



## Comparison of Antinociceptive activity of *Origanum majorana* L. methanol leaf extract in mice in different models

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### ABSTRACT

*Origanum majorana* L. widely used in Jordan and other Mediterranean countries as a flavoring and herbal spice. An infusion of the plant is used for treatment of internal diseases, haemorrhoids, pains, and animal bites and poisons. In this study, we attempted to identify the possible antinociceptive action of *Origanum majorana* methanol leaf extract using tail immersion, hot plate, and writhing tests. The antinociceptive effect of the methanol extract of *Origanum majorana* (MEO) leaves was assessed after intraperitoneal administration into mice. Morphine sulfate (5 mg/kg; i.p.) and diclofenac (10 mg/kg; i.p.) were used as reference analgesic agents. Naloxone (5 mg/kg; i.p.) was also tested. MEO was studied at the doses of 50, 100, and 200 mg/kg (i.p.) and exhibited significant antinociceptive activities in all tests used. The above-mentioned doses of the extract reduced the writhing responses by 24.51, 52.11, and 72.43%, respectively. MPE% was increased by 10.51, 15.07, 22.40% in the tail immersion, and 12.38, 14.31, 22.14 % in the hot plate test at the tested doses, respectively. Naloxone antagonized antinociceptive effect at the doses of 100 and 200 mg/kg whereas partially antagonized the effect of MEO at the dose of 50 mg/kg. Based on the results obtained, it can be concluded that MEO has antinociceptive effects both at the peripheral and central levels.

**Keywords:** *Origanum majorana*, Antinociceptive, Opioid, methanol extract, mice.

### INTRODUCTION

*Origanum majorana*, commonly known in our region as " Mardakoush ", is an aromatic, perennial, herbaceous plant native to southern Europe and the Mediterranean area and belonging to the family Lamiaceae [1]. The plant has been used as a flavoring and herbal spice from time immemorial.

*Origanum majorana* has a wide use in traditional medicine in Jordan and other arebian countries. *Origanum majorana* tea (extract of its leaves and flowers) has been prescribed in folkloric medicine for relieving the symptoms of hay fever, sinus congestion, indigestion, asthma, stomach pain, headache, dizziness, colds, coughs, internal diseases, haemorrhoids, animal bites and poisons, and nervous disorders. The unsweetened tea can also be used as a mouthwash or gargle. Further, *Origanum majorana* leaves can be used in folkloric medicine as a poultice for the pain of rheumatism and for sprains. The oil from the leaves is also used to relieve toothache pain [5,6,2-4].

Many studies demonstrated that *Origanum majorana* extract has a potent antioxidant, antiproliferative, antimicrobial, anti-hepatoma, and antimutagenic activities [6, 7-12].

Phytochemical study of the *Origanum majorana* revealed the presence of terpenoids (thymol and carvacrol), flavonoids (diosmetin, luteolin, and apigenin), tannins, hydroquinone, phenolic glycosides (arbutin, methyl arbutin, vitexin, orientin, and thymonin), and triterpenoids (ursolic acid and oleanolic acid) [13-16].

In the present study, *Origanum majorana* was selected because

it is one of the medicinal plants commonly used in remedies to treat abdominal pain, toothache and rheumatism in Jordan traditional medicine and other countries in Middle East??. However, up to date no ethnopharmacological data have previously been systematically conducted to evaluate the antinociceptive action supporting traditional uses of this plant in folklore medicine. In this work we evaluate the antinociceptive activity of *Origanum majorana* methanol extract in different models of acute pain in mice to discriminate between central and peripheral pain components. The reason to use methanol extract in this investigation is that methanol is more nonpolar than water, therefore, several substances including volatile oils, the major chemical constituents of *Origanum majorana*, would be expected to be more soluble in methanol fraction than in water extract

## MATERIALS AND METHODS

### *Plant material*

Leaves of *Origanum majorana* were collected during April from Sama-Alsarhan (Northern Badia-Jordan). The plant material was identified and authenticated taxonomically at the Hashemite University herbarium. A voucher specimen was deposited under the number HU-625 at the Hashemite University herbarium, Zarka, Jordan, for future reference. Powdered dried leaves of *Origanum majorana* (100 g) were extracted with 2500 ml methanol using the Soxhlet apparatus. The solvent was removed from resulting solution under vacuum in a rotary evaporator. The dried extract yield was calculated as 18 g/100 g (18%).

### *Drugs and chemicals*

The following drugs and chemicals were used in this study: methanol (Merck, Darmstadt, Germany), DMSO (Merck, Germany), morphine sulfate (Sigma, St. Louis, USA), diclofenac (Sigma, St. Louis, USA), naloxone (Sigma, St. Louis, USA), and acetic acid (Merck, Germany).

### *Drugs and treatment*

All drugs, administered intraperitoneally (i.p.) 30 min before the procedure, were dissolved in the vehicle (ratio DMSO saline 1:4). Methanol extract of *Origanum majorana* (MEO) was given to the animals at the doses of 50, 100, and 200 mg/kg in fixed volume of 0.1 ml. The vehicle at the same volume was injected into the control group. Morphine sulfate (5 mg/kg, [17], Sigma) and diclofenac (10 mg/kg, [18], Sigma), a non-steroidal anti-inflammatory drug, were used as the reference analgesic agents. In order to investigate possible involvement of opioid system in the antinociceptive effect of MEO, mice were pre-treated with the nonselective opioid receptor antagonist, naloxone (5 mg/kg, [17], Sigma) and injected 10 min before the administration of the extracts (50, 100, and 200 mg/kg). The doses were selected based on the literature review and results of acute toxicity. If the literature is devoid of any such reference then the 10 times less dose of maximum concentration (2000mg/kg) used in acute toxicity test was consider as the maximum dose (200mg/kg) and two more doses (50 and 100mg/kg) below the maximum dose level were selected for study.

### *Phytochemical screening*

MEO was tested for the presence of alkaloids, tannins, reducing sugar, and phenolics by using standard phytochemical screening procedures. In each test, we used 10% (w/v) methanol solution of extract used [19]. The total phenolic content of extracts was determined using to the Folin–Ciocalteu method [20].

### *Animals*

Swiss albino mice (35–40 g) were obtained from our own animal facility. Animals were maintained in a room with controlled temperature ( $22\pm 2$  °C) for 12 h light/12 h dark cycle with free access to food and water. Twelve hours before each experiment, animals received only water. Animal care and research protocols were based on the principles and guidelines adopted by the Guide for the Care and Use of Laboratory Animals (NIH publication No: 85-23, revised in 1985) and approved by the Animal Care Committee at the Hashemite University.

### *Acute toxicity*

Mice were divided into control and test groups (n = 6). First group served as control. MEO was administered i.p. to different groups at the increasing doses of 200, 400, 500, 1000, and 2000 mg/kg. After injections of extracts, mice were allowed food and water *ad libitum*. Animals were observed for possible mortality cases and behavioral changes for 72 h [21].

### *Tail immersion*

The tail immersion method was used to evaluate the central MEO mechanism of analgesic activity. The painful reactions in animals were produced by thermal stimuli through dipping the tail tips into hot water [22]. An area (the lower 5 cm portion) of the tail was marked and immersed in the water bath thermostatically maintained at  $52.5\pm 0.2$

°C (GFL water bath, Germany). The withdrawal time of the tail from hot water (in seconds) was noted as the reaction time. The reaction time was measured at 30 min after treatment. The maximum cutoff time for immersion was 15 s to avoid the injury of the tissues of tail.

#### **Hot plate test**

Pain reflexes in response to a thermal stimulus were measured using a Hot Plate Analgesia Meter from Ugo Basile Instruments (No.7280). Mice were put on the hot plate at  $55\pm 0.5$  °C for testing. Their action time (hind paw licking, hindpaw flicking or jumping) of the pain response at 30 min after drug or extract injection was measured. The cutoff time was 30 s for analgesic assays [23].

#### **Writhing test**

The method was chosen to evaluate the peripheral antinociceptive effect. Writhing test, a chemical visceral pain model, was performed as described by Koster *et al*, [24]. Mice were injected with 10 ml/kg of 0.6% acetic acid solution (i.p.) 30 min after the administration of the drugs or extracts. Five minutes after the administration of acetic acid, the number of writhes was counted for 10 min.

#### **Statistical analysis**

The statistical analyses were performed by one-way ANOVA, followed by Tukey's multiple comparison tests. The statistical analyses were carried out using GraphPad Prism version 5.0. The results were expressed as the mean $\pm$ S.E.M. to show variation in groups. Differences were considered significant when  $P\leq 0.05$ . The results of the tail immersion and hot plate tests were given as a percentage of the maximal possible effect (MPE% $\pm$ S.E.M.), which was calculated as follows [25]:  $MPE\% = [(post\text{-}drug\text{ latency}) - (pre\text{-}drug\text{ latency}) / (cutoff\text{ time}) - (pre\text{-}drug\text{ latency})] \times 100$ .

## **RESULTS**

#### **Phytochemical screening**

Phytochemical investigations of MEO showed the presence of phenolics, tannins, and reducing sugars. Total phenols were calculated as  $21.22\pm 0.54$  mg GAE/g extract.

#### **Acute toxicity**

MEO, at the doses of 200–2000 mg/kg i.p. given to mice, had no effect on their behavioral responses and no mortality during the observation period of 72 h after administration. Therefore, it can be indicated that MEO has low toxicity profile.

#### **Tail immersion**

The antinociceptive activities of MEO and reference drug on the tail immersion test are shown in Fig. 1. The MEO showed a dose-dependent and significant ( $P < 0.001$ ) increase in the pain threshold at 30 min post-treatment. Three doses (50, 100, and 200 mg/kg) of MEO increased the reaction time in the thermal stimulus. MPE% values were observed as  $1.41\pm 0.15\%$  (control),  $32.15\pm 2.73\%$  (morphine),  $10.51\pm 1.76\%$  (MEO 50 mg/kg),  $15.07\pm 2.1\%$  (MEO 100 mg/kg) and  $22.40\pm 2.11\%$  (MEO 200 mg/kg), respectively. The effects of the extract were significantly ( $P < 0.001$ ) lower than those produced by morphine (10 mg/kg) in the same tests. As can be seen in Fig. 1, naloxone-induced reversal effect against morphine (5 mg/kg) in tail-immersion experiments was found to be significantly greater than those against MEO doses (100 and 200 mg/kg).

#### **Hot plate test**

Treatment of animals with the MEO caused a significant ( $P < 0.05$  and  $P < 0.001$ ) increase in the MPE% (Fig. 2). MPE% values were calculated as  $3.35\pm 0.71\%$  (control),  $34.12\pm 3.70\%$  (morphine),  $12.38\pm 2.41\%$  (MEO 50 mg/kg),  $14.31\pm 2.70\%$  (MEO 100 mg/kg) and  $22.14\pm 3.10\%$  (MEO 200 mg/kg), respectively. Naloxone counteracted the antinociceptive activity of MEO at the doses of 100 and 200 mg/kg ( $P < 0.01$  and  $P < 0.001$ ) and morphine ( $P < 0.001$ ).

#### **Writhing test**

The administration of MEO (50, 100, and 200 mg/kg) caused a dose-dependent significant reduction in the number of writhing episodes induced by acetic acid compared to the control ( $P < 0.001$ ). The results are shown in Fig. 3. The percentage inhibition of constrictions was calculated as 64.25% (diclofenac), 94.50% (morphine), 24.51% (MEO 50 mg/kg), 52.11% (MEO 100 mg/kg), and 72.43% (MEO 200 mg/kg). The 200 mg/kg dose of MEO was statistically similar to the reference drug diclofenac. The antinociceptive effect of MEO was antagonized by pretreatment with naloxone at the doses of 100 and 200 mg/kg ( $P < 0.05$  and  $P < 0.01$ ). This reversal effect of naloxone on the antinociceptive effect of MEO was lower than its effect on morphine.

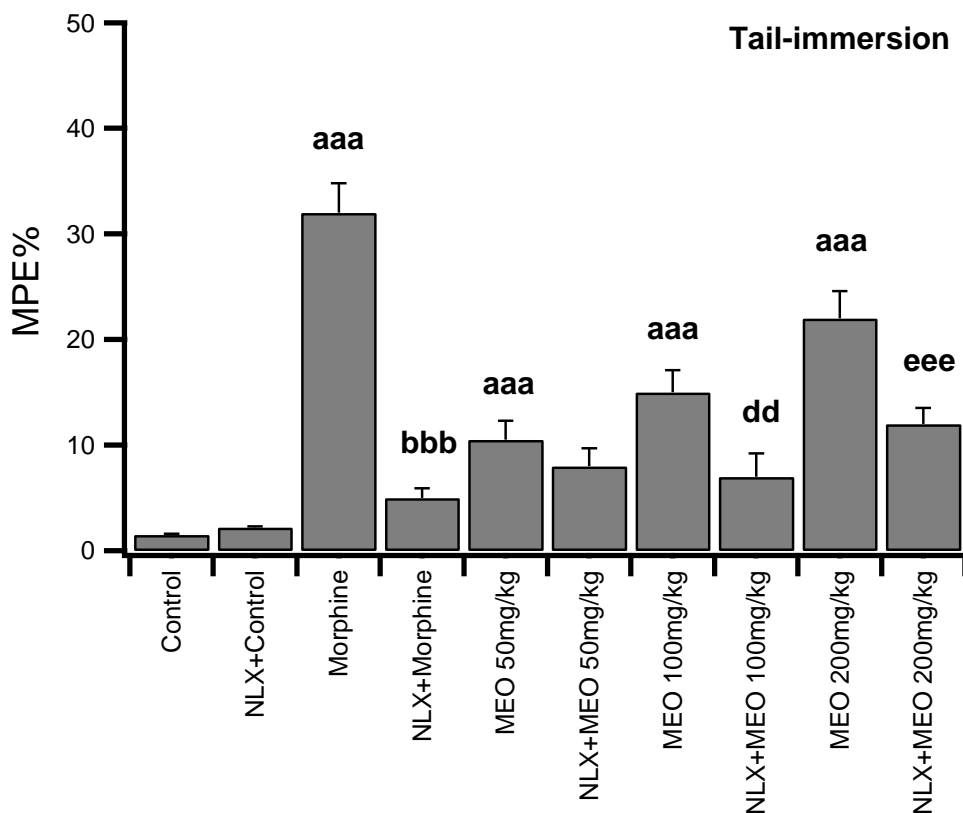


Fig. 1. The antinociceptive effect of *Origanum majorana* leaves extracts, morphine, and reversal effect of naloxone as observed in tail-immersion tests. Values are presented as the mean±S.E.M. (n = 6–7) (NLX; naloxone); aaaP < 0.001, significant difference from control; bbbP < 0.001, significant difference from morphine alone; ddP < 0.01, significant difference from MEO 100 mg/kg alone; eeeP < 0.001, significant difference from MEO 200 mg/kg alone

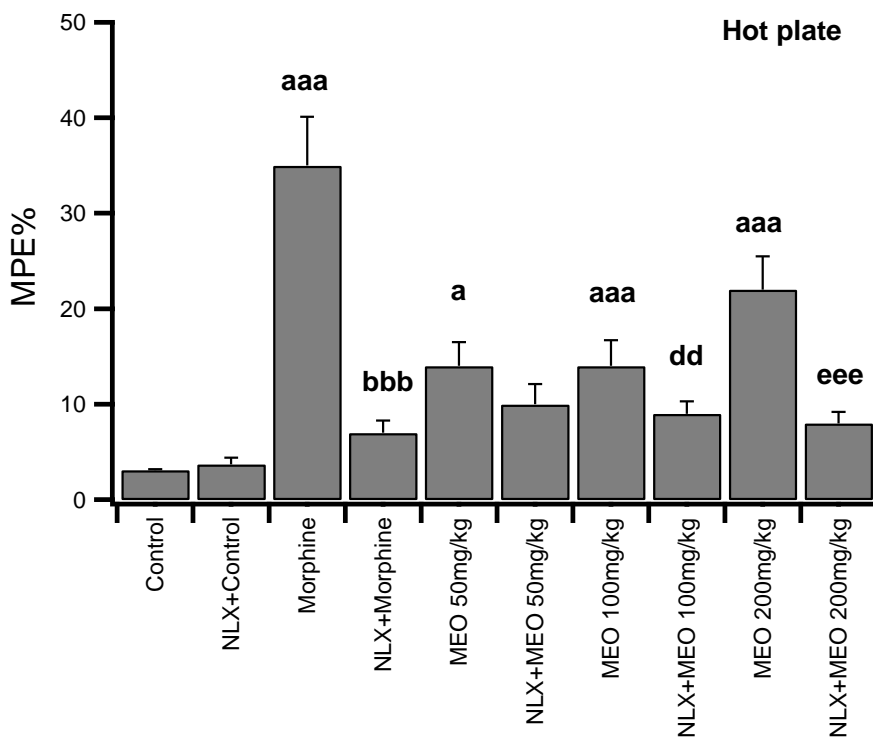


Fig. 2. The antinociceptive effect of *Origanum majorana* leaves extracts, morphine, and reversal effect of naloxone as observed in hot-plate test. Values are presented as the mean±S.E.M. (n = 6–8) (NLX; naloxone); aP < 0.05 and aaaP < 0.001, significant difference from control; bbbP < 0.001, significant difference from morphine alone; ddP < 0.01, significant difference from MEO 100 mg/kg alone; eeeP < 0.001, significant difference from MEO 200 mg/kg alone

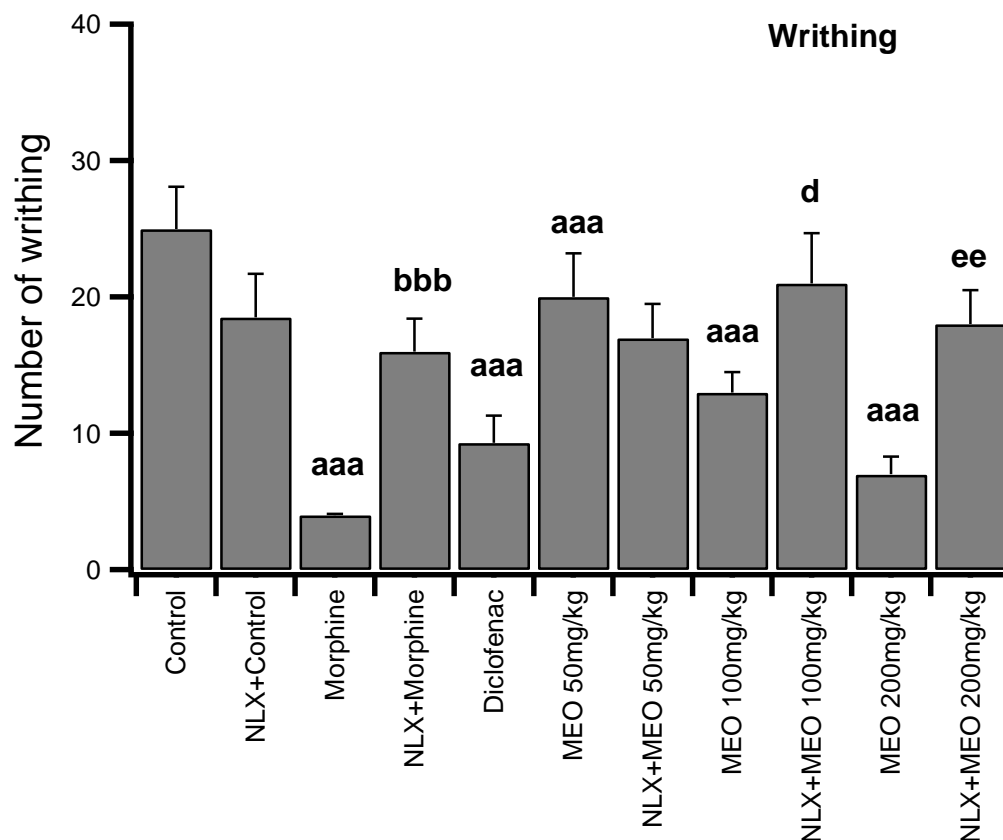


Fig. 3. The antinociceptive effect of *Origanum majorana* leaves extracts, morphine, diclofenac, and reversal effect of naloxone as observed in acetic acid-induced writhing test. Values are presented as the mean±S.E.M. (n = 6–7) (NLX; naloxone); aaaP < 0.001, significant difference from control, bbbP < 0.001, significant difference from morphine alone; dP < 0.05, significant difference from MEO 100 mg/kg alone; eeP < 0.01, significant difference from MEO 200 mg/kg alone

## DISCUSSION AND CONCLUSION

In the present study, we did not observe any mortality case up to the dose of 2 g/kg of MEO (i.p.). Therefore, we may suggest that the extract has no lethal toxicity in mice. Based on the findings of one chemical and two thermal pain models employed in this study, MEO was found to possess antinociceptive activities.

The hot plate and tail immersion tests are widely used for assessing central antinociceptive activities. Furthermore, these two tests are distinguished by their tendency to respond to the pain stimuli conducting through neuronal pathways, as the tail immersion mediates spinal reflexes to nociceptive stimuli, whereas the hot plate is a supraspinally organized response of pain [26,27]. Opioid agents exhibit their analgesic effects both via supraspinal ( $\mu 1$ ,  $\kappa 3$ ,  $\delta 1$ ,  $\sigma 2$ ) and spinal ( $\mu 2$ ,  $\kappa 1$ ,  $\delta 2$ ) receptors [28,29]. In our experiments, MEO exhibited a statistically significant, but lesser antinociceptive activity than morphine in hot plate and tail immersion tests. It seems quite possible that the higher doses of the extract have more potent central antinociceptive effect. It has been suggested that the opioid MEO mechanisms mediate antinociceptive effect of MEO.

In general, acetic acid writhing test are used to evaluate the compounds for peripheral antinociceptive activities. The writhing test is useful to discriminate central and peripheral nociception [30]. Acetic acid injection produces peritoneal inflammation, which triggers a response characterized by writhing [24]. Related studies have demonstrated that acetic acid indirectly induces the release of endogenous mediators of pain (such as prostaglandins, kinins, histamin, etc.) that stimulate the nociceptive neurons, which are sensitive to nonsteroidal anti-inflammatory drugs and opioids [31-33]. When compared antinociceptive activities, MEO was relatively more potent in acetic acid writhing test indicating peripheral antinociception. MEO did not demonstrate antinociceptive effect as potent as reference drug, morphine (5 mg/kg; i.p.). In contrast, the extract (200 mg/kg) exhibited an action similar to that of diclofenac (10 mg/kg; i.p.), a reference drug for peripheral antinociception.

The antinociceptive activities of all substances used in the pain models were antagonized by naloxone. The antagonistic effect of naloxone on the antinociceptive effect of MEO (50, 100, and 200 mg/kg) was lower than its

effect on morphine. The reversing effects of naloxone in the hot plate and tail immersion tests were higher than the writhing test. By considering all these results, it was concluded that inhibition of releases of endogenous mediators like bradykinin, serotonin, histamine, substance P and prostaglandins may play a pivotal role in the peripheral antinociceptive effect of MEO. As the activity of MEO in writhing test was antagonized partially by naloxone, opioid MEO mechanisms may only partially involve in the antinociceptive effect of extract.

Several phytochemical screening studies have reported that species belonging to Lamiaceae family contain various active compounds such as flavonoids, glucosinolates, alkaloids, etc. [34-36]. Furthermore, phytochemical study of the *Origanum majorana* revealed the presence of luteolin, apigenin, thymol, carvacrol, ursolic acid, and vitexin). It is, therefore, possible that the antinociceptive activity of MEO observed may be attributable to its phenolics, reducing sugars, and tannins, shown to be present during phytochemical analysis. In accordance with this finding, the phenolic compound vitexin (which has been isolated from *Origanum majorana*) was reported to reduce the edema formation induced by carrageenan and have antinociceptive activity [37]. Hence, it was suggested that the antinociceptive action of MEO may be related to this constituent. In addition, there is probably an involvement of luteolin, thymol, carvacrol and ursolic acid, which have been isolated from *Origanum majorana* and shown to have antinociceptive and anti-inflammatory activity [38-42]. In conclusion, results of the present study indicated that all doses of MEO exhibited central and peripheral antinociceptive effects. However, the MEO mechanisms behind the central and peripheral antinociceptive activity of MEO are not completely understood and may need further studies with different antagonists (such as adrenergic, serotonergic, etc.). Taking these findings into account, it seems quite possible that *Origanum majorana* contains constituents with antinociceptive activity, which may lead for the development of new natural products having analgesic effect. In further investigations, the different fractions of *Origanum majorana* will be evaluated and the structural characterization of responsible components will be clarified.

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