase (MPO) activity in the rats' paws (B). Formalin (50 µL of 2.5%) was injected into the plantar surface of rat. Sixty min. before formalin injection, the animals were pretreated with *S. securidaca* seed oil (250-4000 µl/kg; i.p), DMSO (control) or paracetamol (APAP; 100 mg/kg, i.p.). Score of pain was detected in 0-5 and 15-45 min. after formalin injection. MPO activity was detected in the paw tissue after 4 h of formalin injection. The values are expressed as the mean±S.D (n = 6-8). * p< 0.05 and ** p< 0.01 indicate statistically significant differences from the formalin group.

Additionally, other herbs of this family such as *Trigonella foenum-graecum* has been shown to reduce inflammation levels significantly [36]. Many studies have indicated that the lipophilic components of the Fabaceae plants such as the steroids, fatty acids, and triterpenes are involved in the anti-inflammatory activity of these plants [37]. During acute inflammation, polymorphonuclear neutrophils (PMNs) including neutrophil, eosinophils, basophils, and mast cells play an important role in producing

inflammatory factors [38]. The activated PMNs release myeloperoxidase (MPO), which acts as a primary inflammatory process in the body and as a defensive reaction in the damaged tissues to act on the concentration of nitric oxide. This reaction could affect the nitric oxide-dependent signaling pathways by nitric oxide degradation, expression, and decreasing bioavailability [39,40]. In addition to the role of nitric oxide in the outbreak of acute inflammation, the oxidative intermediates of arachidonic acid (AA) and

linoleic acid (LA) could also exhibit potent inflammatory mediators [41,42]. Investigations suggest that the MPO plays an important role in producing the AA and LA epoxides and hydroxy intermediates. Among these metabolites, some constitutions such as epoxyeicosatrienoic acid, hydroxyeicosatetraenoic acid, and hydroxyoctadecadienoic acid can play a dual role to stimulate both, pro- and anti-inflammatory pathways [43,44]. On the other hand, some studies have shown that an increased intake of dietary α -linolenic acid can exert anti-inflammatory activity via a decrease in production of inflammatory cytokines. This pro- and anti-inflammatory stimulation depends on various cellular and chemical factors including the cell type, conformational, and positional isomerism of the metabolites [45,46]. LA as the AA biosynthesis initiator has a key role in the regulation of eicosanoids' production [47,48]. These eicosanoids which include prostaglandins (PGs), thromboxanes, leukotrienes (LTs), and other oxidized derivatives are responsible for the intensity and duration of an inflammation. This physiological (or pathophysiological) outcome is dependent upon the concentration of the different eicosanoids in tissues, the sensitivity of the different cells and tissues to the various eicosanoids and the timing of the mediators' generation [49,50]. In most of the studies which indicated the anti-inflammatory properties for PUFA, the fatty acid intake was chronic. Although the present study showed that *S. securidaca* seed oil contained a high percentage of unsaturated fatty acids, it didn't show a significant effect on the acute inflammation model.

S. securidaca seed oil is a rich source of linoleic acid (an essential polyunsaturated fatty acid) which makes it a good candidate for use in the pharmaceutical industry. This oil failed to exhibit any significant effect on acute inflammation, however considering its chemical composition, the impact of its prolonged administration on inflammation should be investigated in further studies.

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Author contributions

Azadeh Hamed and Ardalan Pasdaran wrote the draft and contributed in guidance, analyzing data and revisions of the final version of the article. Akram Jamshidzadeh and Reza Heidari contributed in data collection and analyzing data.

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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Abbreviations

APAP: paracetamol; DMSO: dimethyl sulfoxide; DSC: differential scanning calorimetry; GC-MS: chromatography–mass spectrometry; HPTLC: high performance thin layer chromatography; HTAB: hexa-decyltrimethyl-ammonium bromide; MPO: myeloperoxidase; mU: milliunits; PUFA: polyunsaturated fatty acid; PMNs: polymorphonuclear neutrophils; UV: ultraviolet; Vis: visible light