

Citrus aurantium (bitter orange) seed oil: pharmacognostic, anti-inflammatory, and anti-nociceptive properties

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Abstract

The present investigation was carried out to evaluate the pharmacognostic properties of *Citrus aurantium* seeds as well as its anti-inflammatory activities. The n-hexane fraction of seed oil was extracted via a Soxhlet extractor and subjected to pharmacognostic assays, HPTLC, and GC-MS analysis for the determination of fatty acids and sterols. The possible anti-inflammatory and antinociceptive activities in rats were evaluated using formalin-induced paw licking, oedema, and myeloperoxidase activity assessment. Total ash, acid insoluble and water soluble ash values were determined as 35.83±4.92, 6.67±2.89, and 28.33±5.77 mg/g, respectively. HPTLC assessment revealed the presence of different fatty acids and steroidal triterpenes. The principal fatty acids of the seed oil were linoleic acid (C18:2, 50.10±2.58 %) and oleic acid (C18:1, 30.14±0.39). Esterified (2.40 mg/g) and free β-sitosterol (32.90 mg/g), free campesterol (3.9 mg/g), and free stigmasterol (10.165 mg/g) were detected in the oil. Seed oil exhibited anti-inflammatory properties in the first and the second phases of formalin test. Moreover, it showed anti-edematogenic effects but exerted no effects on myeloperoxidase activity.

Keywords: Anti-inflammatory, Bitter orange, *Citrus aurantium* seed oil, linoleic acid, phytosterols.

1. Introduction

Rutaceae, the family of flowering plants, is commonly named as rue or citrus family. This family encompasses around 155 genera and more than 1600 species (1). Rutaceae is also well known due to its various secondary metabolites. Various flavonoids, coumarins, volatile oils, and alkaloids have been isolated from different Rutaceae genera (2).

As a small citrus tree, Bitter orange, sour

orange, or Seville orange with the scientific name of *Citrus × aurantium* L. (*Syn: Aurantium × bigarrella* Poit. & Turpin, *Citrus × bigaradia* Loisel., *Aurantium × acre* Mill., *Aurantium × myrtifolium* Descourt.) is a popular plant from the family Rutaceae (3). Different parts of the plant such as peel, flower, leaves, fruit, and seeds are traditionally used for dietary purposes, gastrointestinal disorders, and neurological complications (4-5). In addition, bitter orange (called *Naranj* in Persian) was introduced as an important medicinal plant in Traditional Persian Medicine. Early physicians of Persian medicine have used the fruits and seeds of the plant as anti-irritant, analgesic, and antiemetic

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as well as antidote for toxins and poisons, either in oral or topical dosage forms (6).

Numerous investigations have been performed on the pharmacological activities of Bitter orange. The central effects of fruit essential oil including reduction in the emotionality level of animal models (7), antioxidant activity of seeds and peels' phenolic composition (8), anxiolytic effects of the total essential oil (4), antimutagenic activity of polymethoxyflavonoids in a methanol extract of fruits (9), and weight reducing effects (10) have been proved.

Other than pharmacological assessments, the phytochemical analysis of fruits and seeds of Bitter orange is considered crucial. Studies on the chemical constituents of peel essential oil resulted in the identification of limonene as the major compound (11). Other compounds including phenethylamine alkaloids (12-13) and flavonoids such as isonaringin, naringin, hesperidin, neohesperidin, and tangeritin (14) have also been isolated and identified. On the other side, the analysis of seed fixed oil has been done by some researchers, which resulted in the identification and quantification of palmitic, oleic, linoleic, and stearic acids (15-16).

Considering the wide cultivation of the bitter orange for nutritional purposes in some Asian countries and its application in Persian traditional medicine, the current study focused on the determination of phytosterol and fatty acids content and the *in vivo* anti-inflammatory activity of the seeds.

2. Materials and methods

2.1. Plant collection

Seeds of *Citrus aurantium* were collected from a garden in Shiraz (center of Fars province, south of Iran) and authenticated by Ms. Sedigheh Khademian, the botanist of Department of Phytopharmaceuticals, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran. A voucher specimen (PM-188) was specified for the sample. The plant material was powdered, passed through a sieve with a dense mesh (≈ 100) and kept in a dark closed container.

2.1.1. Physicochemical analysis

Determination of total ash, water soluble

ash, and acid insoluble ash were performed by employing standard methods of the analysis described in WHO guidelines for the quality control of herbal medicines (17). To detect the extractive values and perform HPTLC analysis, the seed powder (100 g) was extracted with n-hexane in a Soxhlet apparatus (6 h). The residuum was subsequently dried and macerated in dichloromethane, then in ethanol (48 h for each). Hexane, dichloromethane, and ethanol fractions were concentrated with a rotary evaporator and dried in a speed vacuum apparatus at 40 °C for 24 h. The solvent free n-hexane fraction was considered as the seeds' fixed oil. Dried fractions were weighed out and kept in Teflon capped tubes at -20 °C.

The refractive indices of seed oil were measured at 40 °C via an automatic ATAGO Rx7000- α digital refractometer (ATAGO, Tokyo, Japan). Seed oil thermal behavior was evaluated by differential scanning calorimetry (DSC) on a Bahr DSC 310 calorimeter (Germany). The temperature was correlated with indium. The oil was cooled down from room temperature to -20 °C with a rate of 5 °C/min, maintained at this temperature for 5 min, and subsequently heated back to 300 °C. An empty DSC pan was used as an inert reference to balance the heat capacity of the sample pan (18).

2.1.2. High performance thin layer chromatography

To screen the seeds' primary and secondary metabolites, 10 μ l of dichloromethane and ethanol fractions (5 mg/ml) as well as 2 μ l of the oil were applied to HPTLC (CAMAG) on a silica gel plate 60F254 (10 \times 20 cm, Merck). Plates were run in non-polar (toluene- acetone; 80:20, MP1), semi-polar (toluene- chloroform- acetone, 40:25:35, MP2), and polar (n-butanol- glacial acetic acid- water, 50:10:40, MP3) mobile phases. The TLC of the oil was run in chloroform- acetone- water (98:1.99:0.01, MP4). Chromatographic spots were visualized firstly by using ultra-violet lamps emitting at 254 and 365 nm and subsequently different reagents including phosphomolybdic acid reagent (vis.), Dragendorff, 5% potassium hydroxide (vis. & UV365 nm), orcinol, NP (ethanolamine diphenylborate)/PEG ,UV365 nm), Liebermann Burchard (UV365 nm or vis.), 3% FeCl₃ (vis.), van-

illin-sulfuric acid, and anisaldehyde-sulfuric acid were sprayed to the plates (19, 20). All chemicals and solvents were of analytical grade purchased from Merck (Darmstadt, Germany) or Sigma Aldrich (St. Louis, Mo., United States).

2.2. Fatty acid analysis of the seed oil

Fatty acid methyl esters were prepared in accordance with the procedure explained by AOAC (21). Oil or standard fatty acids (0.2 g) were transferred to a Teflon capped test tube. Toluene (1 ml) and sulfuric acid in methanol (1%, 2 ml) were added to the samples followed by adding 0.1 ml of hexadecanoic acid (2 g/l), as the internal standard. Tubes were incubated at 50 °C overnight. Subsequently, NaCl 5% solution was added and the required esters were extracted with n-hexane (2×5 ml). The yielded extract was washed with sodium bicarbonate (2%) solution and dried by anhydrous Na₂SO₄. The tubes were centrifuged for 10 min at 3000 rpm. The upper layer was transferred to a test tube and solvent was then removed under a stream of nitrogen and kept at -20 °C. Prior to the gas chromatography-mass spectrometry (GC-MS) analysis, 500 µl of pure n-hexane was added to dissolve the samples.

2.2.1. Isolation and TMS derivatization of sterols

After addition of 0.2 mg of free cholesterol and cholesteryl heptadecanoate as the internal standards, the oil (300 µl) was applied to a Pasteur pipette (7 mm external diameter) filled and packed with Silica gel and sequentially fractionated with A: 10 ml hexane- diethyl ether (200:1, v/v, fraction 1), B: 10 ml hexane- diethyl ether (96:4, v/v, fraction 2) and C: 10 ml diethyl ether- acetic acid (100:0.2, v/v, fraction 3). Wax esters as well as sterol esters, triacylglycerols, and free sterols were eluted in fractions 1, 2, and 3, respectively. Solvent free fractions were yielded after drying under a stream of nitrogen. Sterol esters were saponified in a tube containing 1 ml of KOH (33%) and 4 ml of ethanol (96%). The mixture was refluxed for 1 h in 80 °C and subsequently cooled to the room temperature. The unsaponifiables were extracted with 3 ml n-hexane (3 times) after washing the mixture with 2.5 ml distilled water. Pooled n-hexane fractions were dried under a stream of nitrogen.

Trimethylsilyl- (TMS) derivatives of sterols were prepared by incubating the sterols in 200 ml of N, O-bis (trimethylsilyl) trifluoroacetamide at 60 °C for 2 h (22).

2.2.2. GC-MS analysis of fatty acids and sterols

Derivatized fatty acid and sterols were analyzed via GC-MS analysis carried out using a Hewlett-Packard 6890. The gas chromatograph was equipped with an HP-5MS capillary column (phenylmethylsiloxane, 25 m, 0.25 mm i.d.). The injector temperature was set at 250 °C and positive ion electron impact mass spectra were recorded at ionization energy of 70 eV. Helium (1 ml/min) was applied as the carrier gas. In regard to the fatty acid methyl esters, the oven temperature was programmed from 160 °C (2 min) to 230 °C at a rate of 8 °C/min, then held at 230 °C for 20 min. In order to analyze the TMS-derivatives of sterols, the column temperature was held at 230 °C for 1 min, increased to 275 °C at a rate of 1 °C/min, then finally held at 275 °C for 30 min. The injector temperature was set at 275 °C (23).

Reference compounds as β-sitosterol, stigmasterol, campesterol, and standard fatty acids were purchased from Sigma (St. Louis, Mo., United States). Fatty acids and sterols were identified by comparing the mass spectra and retention times with those of the reference compounds, or with the mass spectra cited in the literatures. Chemical structures of the compounds for which neither standard compounds nor reference spectra were available, were postulated according to the general patterns of mass spectrometric fragmentation of different sterols.

2.3. Experimental animals

Sprague-Dawley female rats (180-220g) were sampled from the laboratory animal center of Shiraz University of Medical Sciences, Shiraz, Iran. The animals were acclimated for one week under 12 h light and 12 h dark cycle at the room temperature of 25-30 °C. Chow diet and water were provided ad libitum. The animal care and treatment procedures conformed to the Institutional Guidelines and Animal Ordinance (Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran). Rats had free access to water

but fasted overnight (12 h) and were randomly divided into six groups (n dark cycle=5). Treatments were as follows: (I) control (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 group rats received the standard drug paracetamol (APAP, 100 mg/kg, i.p), and groups (IV-VIII), got *S. aurantium* seed oil at doses of 250, 500, 1000, 2000, and 4000 µl/kg, i.p, respectively.

2.3.1. Formalin-induced paw licking in the rat

Analgesic activity was assessed by observing the reaction time in the test groups. Sixty min after vehicle, APAP, and seed oil treatment, formalin was subcutaneously injected into the plantar surface of the rats' right hind paw. Nociception was recorded and rated using the original formalin test protocol (23). Scoring the pain was as follows: 0: no response behavior of the injected paw; 1: limping during locomotion or resting the paw lightly on the floor; 2: elevation of the injected paw; 3: licking or biting of the injected paw, or grooming (24).

Behavioral responses were recorded for 45 min after the formalin injection. The first 5 min reflected the early phase and the time period between 15 and 45 min as the second phase. Immediately after sub-plantar injection of formalin, the animals were placed in a chamber with a mirror placed under it, with a 45° angle underneath the floor to allow an unobstructed view of the rats paw. All the rats were brought to the test chamber 1 h prior to the experiment.

2.3.2. Formalin -induced paw thickness

Immediately prior to the sub-plantar injection of formalin in the right hind paw and also at 4 h afterward, the paw thickness was measured from the ventral to the dorsal surfaces using a dial caliper (25). Data are expressed as mm and 4 h post-injection values were compared with the pre-injection values.

2.4. Myeloperoxidase (MPO) activity

MPO is an enzyme found primarily in the azurophilic granules of the neutrophils (26). It has been widely used as a biochemical marker of

granulocyte infiltration into various tissues. Intra-peritoneal injection of dimethyl sulfoxide (DMSO, 250 µl/kg, i.p. control, n=5) or the oil (250, 500, 1000, 2000 and 4000 µl/kg, i.p, n=6) was performed 60 min prior to the intra-plantar injection of formalin. Four h later (when the inflammation was at the maximum) paw tissues were collected under pentobarbital anesthesia (50 mg/kg, i.p.) and homogenized (IKA Homogenizer, Germany) in a solution containing 0.5% hexa-decyltrimethylammonium bromide (HTAB) dissolved in 50 mM, pH 6 potassium phosphate buffer, then centrifuged (3000 rpm, 20 min, 4 °C). 0.1 ml of the supernatant or standard (Sigma, Germany) were let to react with a 2.9 ml solution of potassium phosphate buffer (pH 6, 50 mM) containing O-dianisidine hydrochloride (0.167 mg/ml) and H₂O₂ (0.0005%). After 5 min, the reaction was stopped using 0.1 ml of hydrochloric acid (1.2 M). The absorbance changes was measured by a spectrophotometer (Cecil 9000, UK) at 400 nm. MPO activity was expressed in milliunits (mU) per 100 mg weight of wet tissue (27).

2.5. Statistical analysis

All data were expressed as means±SEM. Computer Statistical Package for the Social Sciences (SPSS, version 15) was used to perform statistical analyses. Analysis of Variance (ANOVA) followed by Tukey's multiple comparisons was used to analyze the data. $P < 0.05$ was considered as statistically significant.

3. Results and Discussion

3.1. Physicochemical properties of the seed

Total ash was determined as 35.83±4.92 (mg/g), which represents the physiological ash derived from seed tissues and non-physiological ash derived from soil or sand (environmental contamination). Acid insoluble and water soluble ash values were determined as 6.67±2.89 and 28.33±5.77 (mg/g), respectively.

The extractive values for n-hexane, dichloromethane, and ethanol fractions of *C. aurantium* seeds were 14.92%, 1.66%, and 1.78% (w/w).

The high performance thin layer chromatography (HPTLC) finger print of different fractions of the seeds visualized using UV lamps and

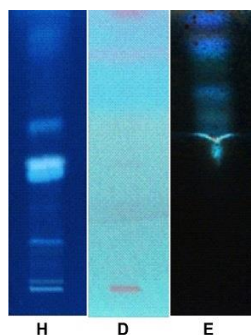


Figure 1. HPTLC fingerprints of n-hexane (H), dichloromethane (D), and ethanolic (E,) fractions, *C. aurantium* seeds fractions run in MP2 (D), MP3 (E), and MP4 (H), treated with Liebermann Burchard reagent (under UV365 lamp, H and E), anisaldehyde-sulfuric acid (visible light, D).

variety of chemical reagents represented the classes of primary and secondary metabolites and can be used for authenticating of the seeds (Figure 1).

Different fatty acids and steroidal triterpenes can be detected in HPTLC fingerprint of the fixed oil (n-hexane fraction), which was chromatographed in MP4 and sprayed with Liebermann Burchard (Figure 1-H). Different polyphenolics, glycosides, and alkaloids could be identified in ethanol and dichloromethane fractions (HPTLCs are not shown here) (18).

3.2. Phytosterols and fatty acid profile of the seed oil

In this study, esterified (2.40 mg/g) and free β -sitosterol (32.90 mg/g), free campesterol (3.9 mg/g), and free stigmasterol (10.165 mg/g) were detected in the oil, but we could not detect esterified stigmasterol or campesterol.

As seen in Table 1, the principal fatty acids of the seed oil were linoleic acid (C18:2, 50.10 \pm 2.58 %) and oleic acid (C18:1, 30.14 \pm 0.39). Other detected fatty acids (0.5-9%) were stearic

acid (C18:0), cerotic acid (C26:0), palmitoleic acid (C16:1), arachidic acid (C20:0), and palmitic acid (C16:0).

The total unsaturation for this seed oil was determined about 84.45%. Some normal alkanes (2.10%) were also detected in the oil. The result of this study is in a good agreement with the report by Waheed *et al.*, who reported C18:2 as the main fatty acid of *C. aurantium* seed oil (28).

Although *C. aurantium* seed oil cannot be considered as one of the richest sources of linoleic acid such as safflower seed oil and Salicornia oil (68-85%) (29-30), linoleic acid content in *C. aurantium* seed oil is comparable to cottonseed oil (31), walnut oil (32), and soybean oil (33-34) with the linoleic acid content of about 50-55%. The result of the present study showed that the seed oil of CA is a rich source of α -linoleic acid (a PUFA). Given that some PUFAs such as linoleic acid are essential omega-6 fatty acids needed for normal human growth and development, which cannot be produced within the human body, *C. aurantium* seed oil may have the potential to be used as a di-

Table 1. Fatty acids and alkane profile of *C. aurantium* seed oil.

Retention time (min)	Fatty acid name	Common names	Formula	Percentage (mean \pm SD)
4.24	Hexadecane	Cetane	C16H34	1.178 \pm 0.081
6.46	Octadecane	Octadecane	C18H38	0.922 \pm 0.044
7.74	9-Hexadecenoic acid	Palmitoleic acid	C16:1	2.110 \pm 0.551
7.97	Hexadecanoic acid	Palmitic acid	C16:0	0.975 \pm 0.001
9.94	9,12-Octadecadienoic acid	Linoleic acid	C18:2	50.100 \pm 2.579
10.00	9-Octadecenoic acid	Oleic acid	C18:1	30.142 \pm 0.390
10.28	Octadecanoic acid	Stearic acid	C18:0	8.784 \pm 2.189
10.45	Hexacosanoic acid	Cerotic acid	C26:0	4.027 \pm 0.669
12.81	Eicosanoic acid	Arachidic acid	C20:0	1.762 \pm 0.007

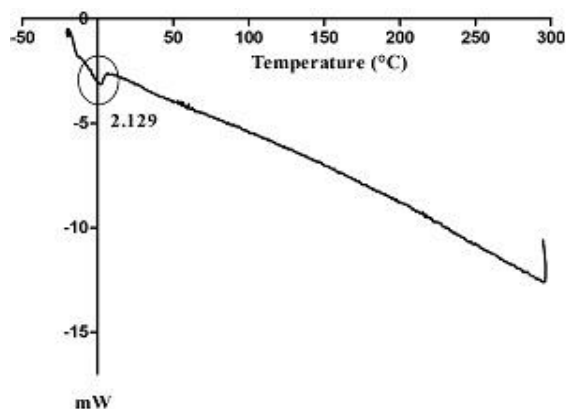


Figure 2. DSC profile of *C. aurantium* seed oil.

etary supplement. Linoleic acid is needed for the biosynthesis of arachidonic acid and thus some prostaglandins, leukotrienes, and thromboxane.

Sesame seed and peas have been introduced as the richest natural sources of β -sitosterol (13.9-19.1 mg/g) (35). According to the results of GC-MS analysis in this study, *C. aurantium* seed oil can be considered as a rich source of phytosterols specially β -sitosterol and stigmasterol. Reports on the phytochemical analysis of seed oils have consistently revealed that the most common phytosterols found in higher plants are β -sitosterol, campesterol, and stigmasterol. Different biological activities have been reported for these phytosterols (36) including lipid lowering (37) and neuro-protective effects (38), anti-inflammatory activity (39), as well as anti-oxidant and anti- mutagenic properties (40).

3.3. Physical properties of the seed oil

The seed oil had a pale yellow color with the refractive index of 1.4634. Figure 2 shows the thermal behavior of the seed oil. The major melting peak at 2.129 °C was exhibited as an endothermic peak. Some minor exothermic peaks can be seen about 60 °C, which may represent the thermal transition of unsaturated fatty acids as the major constituent. The unique DSC profile of the oils prove the presence of adulterations in them. DSC profile of the oils can also give valuable information about their thermal behaviors and chemical profiles (41-42). DSC profile of *C. aurantium* seed oil shows that it has an acceptable thermal stability in high heat (up to 300 °C).

3.4. Anti-nociceptive and anti-inflammatory properties of the seed oil

In the present study, we investigated the possible anti-nociceptive and anti-inflammatory properties of *C. aurantium* seed oil by formalin test and MPO activity.

Our results clearly demonstrate the anti-nociceptive effect of *C. aurantium* seed oil in a model of formalin test in rat. The effect of *C. aurantium* seed oil in early (0-5 min) and late (15-45 min) phases of formalin test are shown in Figure 3. In both early and late phases of formalin test, doses of 2000 and 4000 μ l/kg of *C. aurantium* seed oil showed a significant effect on the nociception compared with formalin group ($P < 0.05$). As compared with the APAP in the early phase, the analgesic effect of APAP was significant. Although there is a statistically significant effect for the oil, this effect was only seen at very high concentrations. Furthermore, even though there was a certain degree of inhibition, it was not total and the effect tended to be inferior to APAP.

The sub-plantar injection of the formalin into the rat hind paw induced an inflammation (swelling and erythema) that was maximal by the 4th h following the formalin administration. Figure 4 shows the paw thickness of *C. aurantium* seed oil and APAP in formalin-induced paw edema. The results demonstrate that formalin increased the thickness of the right paw 4 h after injection.

APAP treatment at a dose of 1000 μ l/kg throughout the experimental period significantly inhibited hind paw swelling ($*P < 0.05$). Moreover, we observed that *C. aurantium* seed oil (500, 1000, 2000 and 4000 μ l/kg) diminished formalin-

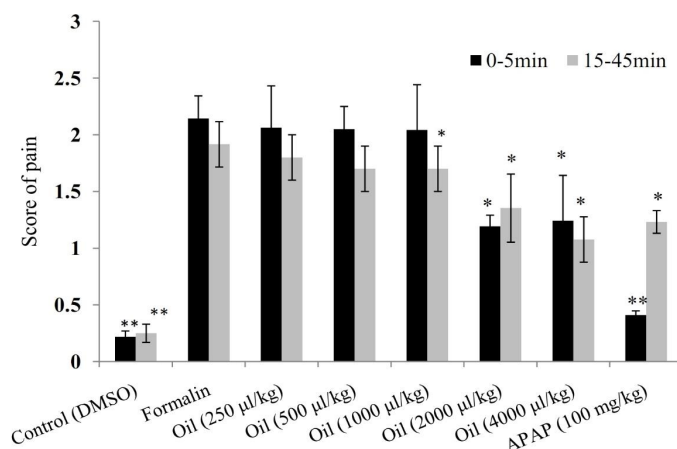


Figure 3. Effects of *C. aurantium* on the inflammatory pain induced by formalin in rats

The values are expressed as the mean±S.D. (n=6-8).

* $P < 0.05$ indicates statistically significant differences from the formalin group.

** $P < 0.001$ indicates statistically significant differences from the formalin group.

induced paw thickness at 4 h. As shown in the figure 4, there was no significant difference between the four doses.

MPO activity is an indication of neutrophil infiltration in this study. Intra-peritoneal injection of different doses of *C. aurantium* seed oil did not show significant decrease in MPO activity compared with APAP. Figure 5 demonstrated that *C. aurantium* seed oil cannot inhibit the neutrophil-induced damage by reducing MPO activity in the paws of the rat.

Formalin test, which produces a local tissue injury to the paw, has been used successively in many studies as a model for tonic pain and localized inflammation pain (43).

In the formalin test, pain occurs in two phases. The first phase is probably a direct result of stimulation of nociceptors in the paw, while the second phase may reflect the inflammation process, and at least to some degree, the sensitization of central nociceptive neurons. Drugs such as opioids act mainly centrally and inhibit both phases of formalin induced pain, while other drugs such as non-steroidal anti-inflammatory are primarily peripherally acting and only inhibit the late phase (44). The second phase of formalin test is related to a peripheral inflammatory process. Our results showed that the administration of 2000 and 4000 µl/kg (i.p.) of *C. aurantium* seed oil caused a decrease in pain responses in the first and the second

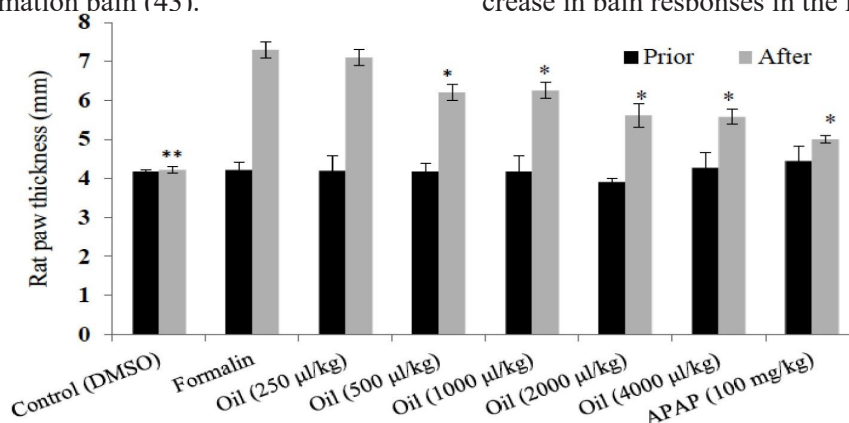


Figure 4. Effects of *C. aurantium* seed oil on formalin-induced paw thickness in rats.

Paw edema was induced by 50 µl of 2.5% formalin injection into the plantar right paw. Paw volume was measured after 4 h. Animals were pretreated with *C. aurantium* seed oil (250-4000 µl/kg; i.p.), DMSO (control), or paracetamol (APAP; 100 mg/kg; i.p.). The values are expressed as the mean±S.D. (n=5).

* $P < 0.05$ indicates statistically significant differences from the formalin group.

** $P < 0.001$ indicates statistically significant differences from the formalin group.

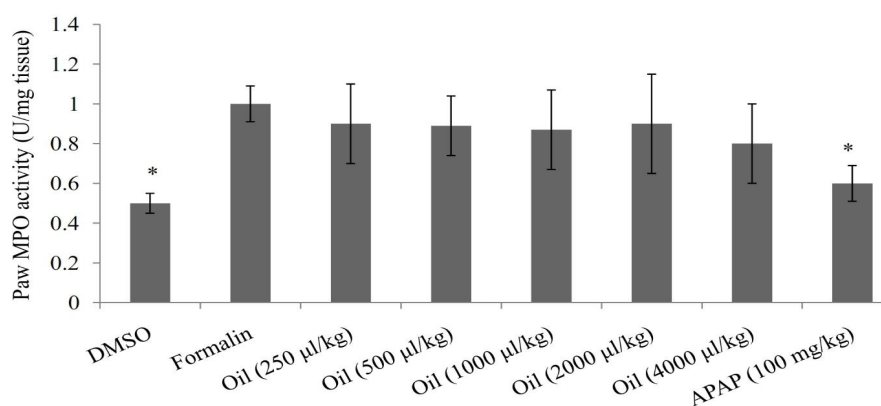


Figure 5. The effect of intraperitoneal injection of *C. aurantium* seed oil on myeloperoxidase (MPO) activity in the rats' paws. Formalin (50µl of 2.5%) was injected into the plantar surface of rat. One hour before formalin injection, the animals were pretreated with DMSO (control), paracetamol (APAP; 100 mg/kg, i.p.), or *C. aurantium* seed oil (250-4000 µl/kg; i.p.). 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).

* $P < 0.05$ indicates statistically significant differences from the formalin group.

phases of formalin test. These findings suggest that central mechanisms may be involved in the antinociceptive activity of this oil. In other words, it seems that the anti-nociceptive effect of *C. aurantium* seed oil is not related to the inflammatory peripheral pain.

As far as we know, there is no report on the anti-inflammatory or anti-nociceptive properties of *C. aurantium* seed oil. But, linoleic acid was the main constituent in the seed oil, which is an omega-6 essential fatty acid. Some studies revealed that n-6 PUFAs may both inhibit and induce pain transmission (45). It was previously reported to exhibit colonic anti-inflammatory properties in ulcerative colitis by down-regulating the generation of inducible eicosanoids (i.e. PGE2 and LTB4) involved in early micro-inflammatory events and modulating the expression of the genes regulated by peroxisome proliferator-activated receptors (46). It was also reported that anti-inflammatory properties are somehow mediated by the nuclear hormone receptor PPAR γ (47). Jäger *et al.* suggested that linoleic acid and alpha-linolenic acid may contribute to the COX-1 and -2 inhibitory activity of rosehip (48).

On the other hand, our findings showed that *C. aurantium* seed oil is a rich source of phytosterols (sitosterol and stigmasterol). In a study, stigmasterol and stigmasterol acetate showed inhibition of both the neurogenic (first phase) and inflammatory phases (second phase) of formalin-

induced pain in mice. Interestingly, both of these compounds were more effective in the second phase of the formalin test. Additionally, both steroids failed to affect the edematogenic response of the formalin test (49). Since the doses, the applied methods, and the source of steroid were different, the results of these studies cannot be compared.

There is an association between the inflammatory process and the development of pain. Acute inflammation is the initial response and is characterized by the increased movement of plasma and innate immune system cells, such as neutrophils and macrophages, from the blood into the injured tissues (50).

The increases of paw thickness are attributed to the leakage of plasma from the blood vessels after formalin-induced inflammation. As demonstrated in Figure 4, *C. aurantium* seed oil at doses 500-4000 µl/kg showed a significant inhibition in paw thickness. Anti-inflammatory activities of *C. aurantium* seed oil were also evaluated by MPO activity test. MPO is an enzyme found primarily in the azurophilic granules of neutrophils, therefore it has been used extensively as a biochemical marker for granulocyte infiltration into various tissues (51). Our results demonstrated that *C. aurantium* seed oil treatment (i.p.; 250-4000 µl/kg) in rat showed no significant inhibition in MPO activity when used 30 min before formalin injection. The results of our experiment suggest that the

anti-inflammatory action of *C. aurantium* seed oil is not dependent on the mechanisms of neutrophil migration. In this study, we did not investigate the effects of the long term administration of *C. aurantium* seed oil on suppression of nociception and inflammatory responses.

4. Conclusion

This study revealed that *C. aurantium* seed oil is a rich source of linoleic acid (C18:2) and phytosterols especially free β -sitosterol and stigmasterol. This oil can exhibit anti-inflammatory properties in the first and the second phases of formalin test. Furthermore, the oil showed anti-edematogenic properties, but had no effects on

MPO activity. The results of the present study suggest that the anti-inflammatory action of *C. aurantium* seed oil is not dependent on the mechanisms of neutrophil migration. In other words, central mechanisms may be involved in the anti-nociceptive activity of this oil.

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Conflict of Interest

None declared.

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