TÜBİTAK

Original Article

Turk J Med Sci
2012; 42 (2): 315-324
© TÜBİTAK
E-mail: medsci@tubitak.gov.tr
doi:10.3906/sag-1011-1304

Antinociceptive, antiinflammatory, and antioxidant activities of the ethanol extract of *Crataegus orientalis* leaves*

Zeynep BOR[1], Rana ARSLAN[1], Nurcan BEKTAŞ[1], Sevda PIRILDAR[2], Ali Aslan DÖNMEZ[3]

Aim: To determine the in vitro antioxidant activity and to investigate the possible antinociceptive and antiinflammatory effects of the ethanol extract of *Crataegus orientalis* leaves. The medicinal use of extracts prepared from the leaves, flowers, and fruits of *Crataegus* (hawthorn) dates back to ancient times. It has been proposed that its antioxidant constituents account for these beneficial therapeutic effects.

Materials and methods: To evaluate these effects, the hot-plate, tail-immersion, writhing, and carrageenan-induced edema tests were used. Moreover, the antioxidant activity of the extract was determined using the 2,2-diphenyl-1-picrylhydrazyl and β-carotene-linoleic acid assay systems. In the tests that were used to determine antinociceptive and antiinflammatory effects, the extract was administered in dose ranges of 50, 100, and 200 mg/kg, intraperitoneally.

Results: In the hot-plate test, the doses of 100 and 200 mg/kg exerted significant antinociceptive effects. In the tail-immersion test, antinociceptive effects were significant in all of the doses. In the writhing test, as the dose increased, a significant decrease in the writhing responses was observed. The antiinflammatory test results showed a dose-dependent antiinflammatory activity. In vivo tests also showed the extract as possessing antioxidant activity.

Conclusion: According to the experimental findings, the ethanol extract of *C. orientalis* displays remarkable antinociceptive, antiinflammatory, and antioxidant activities.

Key words: *Crataegus orientalis*, hawthorn, antinociception, antiinflammation, antioxidant

Crataegus orientalis yapraklarının etanol ekstresinin antinosiseptif, anti-inflamatuvar ve antioksidan etkileri

Amaç: *Crataegus* (alıç) bitkisinin türlerinin yaprak, çiçek ve meyvelerinden hazırlanan ekstrelerinin ilaç olarak kullanımı çok eski zamanlara dayanmaktadır. Alıcın yararlı terapötik etkilerinin antioksidan içeriğinden kaynaklandığı ileri sürülmektedir. Bu çalışmada *Crataegus orientalis* yapraklarının etanol ekstresinin in vitro antioksidan aktivitesinin belirlenmesi ve olası antinosiseptif, ve antiinflamatuvar etkilerinin araştırılması amaçlanmıştır.

Yöntem ve gereç: Ekstrenin bu etkilerinin değerlendirilmesi için hot-plate, tail-immersion, kıvranma ve karragenin ile indüklenen pençe ödem testleri kullanılmıştır. Ekstrenin antioksidan aktivitesi 2,2-difenil-1-pikrilhidrazil ve β-karoten-linoleik asit sistemi kullanılarak tayin edilmiştir. Antinosiseptif ve antiinflamatuvar etkilerin belirlenmesi için uygulanan testlerde ekstrenin 50, 100 ve 200 mg/kg olarak hazırlanan dozları intraperitoneal olarak verilmiştir.

Bulgular: Hot-plate testinde 100 ve 200 mg/kg dozlarında antinosiseptif etkide anlamlılık gözlenmiştir. Tail-immersion testinde tüm dozlarda görülen antinosiseptif etki kontrole göre anlamlılık göstermiştir. Kıvranma testinde ise doz arttıkça kıvranma sayılarında önemli bir düşüş görülmüştür. Antiinflamatuvar etki testinden elde edilen sonuçlar doza bağımlı bir etkinlik göstermiştir. Yapılan in vivo testlerde ekstrenin antioksidan aktiviteye sahip olduğu görülmüştür.

Sonuç: Deneylerden elde edilen bulgulara göre, *C. orientalis* etanol ekstresinin antinosiseptif, antiinflamatuvar ve antioksidan aktivitelerinin dikkate değer olduğu sonucuna varılmıştır.

Anahtar sözcükler: *Crataegus orientalis*, alıç, antinosiseptif, anti-inflamatuvar, antioksidan

Received: 26.11.2010 – Accepted: 26.02.2011

[1] Department of Pharmacology, Faculty of Pharmacy, Anadolu University, Eskişehir - TURKEY
[2] Department of Pharmacognosy, Faculty of Pharmacy, İstanbul University, İstanbul - TURKEY
[3] Department of Biology, Faculty of Science, Hacettepe University, Ankara - TURKEY

Correspondence: *Zeynep BOR, Department of Pharmacology, Faculty of Pharmacy, Anadolu University, Tepebaşı, 26470 Eskişehir - TURKEY*
E-mail: zbor@anadolu.edu.tr

Introduction

Hawthorn (*Crataegus* spp.), belonging to the family Rosaceae, grows in northern temperate regions such as East Asia, Europe, and eastern North America (1). The extracts or tinctures prepared from the leaves, flowers, and/or fruits of *Crataegus* have been used traditionally since ancient times (2,3). The plant is mainly used for the treatment of cardiovascular diseases. In addition, according to pharmacological studies, extracts of *Crataegus* may also be used as antiinflammatory and antioxidant agents (4). Flavonoids and oligomeric proanthocyanidins (OPCs), the main constituents of hawthorn, are responsible for most of the pharmacological activity (1). The flowers contain the highest levels of flavonoids and the leaves contain the highest levels of OPC. It has been reported that the plants that contain these components show antinociceptive and antiinflammatory activities (4). Although some hawthorn species such as *Crataegus monogyna* and *Crataegus pinnatifida* have been studied for their antiinflammatory and antioxidant activities (5,6), there are no scientific studies about the antinociceptive and antiinflammatory activities of *Crataegus orientalis*.

It has been reported that the genus *Crataegus* consists of nearly 21 species in Turkey (7). *C. orientalis*, which is called Anatolian hawthorn and is also locally known as "geyikdikeni" in Turkey, is native to the Mediterranean region, Turkey, Crimea, and western Iran. *C. orientalis* prefers man-made habitats, clearing areas in the steppe, and other open places. The species has many local types and extreme forms. Its fruits are commonly used as food (8,9). The flavonoid content of *C. orientalis* leaves was investigated and the main component was found to be hyperoside, along with apigenin, apigenin-7-glucoside, vitexin, and vitexin-4'-rhamnoside (10).

As *C. orientalis* may have antinociceptive and antiinflammatory actions since it is rich in flavonoids, the aim of the present study was to provide scientific evidence for the analgesic and antiinflammatory activities of the ethanol extract of *C. orientalis* leaves using appropriate models in mice. In addition, the antioxidant capacity of *Crataegus orientalis* ethanol extract (COE) was also evaluated and the total phenolic content was determined.

Material and methods

Drugs and chemicals

The following drugs and chemicals were obtained from the following sources in this study: methanol, dimethyl sulfoxide (DMSO), acetic acid, and Tween 80 from Merck (Darmstadt, Germany); and morphine sulfate, diclofenac, naloxone, Folin-Ciocalteu reagent, β-carotene, gallic acid, butylated hydroxytoluene (BHT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), carrageenan, chloroform, sodium carbonate, Tris-HCl buffer, and linoleic acid from Sigma (St. Louis, MO, USA).

Plant material

C. orientalis M.Bieb. var. *szovitsii* (Pojark.) leaves were collected from Gaziantep in Turkey on 27 May 2002. The plant material was identified by Prof Dr Ali A. Dönmez from the Department of Biology of the Faculty of Science at Hacettepe University. An authenticated voucher specimen (A.A.Dönmez10693) was deposited in the herbarium of the Faculty of Biology of Hacettepe University.

Preparation of the ethanol extract of C. orientalis leaves

The leaves of the plant material were air-dried at room temperature under shade and then powdered. The plant material was extracted in 50% ethanol using a Soxhlet apparatus for 18 h. The ethanol extract was lyophilized, resulting in the crude dry extract (11.82 g, 23.15% yield).

Phytochemical screening

The COE was tested for the presence of alkaloids, tannins, reducing sugar, and phenolics using standard phytochemical screening procedures. In each test, a 10% (w/v) methanol solution of the extract was used (11). The total phenolic content of the extract was determined using the Folin-Ciocalteu method. The total phenolic contents were estimated as gallic acid equivalents, expressed as milligrams of gallic acid per gram of extract (12,13). In a 10.0-mL volumetric flask, 6.0 mL of H_2O and 100 µL of sample were transferred, and 500 µL of undiluted Folin-Ciocalteu reagent was subsequently added to the mixture. After 1 min, 1.5 mL of a solution containing 20% (w/v) Na_2CO_3 was added, and the volume was increased to 10.0 mL with H_2O. After 2 h of incubation at 25 °C, the absorbance

was measured at 760 nm and compared to a gallic acid calibration curve. The data are presented as the averages of triplicate analyses.

Pharmacological procedures

Animals

Swiss albino mice (30-40 g) were obtained from the Anadolu University Experimental Animal Research Center. The animals were maintained in a room with controlled temperature (22 ± 2 °C) for a light/dark photoperiod of 12:12, with free access to food and water. Before each experiment, in order to avoid a possible interaction with food, the animals received only water for 12 h. Animal care and research protocols were based on the principles and guidelines adopted in the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No: 85-23, revised in 1985) and were approved by the local ethics committee of Anadolu University, Eskişehir.

Drugs and administration

All of the drugs, injected intraperitoneally (IP) 30 min before the procedure, were dissolved in a 20% solution of DMSO in saline (v/v). COE was given to the animals at doses of 50, 100, and 200 mg/kg. The same volume (0.1 mL) of the vehicle was administrated to the negative control group. Morphine sulfate (5 mg/kg) and diclofenac (10 mg/kg) were used as the reference analgesic drugs. In order to evaluate possible involvement of the opioid system in the antinociceptive effect of the COE, the mice were pretreated 10 min before the administration of the extracts (50, 100, and 200 mg/kg) with the nonselective opioid receptor antagonist naloxone (5 mg/kg).

Acute toxicity

The mice were divided into the control and test groups (n = 6). COE was administered IP to different groups at increasing doses of 50, 100, 200, 400, 800, and 1000 mg/kg. The mice were allowed food and water ad libitum during the acute toxicity study and all of the animals were observed for possible mortality cases and behavioral changes for 72 h (14).

Hot-plate test

Pain reflexes in response to a thermal stimulus were measured using Hot Plate Analgesia Meter No. 7280 from Ugo Basile Instruments (Comerio, Italy). The hot plate was set to 55 ± 0.5 °C and the mice were put on the plate for testing (n = 6-8). The reaction time of the pain response (hind paw licking, hind paw flicking, or jumping) at 30 min after drug injection was measured. The cut-off time for the hot-plate test was 20 s (15).

Tail-immersion test

To evaluate the central mechanism of analgesic activity, the tail immersion method was used, as described by Schmauss and Yaksh (16). Pain reactions were produced by thermal stimuli, by dipping the tip of the animal's tail into a water bath heated to 52.5 ± 0.2 °C (Heto-Holten, Allerod, Denmark) (n = 6-7). The withdrawal time of the tail from the hot water (in seconds) was noted as the reaction time, and it was measured at 30 min after treatment. The maximum cut-off time for the tail-immersion test was 15 s to avoid injury to the tissues of the tail.

Writhing test

This method is a chemical visceral pain model used to assess the peripheral antinociceptive activity, described by Koster et al. (17). The mice were injected IP with 10 mL/kg of 0.6% acetic acid solution to develop visceral pain after 30 min of the administration of the drugs or extracts (n = 6-7). The intensity of the nociceptive behavior was quantified by counting the total number of writhes for 10 min, 5 min after the administration of the acetic acid. The writhing response consists of a contraction of the abdominal muscles together with a stretching of the hind limbs.

Carrageenan-induced hind paw edema

The carrageenan-induced hind paw edema model was used for determination of antiinflammatory activity (18,19). Each mouse was injected with 40 μL (1%) carrageenan by intraplantar administration in the right hind paw 35 min after the IP injection of the test sample. As the control, 40 μL of saline solution was injected into the left hind paw (n = 7-8). The thicknesses of the injected paws were measured every 90 min during the 6-h period after the induction of inflammation using a digital caliper. The mean values of the treated groups were compared with the mean values of the control group and analyzed using statistical methods. Diclofenac (10 mg/kg) was used as the reference drug.

Antioxidant activity

DPPH free radical-scavenging activity

The ability of the extracts to scavenge DPPH radicals was determined using the method of Gyamfi et al. (20). A 50-µL aliquot of the extract in 50 mM Tris-HCl buffer (pH 7.4) was mixed with 450 µL of Tris-HCl buffer and 1.0 mL of 0.1 mM DPPH in methanol. After 30 min of incubation in darkness at ambient temperature, the resultant absorbance (A) was recorded at 517 nm. The percent of DPPH decolorization of the sample was calculated according to this equation: decolorization% = [1 – ($A_{sample}/A_{control}$)] × 100. BHT and gallic acid were used as positive controls. The values are presented as the means of triplicate analyses.

β-Carotene bleaching assay

In a flask, 1 mL of β-carotene (0.2 mg/mL, dissolved in chloroform) was added to a mixture of linoleic acid (40 mg) and Tween 80 (400 mg). Chloroform was evaporated under a stream of nitrogen and 50 mL of distilled water was added and shaken vigorously. The control was prepared without the sample or standards with the same procedure. Blanks of the control and sample were also prepared without β-carotene. Their absorbance values were measured on a spectrophotometer at 470 nm. The samples were then subjected to thermal autoxidation by keeping them in a constant-temperature water bath at 50 °C for 2 h. The rate of bleaching of β-carotene was monitored by taking the absorbance at 15-min intervals (21). BHT served as the positive control. The assay was repeated 3 times. The antioxidant activity level (AA) of the extract was evaluated as β-carotene bleaching using the following equation:

$$AA\% = 100 \times [1 - (A_s^0 - A_s^{120})/(A_c^0 - A_c^{120})],$$

where A_s^0 and A_c^0 are the absorbance values measured at time 0 of the incubation for the test sample and control, respectively, and A_s^{120} and A_c^{120} are the absorbance values measured in the test sample and control, respectively, after incubation for 120 min.

Analyses of data and statistics

The statistical analyses were carried out using GraphPad Prism Version 5.0. Data obtained from the antioxidant assays were presented as mean values ± standard deviation (SD). Data obtained from the animal experiments were expressed as mean values ± standard error of the mean (SEM) to show variation among the groups. Statistical differences between the treatments and the control were evaluated by one-way ANOVA, followed by Tukey's multiple comparison tests. The results were expressed as the differences and 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) ± SEM, which was calculated as follows:

MPE% = [(postdrug latency) – (predrug latency)/(cutoff time) – (predrug latency)] × 100.

The percentage of analgesic activity of the writhing test was defined using the following equation:

Percentage analgesic activity = [(N – N^1)/N] × 100,

where N is the average number of writhes of the control group and N^1 is the average number of writhes of the test group).

Results

Phytochemical screening

Phytochemical investigations of the COE showed the presence of phenolics, tannins, and alkaloids. The amount of total phenolics in the extract was determined by the Folin-Ciocalteu method. The total phenolic content, expressed as gallic acid equivalents, was 98.24 ± 2.4 mg/g in the COE.

Acute toxicity

Administration of COE at doses of 50-1000 mg/kg did not produce mortality during the observation period of 72 h. During observation of the animals, those administered 400, 800, and 1000 mg/kg of the extract exhibited decreased mobility for almost 10 h, but no sign of convulsions. This result indicates that COE has a low toxicity profile.

Hot-plate test

In the hot-plate test (Figure 1), MPE% values were observed as 3.55 ± 0.69% (control), 30.02 ± 1.79% (morphine), 6.71 ± 0.61% (COE, 50 mg/kg), 9.72 ± 1.25% (COE, 100 mg/kg), and 14.62 ± 1.07% (COE, 200 mg/kg), respectively. When compared with the control value, 100 mg/kg and 200 mg/kg of COE

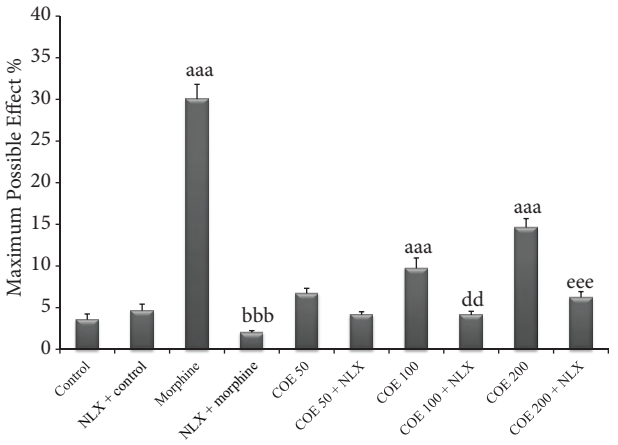

Figure 1. Antinociceptive effects of the COE from leaves, morphine, and the reversal effect of naloxone (NLX) in the hot-plate test. Values are presented as the mean ± SEM (n = 6-8). $^{aaa}P < 0.001$, significant difference from control; $^{bbb}P < 0.001$, significant difference from morphine alone; $^{dd}P < 0.01$, significant difference from COE at 100 mg/kg alone; and $^{eee}P < 0.001$, significant difference from COE at 200 mg/kg alone.

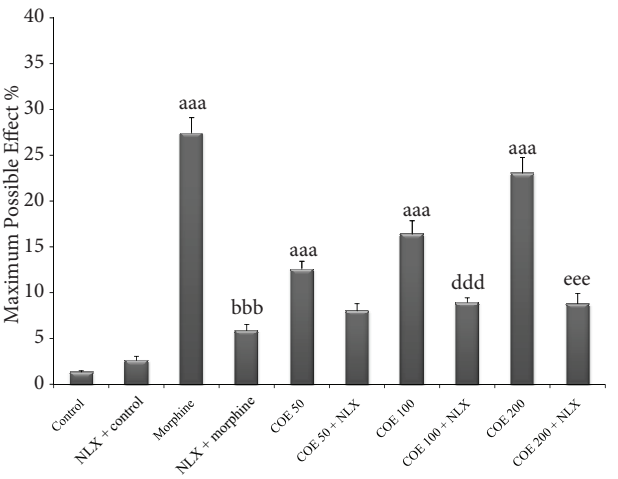

Figure 2. Antinociceptive effects of the COE from leaves, morphine, and the reversal effect of naloxone (NLX) in the tail-immersion test. Values are presented as the mean ± SEM (n = 6-7). $^{aaa}P < 0.001$, significant difference from control; $^{bbb}P < 0.001$, significant difference from morphine alone; $^{dd}P < 0.01$, significant difference from COE at 100 mg/kg alone; and $^{eee}P < 0.001$, significant difference from COE at 200 mg/kg alone.

showed antinociceptive activity ($P < 0.001$). Naloxone influenced the antinociceptive activity of the COE at the doses of 100 mg/kg ($P < 0.05$) and 200 mg/kg ($P < 0.001$).

Tail-immersion test

The inhibitory effects of COE and morphine on the tail-immersion test in mice are shown in Figure 2. The MPE% values were calculated as 1.33 ± 0.15% (control), 27.40 ± 1.73% (morphine, 5 mg/kg), 12.55 ± 0.90% (COE, 50 mg/kg), 16.36 ± 1,50% (COE, 100 mg/kg), and 23.04 ± 1.73% (COE, 200 mg/kg), respectively. Based on these results, the doses of 50, 100, and 200 mg/kg increased the MPE% value for nociception significantly above the control value ($P < 0.001$). There were no significant differences in the antinociceptive effects of 50, 100, and 200 mg/kg of COE when compared with morphine, but the antinociceptive effect of 200 mg/kg was slightly lower than that of morphine. The antinociceptive activity of all of the drugs used in this test was antagonized by naloxone. However, naloxone only significantly decreased the antinociceptive effect produced by morphine ($P < 0.001$) and the doses of 100 and 200 mg/kg of COE ($P < 0.001$).

Writhing test

The results of the acetic acid-induced writhing responses in mice that indicate the analgesic activity of COE are presented in Figure 3. It was found that all of the doses of the extract caused a significant inhibition of the writhing responses induced by

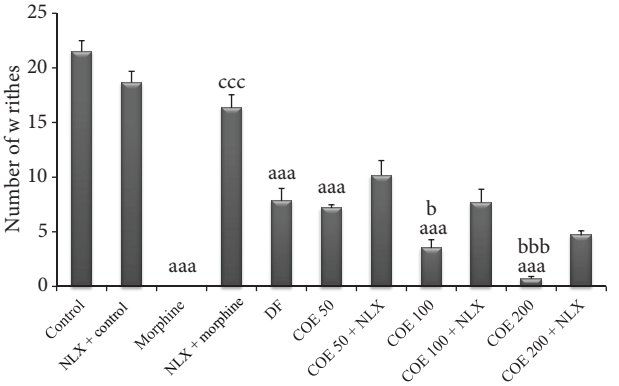

Figure 3. Antinociceptive effects of the COE from leaves, morphine, diclofenac (DF), and the reversal effect of naloxone (NLX) in the acetic acid-induced writhing test. Values are presented as the mean ± SEM (n = 6-7). $^{aaa}P < 0.001$, significant difference from the control; $^{b}P < 0.05$, significant difference from DF; $^{bbb}P < 0.001$, significant difference from DF; and $^{ccc}P < 0.001$, significant difference from morphine alone.

acetic acid when compared to the control ($P < 0.001$), with percentage inhibitions of 100% (morphine), 63.56% (diclofenac), 66.66% (COE at 50 mg/kg), 83.72% (COE at 100 mg/kg), and 96.67% (COE at 200 mg/kg). An increase in the doses of the plant extract resulted in a greater inhibition of writhing. The COE doses of 100 and 200 mg/kg of the extract were statistically more effective than the reference drug diclofenac ($P < 0.05$ and $P < 0.001$, respectively). The antinociceptive effect of COE was antagonized by pretreatment with naloxone. The reversal effect of naloxone on the antinociceptive effect of COE (50, 100, and 200 mg/kg) was lower than its effect on morphine and there was no significant antagonism.

Carrageenan-induced hind paw edema

As shown in Table 1, the COE significantly inhibited carrageenan-induced paw edema in mice and exhibited a dose-dependent antiinflammatory activity. The inhibitory values of edema at 270 min after the carrageenan were 45.2%, 86.6%, and 96.8% for the 50, 100, and 200 mg/kg doses of the extract, respectively. The 200 mg/kg dose of the extract was significant at 180 and 270 min when compared with diclofenac (10 mg/kg).

DPPH radical-scavenging activity

By using a stable DPPH free radical, the leaves of *C. orientalis* were tested for free radical-scavenging activity. In order to compare the DPPH scavenger capacity of the extract, known antioxidative substances such as BHT and gallic acid were used. The antioxidant activity of the reference substances was evaluated at only a concentration of 0.1 mg/mL because the substances possess maximum inhibition at minimum concentrations. The DPPH radical-scavenging activities of the extract at 4 different concentrations and the reference substances are shown in Table 2 as inhibition percentage values. The maximum inhibition percentage value of the extract was 62.91 ± 3.40% at the concentration of 10 mg/mL.

β-Carotene bleaching assay

The antioxidant potential of COE was evaluated by determining the ability of 1 mg/mL of extract to inhibit β-carotene oxidation. The antioxidant activity of the plant extract was compared with that of BHT. The antioxidant activity of the extract, as well as of the positive control and BHT, as measured by the bleaching of β-carotene, is presented in Figure 4. The rate of β-carotene bleaching (oxidation) was reduced by 42.37%, whereas BHT inhibited the rate of oxidation by 92.08%.

Discussion

C. orientalis has wide natural distribution and consumption in Turkey, but the biological potency

Table 1. The effects of *C. orientalis* ethanol extract against carrageenan-induced paw edema in mice (values in parentheses indicate inhibition percentage).

Test samples	Dose (mg/kg)	Swelling thickness ($\times 10^{-2}$ mm) ± SEM (inhibition %)			
		90 min	180 min	270 min	360 min
Control		34.85 ± 0.07	52.42 ± 0.05	62.57 ± 0.07	44.57 ± 0.09
COE	50	33.57 ± 0.06	37.8 5 ± 0.04^{a} (27.78)	34.28 ± 0.03^{aaa} (45.20)	31.71 ± 0.05
	100	25.25 ± 0.03	16.37± 0.02$^{aaa, b}$ (68.76)	83.75 ± 0.01^{aaa} (86.61)	38.75 ± 0.01^{aaa} (91.30)
	200	11.14 ± 0.02^{a} (68.02)	9.71 ± 0.02$^{aaa, bb}$ (81.46)	2.00 ± 0.01$^{aaa, bb}$ (96.80)	2.85 ± 0.01^{aaa} (93.58)
Diclofenac	10	31.28 ± 0,05	29.85 ± 0.03^{aaa} (43.04)	22.57 ± 0.03^{aaa} (63.92)	11.71 ± 0.03^{aaa} (73.71)

$^{a}P < 0.05$, significant from the control value. $^{b}P < 0.05$, significant from the diclofenac value.
$^{aaa}P < 0.001$, significant from the control value. $^{bb}P < 0.01$, significant from the diclofenac value.

Table 2. Scavenging activity of DPPH radicals of *C. orientalis* ethanol extract at 4 different concentrations.

Test samples	Inhibition % ± SD				
	0.1 mg/mL	1 mg/mL	3 mg/mL	5 mg/mL	10 mg/mL
COE		51.37 ± 4.47	54.12 ± 6.60	62.09 ± 3.50	62.91 ± 3.40
Gallic acid	90.00 ± 2.21	_	_	_	_

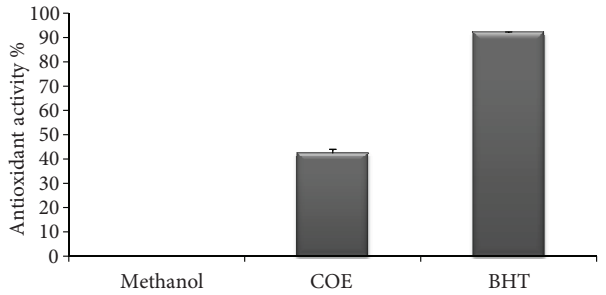

Figure 4. Antioxidant activities (%) of the *C. orientalis* ethanol extract by the β-carotene bleaching method. Each value is expressed as mean ± SD (n = 3).

of *C. orientalis* has not been investigated sufficiently, especially in animal models. Hence, some of the pharmacological effects of the extract were evaluated here. The present study demonstrates that the COE from leaves has antinociceptive, antiinflammatory, and antioxidant activities and shows no lethal toxicity at up to a dose of 1 g/kg in mice.

For the evaluation of the central antinociceptive effects of COE, hot-plate and tail-immersion tests were used to evaluate central pain at the supraspinal and spinal levels, respectively (22). It is known that the opioidergic system and descending inhibition pathways, which involve the noradrenergic and serotonergic systems, play an important role in the regulation/modulation of pain at the spinal and supraspinal levels (23,24).

The COE exerted significant antinociceptive effects in the tail-immersion test in comparison to the hot-plate test, which indicated that the extract has more potent analgesic activities at the spinal level than the supraspinal level. The obtained experimental findings suggest that the pain systems mentioned above may have a role in the antinociceptive activities of the COE. In both tests, the antinociceptive effects of the extract (100 and 200 mg/kg) were partially inhibited by the opioid antagonist naloxone. Thus, it is possible that the opioid mechanisms may mediate the antinociceptive effects of COE. It is known that the analgesic effects of opioid agents are due to the inhibition of supraspinal receptors μ_1, κ_3, δ_1, and σ_2 and spinal receptors μ_2, κ_1, and δ_2 (25,26). In light of this information, it was thought that COE may have higher affinity to spinal receptors μ_2, κ_1, and δ_2. However, since the antinociceptive effects were partially inhibited by naloxone, involvement of the noradrenergic and serotonergic pathways should also be considered. In addition, intraperitoneal administration of COE produced a dose-related antinociception. It seems quite possible that the higher doses of the extract may have more potent central antinociceptive effects.

The writhing test is a typical model that has been used as a screening tool for the assessment of both peripherally and centrally acting analgesic or antiinflammatory properties of new agents. Induction of writhing by acetic acid causes the release of endogenous substances such as bradykinin, substance P, histamine, serotonin, and prostaglandins, which excite the pain nerve endings. These nerve endings are sensitive to analgesics such as narcotics and nonsteroidal antiinflammatory drugs (NSAIDs) (27-29). The writhing test results indicated that COE significantly reduced the number of writhes. The obtained responses were similar to that of morphine at the COE dose of 200 mg/kg; moreover, at the doses of 100 and 200 mg/kg, the activity of the extract was more potent than that of diclofenac (10 mg/kg). However, unlike morphine, the analgesia of the extract in the acetic acid-induced writhing test was partially naloxone-reversible, suggesting a different mechanism of action, which may include a possible inhibition of cyclooxygenase enzymes.

The carrageenan-induced hind paw edema is a commonly used in vivo model for prediction of antiinflammatory drug activity (30). Subcutaneous

injection of carrageenan into the paw in order to develop edema is a biphasic event. Release of proinflammatory agents such as histamine and serotonin is attributed to the initial phase, which can cause the sensitization of the central nociceptive neurons. The following phase of edema is due to the release of prostaglandins, protease, and lysosome, which are sensitive to most clinically effective antiinflammatory drugs (31,32). The COE administration resulted in a marked inhibition of important events related to the inflammatory response induced by carrageenan. The COE, at the dose of 200 mg/kg, showed an antiinflammatory effect after 90 min. All of the doses of COE possessed an antiinflammatory effect at 3 h, and the edema produced at this peak is thought to be due to the release of kinin-like substances, especially bradykinin (33,34). This finding also supports the antinociceptive activities that were seen in the writhing test.

The first step in investigating the antioxidant potential of the COE involved defining its ability to scavenge free radicals to inhibit the oxidative process. According to the antioxidant activity results, COE was shown to possess a moderate inhibition of free radicals (51.37%-62.91%) in DPPH radical-scavenging activity. However, the scavenging effects of BHT and gallic acid were much more effective at an extremely low concentration.

The absence of an antioxidant results in the rapid discoloration of β-carotene in the β-carotene bleaching assay. This discoloration occurs as a consequence of free radical formation by the coupling of β-carotene and linoleic acid (35). Although the antioxidant activity of COE was lower than that of synthetic BHT (92.08%), it showed almost 50% activity. Therefore, it is thought that the extract was effective at inhibiting lipid peroxidation.

Flavonoids and OPCs are the main constituents responsible for the biological activity of *Crataegus* species. The phytochemical analysis showed that *C. orientalis* leaves contain flavonoids, mainly hyperoside (quercetin-3-O-galactoside) and OPCs (4,10). Rylski et al. reported that hyperoside and OPC exerted analgesic action in the hot-plate test (36). In addition, various studies have suggested that plant materials that contain tannins, alkaloids, OPCs, flavonoids, and phenolic acids have antinociceptive and antiinflammatory effects in experimental animals (4,37,38). Since the extract is known to contain flavonoids and has antioxidant activity, it is possible to speculate that these compounds might be responsible for the antinociceptive and antiinflammatory effects of *C. orientalis* leaves.

In conclusion, the present study reports, for the first time, the antinociceptive and antiinflammatory activities of the ethanol extract of *C. orientalis* leaves. Since some of the current opioids and NSAIDs have side effects and low potency, they may not be useful in all cases. Thus, the potent antiinflammatory effect and remarkable antinociceptive effect of the *C. orientalis* extract may provide support in the treatment of painful and inflammatory diseases. Moreover, the extract has a low toxicity profile, which indicates therapeutic safety for the pharmacologically active doses. The mechanisms of these activities need to be further studied with different antagonists, such as adrenergic or serotonergic. In future investigations, the different fractions of *C. orientalis* should be evaluated and the structural characterization of responsible components should be clarified.

Acknowledgments

The authors are grateful to Prof Dr Yusuf Öztürk, Professor of Pharmacology, Faculty of Pharmacy, Anadolu University, Eskişehir, Turkey, and Prof Dr Ali Hikmet Meriçli, Professor of Pharmacognosy, Faculty of Pharmacy, İstanbul University, İstanbul, Turkey, for their suggestions on this paper. Financial support for this work through the Scientific Research Projects Foundation of Anadolu University, Eskişehir, Turkey, is gratefully acknowledged (Project Code No: 090331).

References

1. Yao M, Ritchie HE, Brown-Woodman PD. A reproductive screening test of hawthorn. J Ethnopharmacol 2008; 118: 127-32.

2. Barnes J, Anderson LA, Phillipson JD. Herbal medicines: a guide for health care professionals. London: Pharmaceutical Press, Royal Pharmaceutical Society; 2002.

3. Bahorun T, Aumjaud E, Ramphul H, Rycha M, Luximon-Ramma A, Trotin F et al. Phenolic constituents and antioxidant capacities of *Crataegus monogyna* (Hawthorn) callus extracts. Nahrung 2003; 47: 191-8.
4. Mills S, Bone K. Principles and practice of phytotherapy. Edinburgh: Churchill Livingstone; 2000.
5. Vibes J, Lasserre B, Gleye J, Declume C. Inhibition of thromboxane A_2 biosynthesis in vitro by the main components of *Crataegus oxyacantha* (Hawthorn) flower heads. Prostaglandins Leukot Essent Fatty Acids 1994; 50: 173-5.
6. Kao ES, Wang CJ, Lin WL, Yin YF, Wang CP, Tseng TH. Anti-inflammatory potential of flavonoid contents from dried fruit of *Crataegus pinnatifida* in vitro and in vivo. J Agr Food Chem 2005; 53: 430-6.
7. Sözer U, Dönmez AA, Meriçli AH. Constituents from the leaves of *Crataegus davisii* Browicz. Sci Pharm 2006; 74: 203-8.
8. Dönmez AA. The genus *Crataegus L* (Rosaceae) with special reference to hybridisation and biodiversity in Turkey. Turk J Bot 2003; 28: 29-37.
9. Horoz M, Gok E, Genctoy G, Ozcan T, Olmaz R, Akca M et al. *Crataegus Orientalis* associated multiorgan hypersensitivity reaction and acute renal failure. Inter Med 2008; 47: 2039-42.
10. Melikoglu G, Mericli F, Mericli AH. Flavonoids of *Crataegus orientalis*. Boll Chim Farm 1999; 138: 351-2.
11. Evans WC. Trease and Evans' pharmacognosy. Edinburgh: Saunders Ltd.; 2002.
12. Singleton VL, Orthofer R, Lamuela-Raventós RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. In: Packer L, editor. Methods in enzymology. San Diego (CA): Academic Press; 1999. p.152-315.
13. Akkol EK, Göger F, Koşar M, Başer KHC. Phenolic composition and biological activities of *Salvia halophila* and *Salvia virgata* from Turkey. Food Chem 2008; 108: 942-9.
14. Lorke D. A new approach to practical acute toxicity testing. Arch Toxicol 1983; 54: 275-87.
15. Eddy NB, Leimback D. Synthetic analgesics II. Dithienylbutenyl and dithienylbutylamines. J Pharmacol Exp Ther 1953; 107: 385-93.
16. Schmauss C, Yaksh TL. In vivo studies on spinal receptor systems mediating antinociception II. Pharmacological profiles suggesting a differential association of mu, delta and kappa receptors with visceral chemical and cutaneous thermal stimuli in the rat. J Pharmacol Exp Ther 1984; 228: 1-12.
17. Koster R, Anderson M, Beer EJ. Acetic acid for analgesic screening. Fed Proc 1959; 18: 412.
18. Kasahara Y, Hikino H, Tsurufiji S, Wanatabe M, Ohuchi K. Antiinflammatory actions of ephedrines in acute inflammations. Planta Med 1985; 51: 325-31.
19. Yeşilada E, Küpeli E. *Berberis crataegina* DC. root exhibits potent anti-inflammatory, analgesic and febrifuge effects in mice and rats. J Ethnopharmacol 2002; 79: 237-48.
20. Gyamfi MA, Yonamine M, Aniya Y. Free-radical scavenging action of medicinal herbs from Ghana: *Thonningia sanguinea* on experimentally-induced liver injuries. Gen Pharmacol 1999; 32: 661-7.
21. Oomah B, Mazza G. Flavonoids and antioxidative activities in buckwheat. J Agr Food Chem 1996; 44: 1746-50.
22. Wong CH, Day P, Yarmush J, Wu W, Zbuzek UK. Nifedipine-induced analgesia after epidural injections in rats. Anesth Analg 1994; 79: 303-6.
23. Pinardi G, Sierralta F, Miranda HF. Adrenergic mechanisms in antinociceptive effects of non steroidal anti-inflammatory drugs in acute thermal nociception in mice. Inflamm Res 2002; 51: 219-22.
24. Bardin L, Lavarenne J, Eschalier A. Serotonin receptor subtypes involved in the spinal antinociceptive effect of 5-HT in rats. Pain 2000; 86: 11-8.
25. Reisine T, Pasternack G. Opioid analgesics and antagonists. In: Hardman JG, Limbird LE, editors. Goodman and Gilman's The pharmacological basis of therapeutics. New York: McGraw-Hill; 1996. p.521-6.
26. Hosseinzadeh H, Ramezani M, Salmani GA. Antinociceptive, anti-inflammatory and acute toxicity effects of *Zataria multiflora* Boiss extracts in mice and rats. J Ethnopharmacol 2000; 73: 379-85.
27. Nguemfo EL, Dimo T, Azebaze AG, Asongalemc EA, Alaoui K, Dongmo AB et al. Anti-inflammatory and anti-nociceptive activities of the stem bark extracts from *Allanblackia monticola* STANER L.C. (Guttiferae). J Ethnopharmacol 2007; 114: 417-24.
28. Taesotikul T, Panthong A, Kanjanapothi D, Verpoorte R, Scheffer JJC. Anti-inflammatory, antipyretic and antinociceptive activities of *Tabernaemontana pandacaqui* Poir. J Ethnopharmacol 2003; 84: 31-5.
29. Collier HO, Dinneen LC, Johnson CA, Schneider C. The abdominal constriction response and its suppression by analgesic drugs in the mouse. Br J Pharmacol Chemother 1968; 32: 295-310.
30. Ismail TS, Gapalakrisan S, Begum VH, Elango V. Anti-inflammatory activity of *Salacia oblonga* Wall. and *Azima tetracantha* Lam. J Ethnopharmacol 1997; 56: 145-52.
31. Ridtitid W, Sae-Wong C, Reanmongkol W, Wongnawa M. Antinociceptive activity of the methanolic extract of *Kaempferia galanga* Linn. in experimental animals. J Ethnopharmacol 2008; 118: 225-30.
32. Olajide OA, Makinde JM, Awe EO. Effect of the aqueous extract of *Bridelia ferruginea* stem bark on carrageenan-induced oedema and granuloma tissue formation in rats and mice. J Ethnopharmacol 1999; 66: 113-7.

33. Van Arman CG, Begany AJ, Miller LM, Pless HH. Some details of the inflammation caused by yeast and carrageenan. J Pharmacol Exp Ther 1965; 150: 328-34.

34. Crunkhorn P, Meacock SER. Mediators of the inflammation induced in the rat paw by carrageenan. Br J Pharmacol 1961; 42: 392-402.

35. Tosun M, Ercisli S, Sengul M, Ozer H, Polat T, Ozturk E. Antioxidant properties and total phenolic content of eight *Salvia* species from Turkey. Biol Res; 2009; 42: 175-81.

36. Rylski M, Duriasz-Rowińska H, Rewerski W. The analgesic action of some flavonoids in the hot plate test. Acta Physiol Pol 1979; 30: 385-8.

37. Morteza-Semnani K, Mahmoudi M, Heidar MR. Analgesic activity of the methanolic extract and total alkaloids of *Glaucium paucilobum*. Methods Find Exp Clin Pharmacol 2006; 28: 151-5.

38. Uchida S, Hirai K, Hatanaka J, Hanato J, Umegaki K, Yamada S. Antinociceptive effects of St. John's wort *Harpagophytum procumbens* extract and grape seed proanthocyanidins extract in mice. Biol Pharm Bull 2008; 31: 240-5.