Anti-inflammatory and antinociceptive effect of *Symphytum officinale* root

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Abstract

The present work evaluated the anti-inflammatory and antinociceptive activity of a Symphytum officinale root extract standardized in rosmarinic acid. The anti-inflammatory effect of the hydro-glycero-alcoholic extract administered orally was assessed by the carrageenan-induced rat paw oedema method while the antinociceptive effect was determined by the acetic acid induced writhing test in mice and by Randall Selitto test in rats. Content in rosmarinic acid quantified by a HPLC-MS method was 74.77 µg/mL. In the carrageenan-induced rat paw oedema method administration of S. officinale extract (500 mg/kg, orally) reduced significantly and dose-dependently oedema by 55.6% at 1h after inflammation was induced. In the writhing test, S. officinale extract (500 mg/kg, orally) inhibited by 45.25% the abdominal constrictions induced by acetic acid in mice. In the Randall Selitto test in rats, the oral administration of S. officinale extract (500 mg/kg, orally) demonstrated a strong peripheral antinociceptive effect in the first two hours after administration increasing the pain threshold by 58%. Our study demonstrated significant anti-inflammatory and antinociceptive effects of S. officinale extract probably mediated by peripheral mechanisms which may justify the traditional use in the treatment of some inflammatory diseases.

1. Introduction

Symphytum officinale (Boraginaceae family) (comfrey) is a well known remedy used in folk medicine as poultices for topic applications according to the wound healing effects, to reduce joint inflammation in rheumatic and arthritic diseases, in broken bones and tendon damages, and for internal use for curing ulcers. Previous experimental data reported a wide range of pharmacologic activities of various *Symphytum* extracts such as antifungal, anti-inflammatory, antioxidant, and vasoprotective effects (TALHOUK et al., 2007 [1]; NEAGU et al., 2011 [2]; GOKADZE et al., 2013 [3]).

External preparations of *S. officinale* were widely available and folk medicine suggested that comfrey could be used to treat musculoskeletal disorders, wounds and various other conditions (FROST et al., 2013 [4]).

Chemical composition of comfrey roots revealed 0.6–4.7% allantoin (DENNIS *et al.,* 1987 [5]), mucilage polysaccharides (29%), phenolic acids such as rosmarinic acid (0.2%), chlorogenic acid (0.012%), caffeic acid (0.004%) and α-hydroxy caffeic acid (TEUSCHER *et*

al., 2009 [6]), glycopeptides and amino acids (HIERMANN & WRITZEL, 1998 [7]), proteins, triterpene saponins, pyrrolizidine alkaloids (0.013% to 1.2%), tannins (2.4%), lithospermic acid, carotene (0.63%), choline, asparagine, coniferin, starch, gumiresins, phytosterols, carotenoids, vitamins, mucilage polysaccharides (29%), and high amounts of mineral substances (VOGL et al., 2013 [8]).

Previous studies revealed that allantoin and rosmarinic acid were major compounds responsible for pharmacologic effects (ANDRES *et al.*, 1989 [9]; STAIGER, 2013 [10]; SHIPOCHLIEV *et al.*, 1981 [11]; MASCOLO *et al.*, 1987 [12]; HIERMANN & WRITZEL, 1998 [7]).

Allantoin was also reported as major active compound responsible to promote cell division and growth of the connective tissue, bones and cartilages and to stimulate the wounds healing (KOMMISSION E, 1990 [13]).

Previous papers suggested that the antiinflammatory activity of plants is mostly correlated to triterpenic compounds content, the effects being attributed to inhibition of different stages of inflammatory reaction including histamin release, COX and LOX activity, NO production and also to high polyphenol content due to selective inhibition of COX-2 (GRIGORE et. al, 2015 [14]). Internal use of comfrey is however restricted because of its content in hepatotoxic and carcinogenic pyrrolizidine alkaloids (GOKADZE et al., 2013 [3]).

Previous experimental data showed that emulsion containing 8% extract of comfrey leaves induced the repair of damaged tissue, increased the collagen deposition and reduced cellular inflammatory infiltrate by 46% (ARAUJO et al., 2012 [15]).

In addition, a glycopeptide isolated from the aqueous extract of *S. officinale* roots exerted a dose-dependent antiphlogistic effect on carrageenan-induced rat paw edema. It also inhibited the release of prostaglandins and leukotrienes via a decreased expression of phospholipase A2 (HIERMANN & WRITZEL, 1998 [7]).

It has been clinically proven to relieve pain, inflammation and swelling of muscles and joints in the case of degenerative arthritis and contusions. A monograph of the European Scientific Cooperative on Phytotherapy Monograph (ESCOP) stated that comfrey root was used for tendinitis, knee joint injuries, gonarthrosis, fractures and skin inflammation, although scientific evidence did not always support these uses (ESCOP, 2009 [16]).

The aim of the present study was to explore the anti-inflammatory and antinociceptive potential of a *S. officinale* root extract standardized in a major compound such as rosmarinic acid in order to confirm the traditional use.

2. Materials and Methods

Collection and authentication of plant material

The roots of *S. officinale* L., comfrey (Boraginaceae) were collected from Topa Mica, Salaj, Romania, in October 2015. The plant was authenticated at the Department of Pharmacognosy and Phytotherapy and a voucher specimen was deposited at the herbarium of the Faculty of Pharmacy, "Vasile Goldis" Western University, Arad, Romania (item no. 352/2015).

Preparation of the extract

Dried roots of *S. officinale* were reduced to a fine powder with a mechanical grinder. The powdered plant material (20 g) was extracted by maceration for 10 days at room temperature with 100 mL solvent mixture of water: glycerol: ethanol 1:1:1 (v/v) (FR X, 2009 [17]). After filtration, *S. officinale* extract was added to 100 mL with the same solvent mixture. For the pharmacological studies, the extract was spray-dried and suspended in a mixture of Tween 80 and normal saline solution (1:100 v/v).

Quantification of rosmarinic acid in *S. officinale* extract

Quantification of rosmarinic acid in *S. officinale* extract was carried out by high-performance liquid chromatography method coupled with mass spectrometry (LC/MS/MS) (CONEA et. al., 2014 [18]).

The LC/MS system was an Agilent 1100 Series HPLC system (Agilent Technology Co., Ltd.) consisting of a binary pump, degasser, autosampler, thermostat operating at 48^0C, VL Ion Trap detector and UV detector. Chromatographic separation was performed on a Zorbax SB-C18 column (100mm×3.0mm i.d., 3.5μm) (Agilent). Before use, mobile phase was filtered through a 0.50 μm membrane and dis-aerated in an ultrasonic bath. The mobile phase consisted in acetonitrile and 1 mM ammonium acetate in water, gradient elution: start 5% acetonitrile, at 3.3 min 25% acetonitrile, flow rate 1 mL/min, autosampler injection volume 50 μL. UV detection was performed at 330 nm. The MS was equipped with a Turbo-Ion spray (ESI) interface, negative ion mode. ESI settings were: negative ionization, ion source temperature 360ºC, gas: nitrogen, flow rate 12 L/min, nebulizer: nitrogen at 70 psi pressure, capillary voltage 3000 V. The analysis mode was multiple reaction monitoring (MRM) and single ion monitoring (SIM). The chromatographic data were processed using ChemStation and DataAnalysis software from Agilent, USA. MS spectra of the tested sample was compared to spectra of standard solution of rosmarinic acid. The UV trace was used for quantification. Quantitative determination was performed by external standard method. Calibration curve of rosmarinic acid set for 0.5-50 μg mL^{-1} range had good linearity ($R^2 >$ 0.999) for a five point plot.

Carrageenan-induced rat paw oedema method

The acute anti-inflammatory activity of the *S. officinale* extract was assessed by the carrageenan-induced rat paw oedema method followed by plethysmometric evaluation (CONEA et. al., 2014 [18]).

Animals were provided by the Animal Facility of "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca. All animals were kept in a room with controlled temperature (21±1ºC) and humidity (50-55%) and a 12h light-12h dark cycle and were fed with standard pellet diet (Cantacuzino Institute, Bucharest, Romania) and water *ad libitum.*

Briefly, the *S. officinale* extract was administered orally to three groups of rats (n = 6), by gastric gavage, in increasing doses (125 mg/kg b.w., 250 mg/kg b.w. and 500 mg/kg b.w., 1 hour before inflammation was induced. The rats in the negative control group (n = 6) were treated orally with normal saline solution. The animals from the positive control group (n = 6) were treated orally with a reference anti-inflammatory drug, diclofenac (20 mg/kg b.w.). Oedema was induced by a subplantar injection of 0.1 mL 1 % (w/v) λ-carrageenan into the left hind paw of each rat. The paw volume of each animal was measured before carrageenan injection and afterwards at 1, 2, 3 and 4 h after inflammation was induced with a plethysmometer (model 7140, Ugo Basile, Varese, Italy).

All the experiments were carried out in accordance with the guidelines for the care of laboratory animals and ethical guidelines and were approved by the ethics committee for research on laboratory-animal use of the institution.

The anti-inflammatory effect of the *S. officinale* extract was determined at each time range with the formula: Inhibition of oedema (%) = [1 − (Ot/Oc)] x 100, where Ot was the oedema in the treated group and Oc was the oedema in the negative control group.

Acetic acid induced writhing test in mice

The analgesic effect of the *S. officinale* extract was evaluated by the writhing test in mice, using 1% (v/v) acetic acid solution administered intraperitoneally to induce abdominal constrictions (RIBEIRO et al, 2000 [19]). Briefly, eight groups of male albino mice (n=6)

were provided by the Practical Skills and Experimental Medicine Centre of the "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca (Romania). The mice were housed in polycarbonate type IV-S open-top cages (Tecniplast, Italy) and maintained under standard conditions (22 ± 2 °C, at a relative humidity of 45 ± 10%, and a 12:12-hrs. light: dark cycle). The animals had access to a standard pelleted diet (Cantacuzino Institute, Bucharest, Romania) and filtered water *ad libitum* throughout the experiment, except for the day when the tested substances were administered (BOCSA et. al., 2015 [20]).

Initially, the *S. officinale* extract was administered orally to three groups of mice (n = 6) by gastric gavage of increasing doses (125 mg/kg b.w., 250 mg/kg b.w. and 500 mg/kg b.w.). The mice in the negative control group (n = 6) were treated orally with normal saline solution (10 mL/kg b.w.). The animals from the positive control group (n = 6) were treated orally with a reference anti-inflammatory drug, diclofenac 20 mg/kg b.w. After 30 minutes, all the mice were injected intraperitoneally 0.1 mL of 1% acetic acid solution to induce abdominal writhes. The animals were placed in an observation box, the writhes being counted over a period of 20 minutes. For scoring purposes, a writhe was indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb.

The analgesic activity was evaluated by the percentage of inhibition of the writhes with the formula: % inhibition= (Mean number of writhes for control group - Mean number of writhes for treated group) x 100/ Mean number of writhes for control group.

Randall-Sellito test in rats

The antinociceptive activity of the standardized extract from *S. officinale* root was also assessed by the Randall Selitto test. The pain threshold of the edematous hind paw of the rats subjected to constant force was determined with an analgesimeter (CHITAC et. al, 2015 [21]), Le Bars et. al., 2001 [22]). For this experiment, eight groups of male Charles River Wistar rats (Crl:WI) (n=6) were provided by the Practical Skills and Experimental Medicine Centre of the "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca (Romania). The rats were housed in polycarbonate type IV-S open-top cages (Tecniplast, Italy) and maintained under standard conditions (22 ± 2 °C, a relative humidity of 45 ± 10%, 12:12-h light:dark cycle). The animals had access to a standard pelleted feed (Cantacuzino Institute, Bucharest, Romania) and filtered water *ad libitum* throughout the experiment, except for the day when the test substances were administered.

Briefly, the animals received an intraplantar injection of λ-carrageenan (1% w/v, 0.1 mL) in sterile saline solution. Thirty minutes after inflammation was induced 100 mg/kg, 250 mg/kg and 500 mg/kg b.w. of *S. officinale* extract were administered orally to the test groups, while the normal saline solution (10 mL/kg b.w.) was administered to the negative control group and a reference analgesic, diclofenac (20 mg/kg b.w. p.o.) was administered to the positive control group. After 1, 2, 3, and 4 h, pressure was applied to the inflamed paw using an analgesimeter (model 37215, Ugo Basile, Varese, Italy) generating linearly increasing force, until the animal produced a response characterized by removal of the paw, interpreted as mechanical hypernociception. The instrument recorded the maximal amount of pressure (expressed in grams) withstood by the rats, at each time range.

Statistical analysis

Data were expressed as mean values ± standard error (S.E.) and were statistically analyzed by one way ANOVA method. The differences between the treated groups and the control group were evaluated by Dunnett's 't' test, p values<0.05 being considered statistically significant.
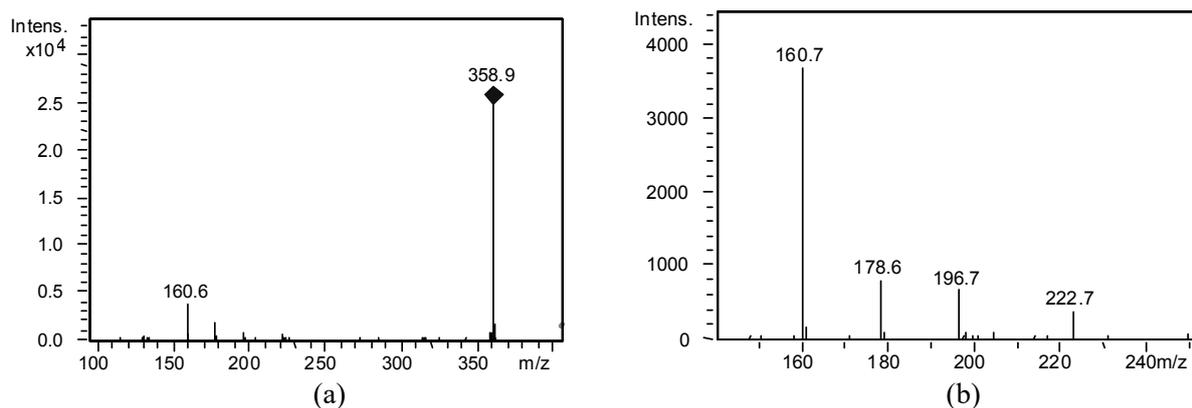
Figure 1. (a) Full-scan ESI-MS spectrum of rosmarinic acid in mobile phase;
(b) MS/MS spectrum of rosmarinic acid in mobile phase
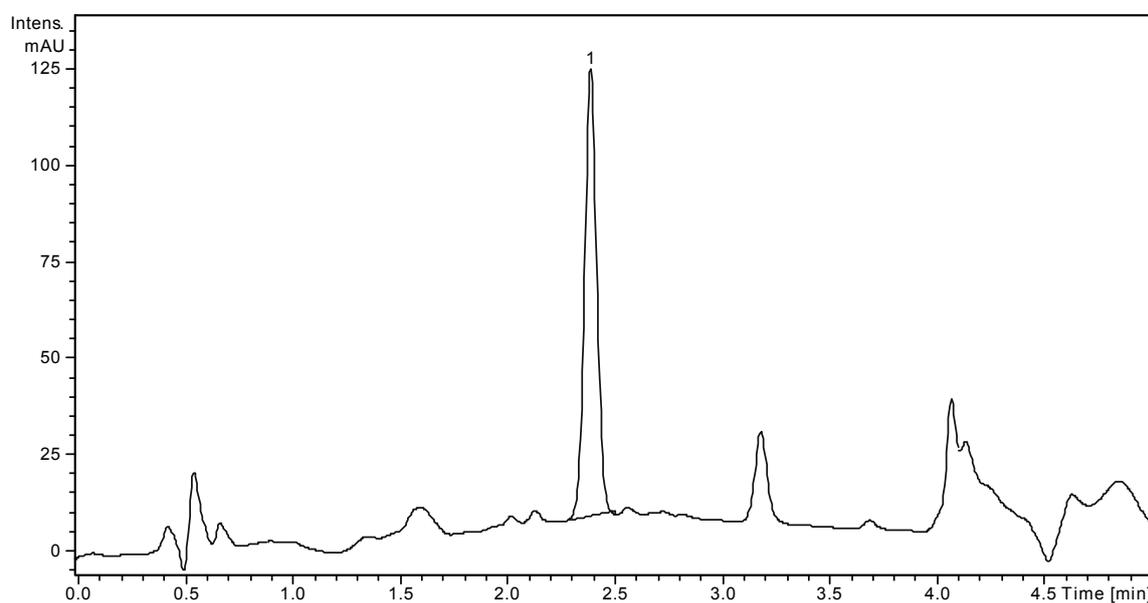

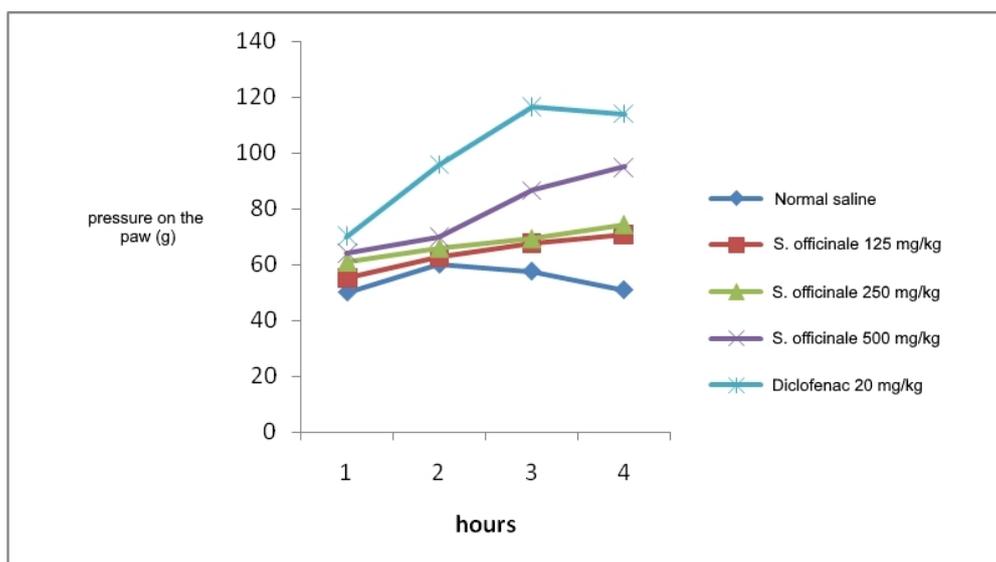**Figure 2.** HPLC chromatogram of *S. officinale* extract

Figure 3. Effects on the standardized extract from *S. officinale* root in the Randall-Sellito test in rats

Table 1. Effect of *S. officinale* extract on carrageenan-induced rat paw oedema

Group	Dose (mg/kg)	Oedema at 1h ± SE (% inhibition)	Oedema at 2h ± SE (% inhibition)	Oedema at 3h ± SE (% inhibition)	Oedema at 4h ± SE (% inhibition)
Control (normal saline)	-	2.23 ± 0.21 (-)	2.68 ± 0.20 (-)	3.01 ± 0.14 (-)	3.47 ± 0.23 (-)
S. officinale	125	1.42 ± 0.17** (36.32)	2.20 ± 0.09* (17.91)	2.92 ± 0.05 (2.99)	2.80± 0.09* (19.42)
S. officinale	250	1.16 ± 0.14** (47.75)	2.00 ± 0.28 (25.37)	2.75 ± 0.12 (8.63)	2.52 ± 0.12** (27.37)
S *officinale*	500	0.99 ± 0.20** (55.60)	1.98 ± 0.34 * (26.11)	2.44 ± 0.28 (19.15)	2.12 ± 0.35** (38.90)
Diclofenac	20	1.63 ± 0.10* (26.90)	1.08 ± 0.09** (59.70)	1.13 ± 0.09** (62.45)	1.53 ± 0.10** (55.90)

($*p \leq 0.05$ vs.control, $**p \leq 0.01$ vs.control, S.E. – standard error)

Table 2. Effect of the *S. officinale* extract in the acetic acid induced writhing test in mice

Group	Dose (mg/kg)	No. of writhes (X±S.E.)	% of inhibition
Control (normal saline)	10 ml/kg	56±2.84	-
S. officinale extract	125 mg/kg	45.83±2.58*	18.16
S. officinale extract	250 mg/kg	39.5±1.8**	29.46
S. officinale extract	500 mg/kg	30.66±1.76**	45.25
Diclofenac	20 mg/kg	21.33±1.64**	61.91

($*p \leq 0.05$ vs.control, $**p \leq 0.01$ vs.control, S.E. - standard error)

3. Results and discussions

The mass spectrometer operated using ESI source in negative mode was set for isolation and fragmentation of deprotonated rosmarinic acid ion with m/z = 359 (Figure 1a). Quantification of rosmarinic acid was based on the sum of ions with m/z = 160.7, 178.6 and 196.7 from the MS spectrum of parent ion (Figure 1b). Calibration curve was linear in the concentration range of 80-640 ng/mL, with a correlation coefficient of 0.999.

A sample chromatogram of rosmarinic acid from *S. officinale* extract is presented in Figure 2 (the UV trace at 330 nm) The retention time for rosmarinic acid was 2.4 min. Content in rosmarinic acid in *S. officinale* root extract was 74.77 μg/mL.

Carrageenan-induced rat paw oedema method

Results of the carrageenan-induced rat paw oedema test were presented in Table 1. The inflammatory oedema developed soon after the subplantar injection of carrageenan, reaching its peak at 4 h (oedema 3.47 ± 0.23 mL). The administration of a reference anti-inflammatory drug, diclofenac 20 mg/kg inhibited the oedema in the treated rats, the effect being maximal at 2-3 h and slightly decreasing afterwards. The administration of the *S. officinale* extract reduced significantly and dose-dependently the oedema formation, especially at dose 500 mg/kg. In the first phase of the oedema formation, the *S. officinale* extract (500 mg/kg) was superior to diclofenac, inhibiting oedema by 55.60%, 1 h after inflammation was induced.

Antinociceptive activity tested by acetic acid induced writhing test in mice

Results of the acetic acid induced writhing test in mice are presented in Table 2. The intraperitoneal injection of 1% acetic acid solution induced 56 writhes in the control group. The oral administration of the *S. officinale* extract produced a significant inhibition of the writhes. The maximum effect was a 45.25% of inhibition of writhes (500 mg/kg). The results

showed a statistically significant and a dose-dependent effect of *S. officinale* extract and inferior to diclofenac which inhibited the writhes by 61.91%.

Antinociceptive activity in the Randall-Sellito test in rats

The results of the Randall-Selitto test in rats are presented in Figure 3. The oral administration of *S. officinale* root extract produced a significant peripheral antinociceptive effect at dose 500 mg/kg, with a pain threshold of 95 g versus control (normal saline) which had the pain threshold of 60 g. The antinociceptive effect increased more in the last two hours after the extract administration which may suggest an inhibition of the release of pro-inflammatory mediators. This antinociceptive effect was lower to diclofenac which had a pain threshold of 116.6 g.

4. Conclusion

The development of oedema in the rat hindpaw following an injection of carrageenan was characterized as a biphasic event in which various mediators are released, producing an inflammatory reaction. The initial inflammatory response to carrageenan (0-1 h) was attributed to the release of histamine, serotonin, bradykinin and also complement and reactive oxygen species. In contrast, the second accelerating phase of swelling (2-4 hrs.) was correlated with the elevated production of prostaglandins in the inflammatory area.

Our experimental data suggested that several mechanisms may be responsible for the anti-inflammatory effect of the *S. officinale* extract. *S. officinale* root extract reduced significantly and dose-dependently the oedema formation in the carrageenan-induced rat paw oedema test, especially at dose 500 mg/kg b.w. Aside from a possible reduction of prostaglandin concentration in the affected tissue, the extract was able to influence also the first phase of carrageenan-induced oedema formation, probably by inhibiting the release of other pro-inflammatory mediators.

Acetic acid is known to trigger an irritative reaction in the peritoneum, which induced the writhing response due to the sensitization of nociceptors by prostaglandins (Le Bars et al., 2001 [22]).

Generally, the effect of the extract against the chemical noxious stimulus may be an indication that it reduced the production of prostaglandins, thereby causing a reduction in the number of writhes. The abdominal constriction response induced by acetic acid was a sensitive procedure to establish peripherally acting antinociceptives.

S. officinale root extract showed significant and dose-dependent antinociceptive effects in the acetic acid induced writhing test in mice (by 45%) and in Randall Selitto test in rats (by 58%). The results indicated that analgesic effect of *S. officinale* extract might be mediated by the peripheral inhibition of prostaglandin synthesis or actions.

Quantification of rosmarinic acid, a major compound in *S. officinale* root, was achieved by a LC/MS/MS method (74.77 μg rosmarinic acid /mL extract). Both analgesic and anti-inflammatory effects of *S. officinale* root extract may be related to the ability of polyphenols such as rosmarinic acid to inhibit the synthesis and release of some pro-inflammatory mediators and cytokines (ERDEMOGLU et al, 2009 [23]) and also to the content in allantoin. Several studies also demonstrated that polyphenols exerted protective effects against inflammatory diseases (BOCSA et al. 2015 [20]).

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