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RESEARCH ARTICLE

Antinociceptive, Anti-inflammatory Effects and Acute Toxicity of Aqueous and Ethanolic Extracts of *Myrtus communis* L. Aerial Parts in Mice

Hossein Hosseinzadeh [1,*], Mohammad Khoshdel [2], Maryam Ghorbani [2]

[1] *Pharmaceutical Research Center, Pharmacodynamy and Toxicology Department, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, I.R. Iran*
[2] *Department of Pharmacodynamy and Toxicology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, I.R. Iran*

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Abstract
Myrtus communis L. aerial parts have been used in traditional medicine for the treatment of inflammatory disease. In this study 350 mice were divided into three main groups: negative (saline), positive (morphine or diclofenac) controls, and test groups. The acute toxicity was assessed for 2 days. Antinociceptive activity was performed using hot plate and writhing tests. The anti-inflammatory effect was investigated using xylene-induced ear edema and a cotton pellet test. According to phytochemical screening, the extracts contained tannins, alkaloids, and flavonoids. The LD50 values of the aqueous and ethanolic extracts were 0.473 and 0.79 g/kg, respectively. In hot plate test, the aqueous and ethanolic extracts showed significant antinociceptive activity that was inhibited by naloxone. The extracts exhibited antinociceptive activity against acetic acid—induced writhing and also showed significant activity against acute inflammation which was dose dependent for aqueous extract. The ethanolic (0.05 g/kg) and aqueous extracts (0.005, 0.015, and 0.03 g/kg) demonstrated anti-inflammatory effects against chronic inflammation. The aqueous and ethanolic extracts of the aerial parts of *M communis* L. showed antinociceptive effects and these may be mediated by opioid receptors.

1. Introduction

Many Iranian plants such as *Zataria multiflora*, *Zhumeria majdae*, *Crocus sativus* L. and *Salvia leriifolia* have antinociceptive and anti-inflammatory effects [1—4]. The Myrtle (*Myrtus*) is a genus of one or two species of flowering plants in the family *Myrtacea*, which is well represented in Australia, the East Indies, and tropical America. They are evergreen shrubs or small trees, growing to 5-m tall spontaneously [5]. *Myrtacea* traditionally have been used as antiseptic, laxative, hypoglycemic, and anti-inflammatory agents [5—7]. The essential oil obtained from the leaves is mainly used in the treatment of lung disorders [5,8], and it has been found to possess antibacterial [9,10] and antioxidant activities [6]. The essential oil obtained from this species has been widely investigated. There is considerable

* Corresponding author. Pharmaceutical Research Center, Pharmacodynamy and Toxicology Department, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, I.R. Iran.
E-mail: hosseinzadehh@mums.ac.ir

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variability in the composition of oil from different locations [11]. The most important constituents of myrtle oil (up to 0.8% in the leaves) are myrtenol, myrtenol acetate, limonene, linalool, pinene, and 1,8-cineole (one of the main constituents of myrtle essential oil) [12].

The ethanolic extract of *Myrtus communis* L. has been assessed for its anti-inflammatory activity on rats by measuring the suppression of carrageenan-induced paw edema for one dose [13]. In other investigations, myrtucommulone from *M communis* L. diminished PGE_2 formation, without significant inhibition of the COX enzymes, and provides an interesting pharmacologic profile suitable for interventions in inflammatory disorders [14].

This study was performed in order to evaluate anti-inflammatory and antinociceptive activity as well as the acute toxicity of *M communis* L. aqueous and ethanolic aerial parts extracts in different doses. Furthermore, the preliminary mechanism of the antinociceptive effect (central or peripheral) and its association with opioid receptors was considered.

2. Materials and methods

2.1. Plants and chemicals

The plant was collected from Fasa, Iran, in late May, dried in shadow, and ground and voucher samples were preserved for reference in the Herbarium of the Department of Pharmacognosy, School of Pharmacy, Mashhad (Voucher no. 153-2613-3). Naloxone hydrochloride and morphine sulfate was obtained from Tolid Daru Co., Tehran, Iran; diclofenac sodium was bought from DarouPakhsh Holding Co., Tehran, Iran; xylene, acetic acid, and chloroform were purchased from Merck Co., Germany; the ampicillin vial was bought from Jaber Ebne Hayya Pharmaceutical Co., Tehran, Iran; and ketamine was obtained from Trittau Co., Germany. All chemicals and solvents used in this study were of analytical grade.

2.2. Preparation of the extracts

The aerial parts of the plant were powdered. For the decoction extract, 1 L water was added to 100-g plant material, soaked for 24 hours, and percolation was performed until the solvent became colorless. The extract was then concentrated in a vacuum to the desired volume (90% solvent out). It was dried completely and weighed. To obtain the ethanolic extract, the plant powder (100 g) was first extracted using the Soxhlet apparatus (Fisher Scientific, Gmbh, Germany) with petroleum ether in order to remove the oil, wax, and colorful substances (24 hours), followed by maceration in 500-ml ethanol (85%, v/v) for 24 hours. The mixture was subsequently filtered and concentrated in the vacuum at 50 °C. The ethanolic extract was suspended in physiologic saline using Tween-80 (Merck Co., Germany) [15].

2.3. Preliminary chemical tests

Phytochemical screening of the extract was performed using the following reagents and chemicals [5]: Alkaloids with Mayer and Bushard's reagent, flavonoids by the use of mg and HCl; tannins with 1% gelatin; and 10% NaCl solutions and saponins with the ability to produce suds.

2.4. Animals

Male albino mice weighing 25—30 g were obtained from a random bred colony and were maintained on a special diet (Khorassan Javane, Mashhad, Iran) in the animal house of Mashhad University of Medical Sciences. The animals were housed in a colony room with a 12:12-h light, dark cycle at 21 ± 2 °C [2]. All animal experiments (i.e., the handling and use of the animals) were carried out in accordance with the institutional current guidelines for the care of laboratory animals in Mashhad University of Medical Sciences, Ethical committee Acts (No. 1024).

2.5. Animal treatment protocols

Male albino mice were divided into different groups ($n = 6$—8). The negative control group received saline (10 ml/kg, i.p.) and the positive control groups for antinociceptive and anti-inflammatory tests received diclofenac (15 mg/kg, i.p.) and morphine (10 mg/kg, i.p.), respectively. Based on maximum tolerated dose (MTD) of the aqueous (0.27 g/kg) and ethanolic (0.47 g/kg) extracts, treatment groups received 0.2, 0.1, 0.03, 0.005, and 0.015 g/kg, (i.p.) of aqueous extract and the 0.35, 0.15, and 0.05 g/kg, (i.p.) of ethanolic extract. For the cotton pellet test, because of the observed mortality, higher doses of extracts were not injected for 7 days. The last group was subcutaneously pretreated with naloxone (2 mg/kg) 20 minutes prior to the injection of the extracts and morphine.

2.6. Acute toxicity

The 0.2-g/kg doses of extracts were injected intraperitoneally into groups of six mice. There was a negative control that received normal saline. The reference dose was determined as the dose in which no death was recorded after 24 hours. By increasing the dose, 100% death was observed at doses of 1.6 g/kg. Then, doses in the range of 0.2 to 1.6 g/kg were divided into six levels and then, according to following formula ($am = a_1.q^{n-1}$, am: dose of 100% death, a_1: reference dose, n: the number of levels, q: coefficient ratio) [1], the coefficient ratio was calculated to obtain the different doses. The number of deaths was counted 48 hours after treatment. The LD50 values and corresponding confidence limits were determined by the Litchfield and Wilcoxon method (PHARM/PCS Version 4, USA).

2.7. Antinociceptive study

2.7.1. Hot plate test

The antinoceceptive activity was assessed using the hot plate test [16]. The temperature of the metal surface was maintained at 55 ± 0.2 °C. Latency to a discomfort reaction (i.e., licking paws or jumping) was determined before and after administration of the drug. The cut-off time was 20 seconds. We used saline and morphine (10 mg/kg i.p.) as negative and positive controls, respectively. Doses 0.005,

0.015, 0.03, 0.1, and 0.2 g/kg from aqueous extract and 0.15 and 0.35 g/kg from ethanolic extract were injected intraperitoneally. Also 2 mg/kg naloxone was injected subcutaneously 15 minutes prior to the administration of morphine or the extracts. The responses were recorded in 0, 60, and 120 minutes after administration.

2.7.2. Writhing test

Thirty minutes after the administration of the extract (0.005, 0.015, 0.03, 0.1, and 0.2 g/kg from aqueous extract and 0.15 and 0.35 g/kg from ethanolic extract), saline (10 ml/kg) , morphine (10 mg/kg i.p.), or diclofenac (10 mg/kg i.p.), the mice were given an intraperitoneal injection of 0.7% v/v acetic acid solution (volume of injection, 0.1 ml/10-g body wt.)[4]. Also, 2 mg/kg naloxone was injected subcutaneously 15 minutes prior to the administration of the morphine or the extracts. The number of writhes produced in these animals was counted for 30 minutes [17].

2.8. Anti-inflammatory study

2.8.1. Xylene-induced ear edema

Thirty minutes after intraperitoneal injection of the extracts (0.005, 0.015, 0.03, 0.1, and 0.2 g/kg from aqueous extract and 0.05, 0.15, and 0.35 g/kg from ethanolic extract), diclofenac (15 mg/kg), and saline (10 ml/kg), 0.03 ml of xylene was applied to the anterior and posterior surfaces of the left ear. The right ear was considered the control. Two hours after xylene application, the mice were sacrificed and both ears were removed. Circular sections were taken using a cork borer with a diameter of 4.5 mm and weighed. The increase in weight caused by the irritant was measured by subtracting the weight of the untreated left ear section from that of the treated right ear sections [18].

2.8.2. Cotton-pellet granuloma

Pellets of dentistry cotton weighing 1 mg each were sterilized in an air oven at 121 °C for 20 minutes and impregnated with 0.4 ml of an aqueous solution of ampicillin (10%). Under ketamine (65 mg/kg body wt.) and xylazine (6.5 mg/kg body wt.) anesthesia, two cotton pellets were implanted subcutaneously in the groin region of each mouse, one on each side. The extracts (0.005, 0.015, 0.03, 0.1, and 0.2 g/kg from aqueous extract and 0.05, 0.15, and 0.35 g/kg from ethanolic extract) and diclofenac (15 mg/kg) or saline (10 ml/kg) were given intraperitoneally once daily for 7 days. On Day 8, the mice were killed and the pellets and surrounding granulation tissue were dried at 60 °C for 24 hours. The weight of granuloma was determined [17].

2.9. Statistical analysis

The data were expressed as mean values ± standard error of the mean (SEM) and tested using an analysis of variance followed by the multiple comparison test of Tukey-Kramer. A difference of $p < 0.05$ was considered significant.

3. Results

The yield of decoction for aqueous extract was 24.50% (w/v) and was about 7.66% (w/v) for the ethanolic extract. The LD50 values of intraperitoneal injection of the aqueous and ethanolic extracts were 0.473 g/kg body wt. [95% confidence level (CL): 0.74, 0.302] and 0.79 g/kg body wt. (95% CL: 0.599, 1.815), and the maximum nonfatal doses were 0.27 g/kg body wt. and 0.47 g/kg body wt, respectively.

Phytochemical screening of the extracts indicated the presence of tannins, alkaloids, and flavonoids. Saponins were detected in ethanolic extract.

In the hot plate test, the administration of the aqueous [at doses of 0.03 g/kg ($p < 0.001$), 0.015 g/kg ($p < 0.01$) and 0.005 g/kg ($p < 0.05$)] and ethanolic extracts in all doses [0.35 g/kg ($p < 0.001$), 0.15 g/kg ($p < 0.01$) and 0.05 g/kg ($p < 0.05$)] showed dose-dependent antinociceptive activity and significantly increased the reaction time compared with the controls.

The antinociceptive effect of 0.03 g/kg of aqueous extracts 60 minutes after injection and the antinociceptive effect of ethanolic extracts in all doses 60 and 120 minutes after injection was more than that of morphine (Figs. 1 and 2).

Naloxone pretreatment (2 mg/kg body wt., subcutaneously) after the intraperitoneal injection of the extracts and morphine (10 mg/kg body wt.) inhibited the antinociceptive activity of aqueous extracts at doses 0.03 and 0.015 g/kg after 60 minutes post-injection of the extract ($p < 0.05$) and the ethanolic extracts at doses 0.35 g/kg ($p < 0.001$) 60 and 120 minutes after injection and 0.15 g/kg ($p < 0.01$ and $p < 0.05$) 60 and 120 minutes after injection as well as morphine (Figs. 1 and 2).

The aqueous extract in all doses (0.2, 0.03, 0.015, 0.005 g/kg) and ethanolic extract in 0.15 and 0.35 g/kg doses significantly reduced the number of mouse abdominal

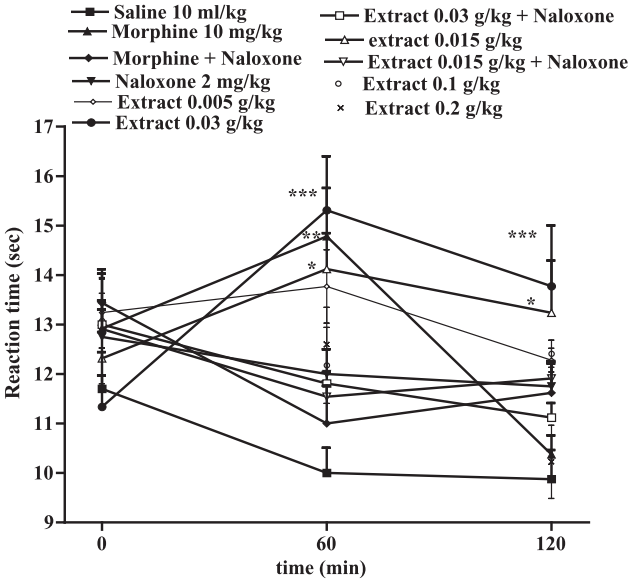

Figure 1 Effect of the aqueous extract of *M communis* L. aerial parts and morphine on the pain threshold of the mice in the hot-plate test. The effect of naloxone on the aqueous extract of *M communis* L. is also shown. Each point represents the mean ± SEM of reaction time for n = 8 experiments on the mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Tukey-Kramer test.

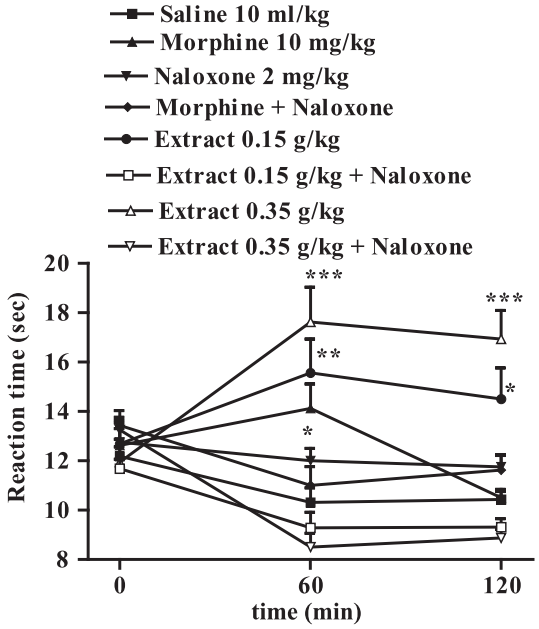

Figure 2 Effect of the ethanolic extract of *M communis* L. and morphine on the pain threshold of the mice in the hot-plate test. The effect of naloxone on the ethanolic extract of *M communis* L. is also shown. Each point represents the mean ± SEM of reaction time for n = 8 experiments on mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Tukey-Kramer test.

constrictions induced by a 0.7% acetic acid solution ($p < 0.001$). Naloxone pretreatment (2 mg/kg body wt., subcutaneously) after the intraperitoneal injection of the extracts did not inhibit the antinociceptive activity of both extracts ($p < 0.05$) (Figs. 3 and 4).

In the xylene-induced ear edema study, the aqueous extract at doses 0.1 ($p < 0.01$), 0.2, and 0.03 g/kg ($p < 0.001$) showed significant anti-inflammatory activity (Table 1).

The ethanolic extract showed activity against acute inflammation, which was not dose dependent (in all doses

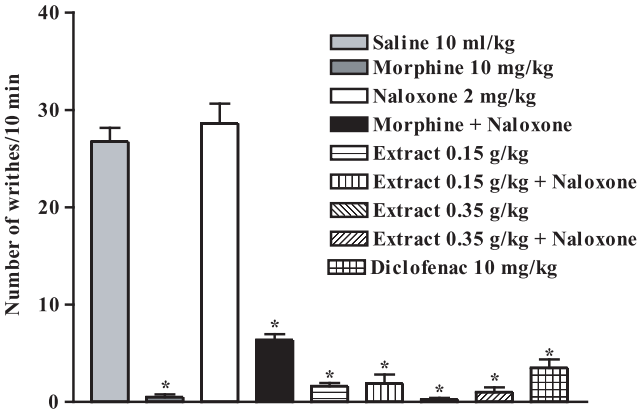

Figure 4 Effect of naloxone on the antinociceptive effect of the ethanolic extract of *Myrtus communis* L. on the acetic acid-induced writhing test in mice. Values are the mean ± SEM for 6 mice. All groups significantly decreased the number of writhes in 10 min; naloxone could not inhibit this effect. $^{*}p < 0.05$, compared with control, Tukey-Kramer test.

0.05, 0.15, and 0.35 g/kg; $p < 0.001$), where 0.15 g/kg dose had the most efficacy [72%, (Table 2)].

In the chronic inflammation (cotton-pellet) test, the aqueous extract exhibited significant and dose-dependent anti-inflammatory activity (0.005, 0.015, and 0.03 g/kg; $p < 0.001$). There was a maximum efficacy for the dose of 0.03 g/kg of the aqueous extract [57.9%, (Table 3)].

Doses 0.2 and 0.1 g/kg were fatal for animals, and there was a 100% and 50% mortality on the last day, respectively. Ethanolic extract in 0.05 g/kg exhibited anti-inflammatory activity 61% ($p < 0.001$), and it was not dose dependent (Table 4).

4. Discussion

The present results indicate that aqueous and ethanolic extracts of aerial parts of *M communis L.* have marked

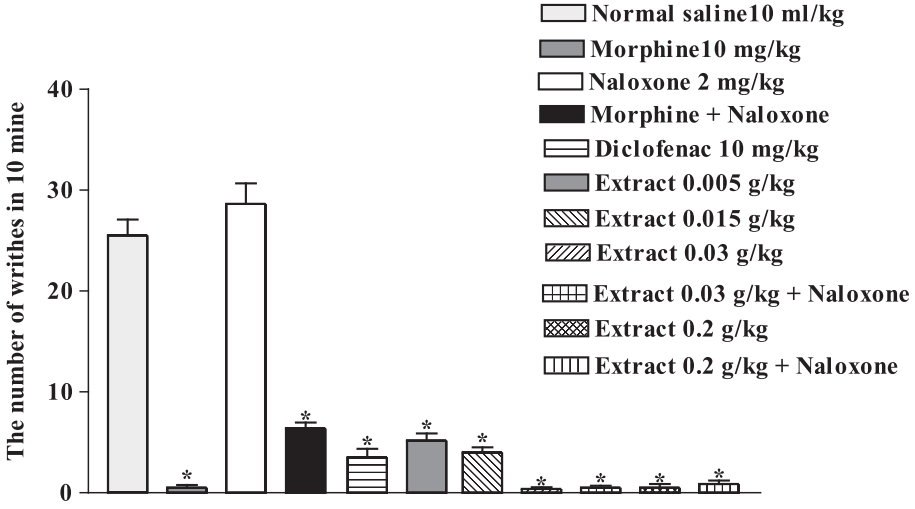

Figure 3 Effect of naloxone on the antinociceptive effect of the aqueous extract of *M communis* L. on the acetic acid-induced writhing test in mice. Values are the mean ± SEM for 6 mice. All groups significantly decrease the number of writhes in 10 min; naloxone could not inhibit this effect. $^{*}p < 0.05$, compared with control. Tukey-Kramer test.

Table 1 Effect of intraperitoneal doses of the aqueous extract of *M communis* L. on xylene-induced ear swelling in mice.

Substance/dose	Weight difference (mg)	Percentage of inhibition
Normal saline (10 ml/kg)	2.8 ± 0.19	—
Diclofenac (15 mg/kg)	$0.4 \pm 0.06^{\dagger}$	83
Aqueous extract (0.005 g/kg)	2.4 ± 0.083	17
Aqueous extract (0.015 g/kg)	2.3 ± 0.066	21
Aqueous extract (0.03 g/kg)	1.8 ± 0.3*	35.8
Aqueous extract (0.1 g/kg)	$1.4 \pm 0.16^{\dagger}$	50.2
Aqueous extract (0.2 g/kg)	$0.9 \pm 0.1^{\dagger}$	66.4

Values are the mean ± SEM. for 8 mice in each group.
*$p < 0.01$.
$^{\dagger}p < 0.001$, compared with control (Tukey-Kramer).

central (hot plate test) and peripheral (writhing test) antinociceptive activity. The extracts also showed activity against acute and chronic inflammations. With respect to LD50 values, the aqueous extract was more toxic than the ethanolic extract. According to a toxicity classification, the aqueous extract is very toxic while the ethanolic extract is relatively toxic [19].

The aqueous and ethanolic extracts showed antinociceptive activity in the hot plate test that was inhibited by naloxone. The hot plate test is a specific central antinociceptive test [20]. Therefore, it is possible that the extracts exerted their effects through the central opioid receptors or promoted the release of endogenous opiopeptides. Antinociceptive activity of opioid agonists and opioid partial agonists on non-steroidal anti-inflammatory agents can be determined using the writhing test [17]. In the writhing test, the antinociceptive activity of the extracts was not inhibited by naloxone; thus, the peripheral effect of the extract is not mediated via the opioid receptors. Other mechanisms of action, such as the inhibition of cyclooxygenase or the inhibition of the release of prostaglandins, are also possible.

The aqueous and ethanolic extract had significant anti-inflammatory effects in acute inflammatory tests with varying efficacy. This plant may have a membrane-stabilizing effect that reduces capillary permeability and/or has inhibitory effects on the release of mediators [17]. The extract effectively and significantly reduced cotton pellet-induced granuloma, thereby suggesting its activity in the proliferative phase of the inflammation.

Table 2 Effect of intraperitoneal doses of the ethanolic extract of *M communis* L. on xylene-induced ear swelling in mice.

Substance/dose	Weight difference (mg)	Percentage of inhibition
Normal saline (10 ml/kg)	2.8 ± 0.19	—
Diclofenac (15 mg/kg)	0.48 ± 0.06*	83
Ethanolic extract (0.05 g/kg)	1.15 ± 0.08*	59.8
Ethanolic extract (0.15 g/kg)	0.8 ± 0.15*	72
Ethanolic extract (0.35 g/kg)	0.98 ± 0.17*	65.5

Values are the mean ± SEM for 8 mice in each group.
*$p < 0.05$, compared with control (Tukey-Kramer).

Table 3 Effect of intraperitoneal doses of the aqueous extract of *M communis* L. (consecutive 7 days) on the weight of granuloma in rats.

Substance/dose	Weight difference (mg)	Percentage of inhibition
Normal saline (10 ml/kg)	10.6 ± 0.299	—
Diclofenac (15 mg/kg)	3.8 ± 0.255*	64.4
Aqueous extract (0.005 g/kg)	8.1 ± 0.365*	19.8
Aqueous extract (0.015 g/kg)	6.5 ± 0.260*	38.9
Aqueous extract (0.03 g/kg)	4.5 ± 0.171*	57.9

Values are the mean ± SEM for 6 mice in each group.
*$p < 0.05$, compared with control (Tukey-Kramer).

It is concluded that the aqueous and ethanolic extracts of aerial parts of *M communis* L. have central and peripheral antinociceptive effects. Opioid receptors and the inhibition of the cyclooxygenase enzyme may mediate these activities. The extracts also have activity against acute and chronic inflammations. As the preliminary phytochemical results indicated, the antinociceptive and anti-inflammatory effects of the extracts may be due to their content of flavonoids and/or tannins. Flavonoids have antinociceptive effects [21], although there are few reports on the role of tannins in antinociceptive and anti-inflammatory activities [22]. Therefore, the antinociceptive and anti-inflammatory effects of the extracts may be due to their content of flavonoids and/or tannins.

Other studies have demonstrated that various components such as 1,8-cineole, α-pinene, limonene and trans-myrtanol acetate are present in *M commonis L.* extracts [12]. It has been shown that 1,8-cineole is present in many plants essential oils such as *Rosmarinus officinalis* and eucalyptus, and it has an anti-inflammatory effect in mice and rats [23,24]. However, the chemical constituents responsible for the pharmacologic activities remain to be investigated. The extracts of *M communis* L. may have good analgesic and anti-inflammatory potential against diseases such as rheumatoid arthritis.

Table 4 Effect of intraperitoneal doses of the ethanolic extract of *M communis* L. (consecutive 7 days) on the weight of granuloma in rats.

Substance/dose	Weight difference (mg)	Percentage of inhibition
Normal saline (10 ml/kg)	2.8 ± 0.19	—
Diclofenac (15 mg/kg)	3.775 ± 0.255*	68.1
Ethanolic extract (0.05 g/kg)	4.6125 ± 0.184*	61

Values are the mean ± SEM for 6 mice in each group.
*$p < 0.05$, compared with control (Tukey-Kramer).

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