ANTINOCICEPTIVE ACTION OF *ONONIS SPINOSA* LEAF EXTRACT IN MOUSE PAIN MODELS

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Abstract: *Ononis spinosa* (spiny restharrow) is a perennial herb that belongs to the Fabaceae family. Among several uses, the aerial parts of *O. spinosa* are used in traditional medicine for toothache. In this study, analgesic activity of the methanolic extract of leaves was studied. In writhing test, the 300 mg/kg and 600 mg/kg doses of *O. spinosa* extract produced 90.1% and 94.5% inhibition of abdominal cramps, respectively compared to 23.6% inhibition produced by 30 mg/kg diclofenac sodium. *O. spinosa* methanolic extract reduced the time of licking in early and late phases of the formalin test. The inhibitory effect of *O. spinosa* extract was more pronounced in late phase. The lowest dose tested (300 mg/kg) of *O. spinosa* extract produced 81.0% inhibition compared to 50.5% inhibition in 30 mg/kg diclofenac sodium in the late phase of the formalin test. This effect was not abrogated by glibenclamide, naloxone or atropine indicating that the action of *O. spinosa* in formalin test is not mediated by ATP-sensitive K^+ channel, opioid or muscarinic receptors. In tail-flick and hot-plate tests, *O. spinosa* increased the latency time at 30 min and 150 min. Naloxone antagonized the action of *O. spinosa* in both tests suggesting an interaction with the opioid receptor as a possible mechanism of *O. spinosa* in thermal pain models at the spinal and supraspinal levels. Phytochemical screening indicated the presence of phenolics, saponins, flavonoids, and terpenoids. Alkaloids, sterols, and anthraquinone glycosides were absent. HPLC analysis confirmed the presence of kaempferol, apigenin and myricetin in the extract.

Keywords: *Ononis spinosa,* analgesic activity, opioid receptor

Ononis spinosa (spiny restharrow) is a perennial herb that belongs to the family Fabaceae with many non-sticky hanging stems, trifoliate serrate leaflets and pink-violet flowers (1). This plant is widespread in the Mediterranean region of Europe, in West Asia and North Africa (2, 3). *O. spinosa* is used for treating kidney stones and inflammatory diseases (4) and healing wounds (5). In fact, ononidis radix; the dried roots of *O. spinosa* are commercially available in different forms for various uses. The herbal tea of this plant is taken mainly as a diuretic and for rheumatism (3).

Among several uses, the aerial parts of *O. spinosa* are used in traditional medicine for toothache (6) and the flowers for abdominal pain (7). Analgesic activity of the aerial parts of this plant was reported earlier using writhing test and tail flick test (4). However, it has not been studied in other pain models. Therefore, this study was performed to investigate the effect of *O. spinosa* in the formalin test and to examine its mechanism of action.

MATERIALS AND METHODS

Drugs

Atropine was purchased from Sigma-Aldrich (USA), diclofenac sodium was from Novartis (Switzerland), glibenclamide (Glibil) was obtained from Hikma Pharmaceuticals (Jordan) and naloxone hydrochloride was purchased from Tocris Bioscience (UK). All drugs were dissolved in sterile normal saline and administered intraperitoneally (i.p).

Collection of plant material

O. spinosa was collected from Subaihi, Jordan in July 2016. The plant was authenticated by Prof. Barakat Abu-Irmaileh/The University of Jordan. A voucher specimen was deposited in the Graduate Studies Laboratory at Al-Ahliyya Amman University.

Preparation of plant extract

Leaves of *O. spinosa* were dried at room temperature away from sunlight. Methanolic extract of

the leaves was prepared by maceration in 96% methanol (Scharlau chemicals, Spain) for 3 days. Rotary evaporator was used to evaporate methanol under reduced pressure at a temperature not exceeding 45°C. The extract was stored at -20°C and was dissolved in sterile normal saline immediately before use. *O. spinosa* extract was administered i.p to the animals in all tests performed.

Experimental animals

All followed procedures comply with the Jordanian Animal Welfare By-Law No. (11) of the year 2010 and the International Association for the Study of Pain (IASP) Guidelines for the Use of Animals in Research and were approved by the ethical committee for research on animals at Al-Ahliyya Amman University. Male BALB/c mice (weight: 20-25 g) were used in all experiments. Mice were brought from the animal house at Al-Ahliyya Amman University, Jordan. Animals were kept at 23 ± 2°C with 12 h lightness and 12 h darkness. Water and pellets of food were available *ad libitum*. Before tests, the animals were acclimatized to the experimental room for at least 120 min.

Writhing test

The writhing test was performed by injecting acetic acid solution (1%, 10 mL/kg, i.p) 30 min after receiving the vehicle, *O. spinosa* extract (300 mg/kg or 600 mg/kg) or 30 mg/kg diclofenac sodium. Each group consisted of 8 mice. The animals were then placed in transparent cages. Ten minutes after acetic acid injection, the number of writhes was counted for 20 min. Writhe was defined as a contraction of the abdominal muscles with elongation of the body and extension of the forelimbs. The percentage inhibition of abdominal cramps was calculated using this formula:

$$\% \text{ inhibition} = \frac{\text{Average number of writhes in control} - \text{average number of writhes in extract-treated animals}}{\text{Average number of writhes in control}} \times 100\%$$

Paw licking test

Animals were divided randomly into groups of 9 mice each. Paw licking test was carried out by injecting twenty microliters of formalin (2.5%) intraplantarly to the left hind paw of the animal. Nociception was determined by counting the time of licking the injected paw, moving while lifting the leg or exhibiting flinching behavior. Counting was performed during the first 5 min after formalin injection (early phase) and during the late phase (25–30 min). The percentage inhibition was calculated using this formula:

$$\% \text{ inhibition} = \frac{\text{Average time of paw licking in control} - \text{average time of paw licking in extract-treated animals}}{\text{Average time of paw licking in control}} \times 100\%$$

Animals were pretreated i.p with vehicle (sterile normal saline, control), *O. spinosa* methanolic extract (300 or 600 mg/kg) or 30 mg/kg diclofenac sodium (standard drug) 30 min prior to performing the experiments. Naloxone (5 mg/kg) or glibenclamide (10 mg/kg) were injected 30 min before a fixed dose of *O. spinosa* extract (300 mg/kg or 600 mg/kg). Atropine (5 mg/kg) was administered 15 min before extract.

Hot-plate test

Animals were divided randomly into 7 groups of 13 mice each. The hot-plate test was used to determine the latencies in pain reaction at 30 min and 150 min. The test was fulfilled only once for every mouse. The animal reaction was assessed by placing the mouse in a glass beaker on a hot-plate at 55 ± 1°C. The time between the animal's placement and first jumping was recorded as an indication for the latency of pain reaction. A cutoff time of 60 sec was determined to avoid tissue damage.

Tail-flick test

Animals were divided randomly into 7 groups of 13 mice each. The tail-flick test was done by immersing the tail in water at 55 ± 1°C. The time starting from immersing the tail in water till showing the first flick was measured after 30 min and 150 min of plant extract administration. A cutoff time of 10 sec was determined.

Qualitative analysis of phytochemicals in *O. spinosa*

For the identification of the main phytochemical groups in *O. spinosa* the following tests were performed as in (8) namely: For alkaloids Dragendorff's test, for phenolics ferric chloride test, for sterols and triterpenes Salkowski test. Flavonoid presence was confirmed by positive alkaline reagent and ferric chloride tests. The presence of anthraquinone glycosides was tested by the addition of ammonia. Saponin presence was checked by shaking the extract vigorously, persistence of foam for 5 min is indicative of saponin presence.

HPLC analysis of *O. spinosa* methanolic leaf extract

The analysis of plant extract was performed using HPLC system Merck-Hitachi equipped with gradient pump L7150, auto sampler L7200, diode array detector L7450. The mobile phase consisted of two mixtures delivered to a reversed phase column (C18 KYA 250 mm × 4.6 mm) in gradient mode, mobile phase A consisted of 95 : 5 phosphate buffer

pH 3.3 to acetonitrile and mobile phase B consisted of 30 : 70 phosphate buffer to acetonitrile. Monitoring wavelength was at 250 nm. Mobile A from 0 to 3 minutes 100% and mobile B 0% at a flow rate of 1.2 mL/min. Mobile A from 3 to 24 minutes 0% and mobile B 100% at a flow rate of 1.2 mL/min. The aim of using diode array detection is to monitor, UV

spectrum of each peak that could help in confirming that it refers to the corresponding standard.

Statistical analysis

Statistical significance of differences between groups was assessed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. GraphPad Prism version 6 was chosen to perform the statistical analysis. Results were represented as the mean $\pm$ standard error of the mean (SEM).

RESULTS

Writhing test

Both doses of *O. spinosa* (300 and 600 mg/kg) decreased the number of writhings in a significant manner compared to the vehicle-treated group (Fig. 1). The i.p administration of 300 mg/kg and 600 mg/kg dose of *O. spinosa* extract resulted in 90.1% and 94.5% inhibition of abdominal cramps, respectively while diclofenac sodium (30 mg/kg) produced only 23.6% inhibition.

Paw licking test

Both doses of *O. spinosa* (300 and 600 mg/kg) inhibited significantly paw licking in the early phase of the formalin test (Fig. 2A). Percentage inhibition in the early phase was 54.9%, 63.1%, and 50.5% for the low dose (300 mg/kg), high dose (600 mg/kg) of *O. spinosa* and diclofenac sodium (30 mg/kg), respectively. Also, in the late phase, both doses of
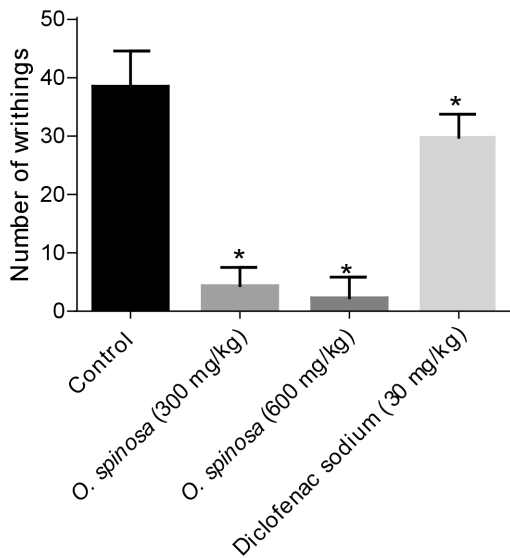

Figure 1. Results of the acetic acid-induced writhing test
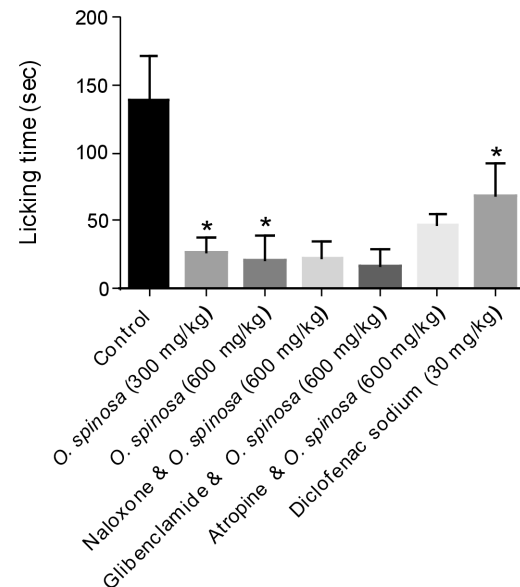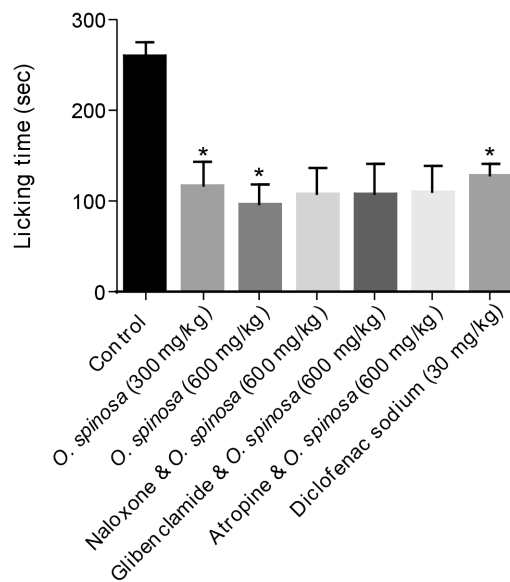* Significant difference from the control (p < 0.05)

Figure 2. Results of paw-licking test. (A) Early phase (B) Late phase of formalin test
* Significant difference from the control (p < 0.05)

O. spinosa produced significant inhibition (Fig. 2B). The percentage inhibition was 81.0%, 85.3% and 50.5% for low dose (300 mg/kg), high dose (600 mg/kg) of *O. spinosa* and diclofenac sodium (30 mg/kg), respectively. The effect of *O. spinosa* was not abrogated by glibenclamide, naloxone or atropine in any of the 2 phases (Fig. 2).

Hot-plate test and tail-flick tests

A significant increase in latency time in hotplate was obtained after 30 min and 150 min of the treatment with *O. spinosa* extracts (300 and 600 mg/kg) (Fig. 3A). The antinociceptive effect of the tested doses of the extract was more pronounced at 30 min compared to 150 min. In tail-
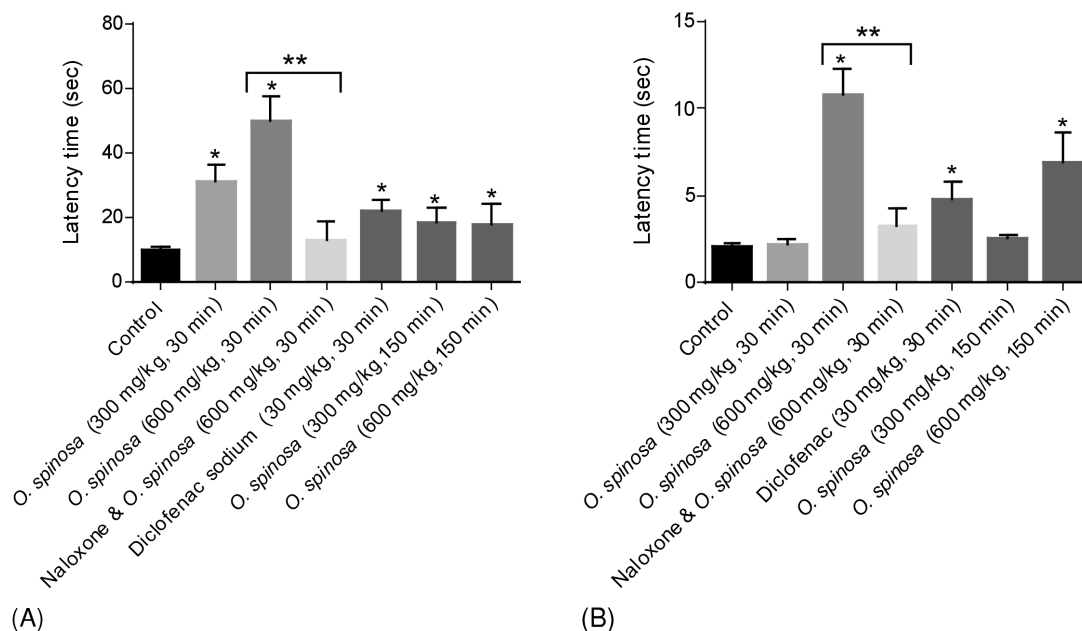
(A)

(B)

Figure 3. Effect of *O. spinosa* extract in thermal pain models (A) Hot-plate test. (B) Tail-flick test
* Significant difference from the control (p < 0.05).
** Significant difference between *O. spinosa* (600 mg/kg) and *O. spinosa* (600 mg/kg) pretreated with naloxone (p < 0.05)
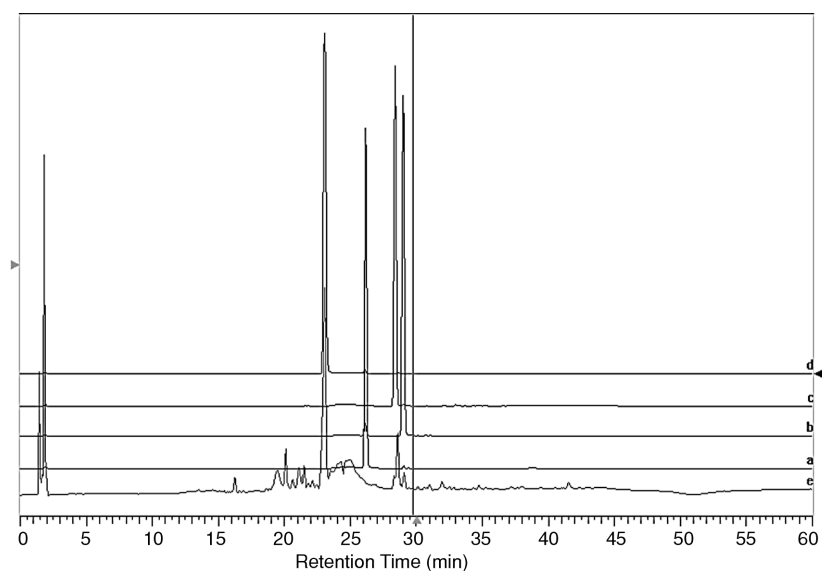
Figure 4. Chromatographic analysis of *O. spinosa* methanolic leaf extract (e). The chromatogram shows the presence of kaempferol (b), apigenin (c) and myricetin (d) but not quercetin (a)

flick test, only the high dose of *O. spinosa* (600 mg/kg) increased latency time after 30 min and 150 min (Fig. 3B). Naloxone antagonized the effect of *O. spinosa* extract in both; hot plate and tail flick tests (Figs. 2, 3).

Qualitative analysis of phytochemicals in *O. spinosa*

Phytochemical screening confirmed the presence of phenolics, saponins, flavonoids, and terpenoids. Alkaloids, sterols, and anthraquinone glycosides were absent in the extracts.

HPLC analysis of *O. spinosa* methanolic leaf extract

The chromatograms show the presence of kaempferol, apigenin and myricetin but not quercetin (Fig. 4).

DISCUSSION AND CONCLUSION

In our study, the administration of 300 mg/kg and 600 mg/kg dose of *O. spinosa* extract produced 90.1% and 94.5% inhibition of abdominal cramps, respectively while diclofenac sodium (30 mg/kg) produced only 23.6% inhibition. Similar results were obtained by Bolle et al. (9) in which the root extract of *O. spinosa* reduced writhing response to phenylquinone when the extract was administered i.p but not orally and in which the extract was more effective than the standard drug.

According to Northover (10), an antinociceptive effect in the abdominal contraction tests (writhing test) cannot easily be distinguished from an anti-inflammatory action. In our study, *O. spinosa* extract inhibited abdominal cramps efficiently. Previous studies have reported the inflammatory activity of *O. spinosa* ethanolic root extract *in vivo* using carragenan-induced inflammatory model (9) and *in vitro* by inhibiting the cytoplasmic phospholipase A_2 enzyme (11). Cytoplasmic phospholipase A_2 catalyzes the release of a fatty acid at the sn_2-position of membrane phospholipids which leads to the formation of lysophospholipid and arachidonic acid; the precursor for eicosanoids (11). In fact, eicosanoid biosynthesis is highly regulated and depends upon the activation of phospholipases A_2 and the subsequent liberation of arachidonic acid, sequestered within the membrane phospholipids (12). Phospholipase A_2 inhibitory activity may explain, at least partially, the antinociceptive action of *O. spinosa* in writhing test in our study.

In the present investigation, *O. spinosa* inhibited significantly paw licking in early and late phases of formalin test. Percentage inhibition in the early phase was 54.9%, 63.1%, and 50.5% for low dose of *O. spinosa* (300 mg/kg), high dose (600 mg/kg) and diclofenac sodium (30 mg/kg), respectively while the inhibition was 81.0%, 85.3% and 50.5% for the three treatments, respectively. Therefore, the inhibitory effect of *O. spinosa* extract was more pronounced in the late phase of formalin test. The lowest dose tested (300 mg/kg) of *O. spinosa* extract was more efficient than 30 mg/kg diclofenac sodium in late phase of formalin test in decreasing paw licking.

The effect of *O. spinosa* was not abrogated by glibenclamide, naloxone or atropine. This indicates that the action of *O. spinosa* is not mediated by ATP-sensitive K^+ channel, opioid or muscarinic receptors. To our best knowledge, this is the first report of antinociceptive action of *O. spinosa* in formalin test.

A significant increase in latency time in hot-plate was obtained after 30 min and 150 min of the treatment with *O. spinosa* extracts. The antinociceptive effect of the tested doses of the extract was more pronounced at 30 min compared to 150 min. Contrary to our results, the administration of *O. spinosa* methanolic root extract orally or i.p produced no significant difference in reaction time in a hot-plate test in mice (9). This can be explained by the presence of different phytochemicals in roots and leaves of this plant which differ in their biological activities.

In our work, naloxone abolished *O. spinosa* effect after 30 min in hot-plate test (Fig. 3A). According to Abid and Khan (13), the hot-plate test is adequate for testing analgesics that act centrally but not peripherally. Therefore, it is expected that *O. spinosa* acts centrally. Even though, the possibility exists that this plant may act also peripherally as it gave positive results in writhing test.

In tail-flick test, only the high dose of *O. spinosa* (600 mg/kg) increased latency time after 30 min and 150 min (Fig. 3B). According to Yılmaz et al. (4), aqueous extract of *O. spinosa* aerial parts showed analgesic activity higher than aspirin in the tail-flick test. In our study naloxone abolished *O. spinosa* effect after 30 min in tail-flick test (Fig. 3B). Up to our best knowledge, this is the first report that *O. spinosa* may interact with the opioid receptor in thermal pain models.

Extensive phytochemical analyses of *O. spinosa* roots were performed while those of the leaves are scarce. Interestingly, ononetin, a deoxybenzoin isolated from the roots of *O. spinosa* inhibited transient receptor potential melastatin-3 (TRPM3)

which is a non-selective calcium permeable channel (14). It is well established that TRPM3 blockade attenuates thermal nociception *in vivo* (14). Recently, it was demonstrated that activation of opioid receptors in mouse paw reduces the pain caused by TRPM3 protein activation (15). Further research is needed to find out if ononetin or other structurally related TRPM3 blockers are present in the aerial parts of *O. spinosa* and whether opioid receptor activation by *O. spinosa* blocks TRPM3 channels.

The present study demonstrated that *O. spinosa* methanolic leaf extract exerts analgesic effects in chemical and thermal pain models in mice. Long term toxicological studies of this plant are needed to ensure its safety since only a short-term study failed to demonstrate any toxic effects with doses up to 2 g/kg (7). Our findings showed that interaction with the opioid receptor is a possible mechanism underlying the thermal antinociceptive action of *O. spinosa* leaf extract. Phytochemical studies are needed for the isolation of TRPM3 blockers from *O. spinosa* aerial parts.

Acknowledgments

Conflicts of interest

The authors declare no conflict of interest.

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