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Investigation of the Antinociceptive Activity of the Hydroethanolic Extract of *Junglas nigra* Leaf by the Tail-Immersion and Formalin Pain Tests in Rats

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Abstract

Background: *Juglans (J.) nigra* leaf is obtained from a plant that is used in traditional medicine in some countries to alleviate inflammatory diseases.

Aim: The aim of this study was to compare the effects of *J. nigra* extract on acute nociceptive and inflammatory pain in rats.

Methods: Antinociceptive activity was examined in Wistar rats by the tail-immersion and formalin tests. Motor function was assessed using the rotarod test. Plant extract was administered intraperitoneally.

Results: In the tail-immersion test, the maximal antinociceptive effect of the plant extract (100–330 mg/kg) was about 24–30% and is the result of the effect of a high concentration of ethanol. In the formalin test, the plant extract (41.3–330 mg/kg) significantly and dose-dependently inhibited nociception in both phases of the test with similar maximal effects of about 76% and 85%. Only the plant extract at the dose of 330 mg/kg caused a significant time-dependent reduction in time spent on the rotarod.

Conclusions: In rats, the preventive systemic administration of the hydroethanolic extract of *J. nigra* leaf reduced chemically but not thermally induced pain. Higher efficacy was obtained in pain associated with inflammation and tissue injury. The antinociceptive effect is dose-dependent and may be limited by motor impairment.

Keywords

Juglans nigra, black walnut, phytocompounds, pain, therapeutic profiling

Introduction

Juglans (J.) nigra is a large, long-living deciduous tree that produces fruit, known as black walnut or *J. nigra*. The extract of leaves of the plant known as black walnut or *J. nigra* is a commonly used herbal folk medicine in Turkey,[1] western[2] and southern[3] Asia, and eastern North America.[4] *J. nigra* leaf extract is used to treat coughs, rheumatism, and fungal infection.[1-4] There is evidence that the extract of *J. nigra* leaf has significant antioxidative,[5,6] anticarcinogenic,[7] antibacterial,[8] and antifungal effects[9] as well as a hypoglycemic effect,[10] which has been confirmed in clinical trials.[11,12] Flavonoids, phenolic acids, and naphthoquinones are the

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main compounds in leaf of *J. nigra*.[5,13,14] Polyphenolic compounds have antioxidant activity.[5,6] An antiinflammatory effect of the extract of *J. nigra* leaf has been reported in some animal models of acute and chronic inflammation, such as carrageenan-induced mice hind paw edema,[15] xylene-induced mice ear edema,[16] and cotton pellet-induced granuloma tests in mice.[17] Also, it was shown that the extract reduced nociception in models of acute pain in mice, such as the hot-plate and the writhing tests,[15,16] but the effect of the extract has not been investigated in other models of pain.

Acute inflammation is associated with increased production of reactive oxygen and nitrogen species, inflammatory mediators, and the activation of enzymes.[17,18] Pain is commonly associated with inflammation, which is the consequence of tissue damage, chemical stimuli, and foreign substances. After tissue injury, nitroxidant dysregulation in the cell promotes the development of inflammation and central sensitization, crucial for the onset of chronic neuropathic or inflammatory pain.[19] Additionally, some components of the plant extract of *J. nigra*, including quercitrin, Mg, and Zn,[14] may contribute to its antinociceptive effect because it is well known that flavonoids[20-22] and minerals alone have an antinociceptive effect in inflammation.

Since standard analgesics, such as nonsteroidal antiinflammatory drugs and opioids may have limited efficacy and/or different dose-dependent side effects, it is necessary to acquire new drugs to relieve pain. Some drugs or herbals can be used parallel with standard analgesics and may influence their efficacy and safety. As herbal medicines can be useful in relieving pain and are often used, it is necessary to find effective and safe herbal medicines with the effects of adjuvant analgesics.

In our previous work, we described the phytochemical analysis of the hydroethanolic extract of leaves of *J. nigra* from the Aleksinac region (located at 43° 32′ 11`` N/21° 42′ 11`` E), in Serbia.[14] The present study aimed to evaluate the antinociceptive activity of the extract in the tail-immersion and formalin pain tests in rats and determine the possible effect of the extract on motor coordination in rats.

Materials and Methods

Preparation of Plant Extract

J. nigra leaves were collected near the town of Aleksinac (located at 43° 32′ 11`` N/21° 42′ 11`` E) in southeastern Serbia in summer. The *J. regia* leaf was botanically identified according to the Flora of Serbia,[23] and the specimen was submitted to the Herbarium of the Department of Botany, Faculty of Pharmacy, University of Belgrade, Serbia (HFF). The voucher specimen was registered under the number 3906HFF.

Fresh leaves were air-dried and extracted with 50% (v/v) ethanol for 4 h under reflux, at a solvent-to-solid ratio of 4:1. After filtration, the resulting liquid residue was labeled as the hydroethanolic extract.[14] The concentration of the total extracted substances in the hydroethanolic extract was 48 mg/cm^3.

Animals

Adult male Wistar rats weighing 220–280 g were used. The animals were housed under laboratory conditions at a room temperature of 22°C and photoperiodicity of a 12-hour light/dark cycle in plastic laboratory cages. Rats had *ad libitum* access to rat food (pellet) and tap water, except during the experiments. *In vivo* experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Ethical Council for the Protection of Experimental Animals of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia (323-07-05650/2021-05). All rats were habituated to handling and experimental procedures for three consecutive days before the experiment.

Drug Administration

On the day of the experiment, the hydroethanolic extract of *J. nigra* leaf was dissolved in saline (.9% NaCl) to prepare the test solution. The final concentrations of the hydroethanolic solutions were 8–50%. Doses of 41.3–330 mg/kg were chosen based on published data and preliminary results. The plant extract was intraperitoneally (i.p.) administered because this route is quick and has a low-stress impact on laboratory animals; this route is also suitable because the aim of the study was to evaluate the pharmacological effects of the extract rather than its pharmacokinetics and/or properties of a drug formulation.[20] Controls were given the vehicle (saline or ethanol) i.p. The vehicle or the plant extract was administered at a 2 mL/kg final volume.

An aqueous stock solution of 37% formaldehyde was diluted in .9% NaCl saline to a final 2.5% formalin solution for subcutaneous (s.c.) administration into the rat paw. The control was given the corresponding volume of the saline by intraplantar (i.pl.) administration. Plant extract or vehicle was administered 30 min before formalin injection.

In vivo Determination of Antinociceptive Activity

The antinociceptive activity was determined in rats by the tail-immersion[21] and the formalin tests.[22,24]

Tail-Immersion Test and Antinociception Assessment

The tail-immersion test is a widely used animal model of thermally induced acute nociceptive pain. During measurement, the rat was placed in a hemicylindrical holder with its tail hanging freely outside the holder. The distal 5 cm of the tail was immersed in a warm water bath (55 ± .5°C). The time for tail-withdrawal was measured as response latency. Rats

that were able to lift the tail from the warm water within 1–3 s were considered to be sensitive to heat and were selected for the test. A cut-off time (time of no response) of 10 s was used to avoid damage to the tail. The basal tail-withdrawal latency was measured before the administration of drugs. Measurements were obtained two times with a 30 min interval and the calculated average value was the pre-drug latency. Post-drug latency was measured after the administration of the test compounds or vehicle in the control group. The measurements were performed at 30, 60, 90, 120, 150, and 180 min time intervals after administration of the compounds.

Antinociceptive activity (AA%) for each rat was calculated according to the formula:[21]

AA% = (post-drug latency - pre-drug latency)/(10 - pre-drug latency) x 100

Formalin Paw Test and Antinociception Assessment

The formalin test is a widely used *in vivo* model of chemically induced persistent pain generated by injured tissue. During the experiment, the rats were placed in individual transparent observation cages. Formalin injection (100 µL of 2.5% formalin) into the plantar surface of the right hind paw induces a spontaneous nociceptive behavior, including licking, flinching, or biting the injected paw and lifting from the surface. A nociceptive reaction was registered as the total time spent in pain-related behavior after the injection of formalin or saline. The nociceptive time was measured in 9 blocks of 5 min and calculated cumulatively during the first phase (0–10 min), intermediate phase (10–20 min), and the second phase (20–45 min) after formalin injection. Phase 1 represents the neurogenic response, while phase 2 represents the inflammatory pain response. The intermediate phase is a quiescent period between phases 1 and 2.

Antinociceptive activity (AA%) for each rat was calculated according to the equation[25]: AA% = (nociceptive time vehicle - post drug nociceptive time)/(nociceptive time vehicle) x 100

In vivo Toxic Test in Rats

Determination of motor coordination in rats. The rotarod test[26] was used to evaluate the effect of the plant extract of *J. nigra* leaf on motor coordination in rats. The test was performed to validate the results obtained during the examination of antinociceptive activity. Rats were placed on a horizontal rod that rotates about its long axis (rotarod apparatus for rats, 47 700, Ugo Basile, Milano, Italy) at a constant speed of 15 r/min. The rats must walk forward to avoid falling. The time at which the rats fell off the rotating cylinder was recorded. The cut-off time was 180 s. Two days before the experiments, the animals were trained to balance on the rotating rod. On the day of the experiment, animals that could remain on the rod for 180 s in 2 separate measurements were selected. In the test groups, rats were administered i.p. the extract. The control rats received an i.p. injection of the same volume of vehicle. The post-treatment latency to remain on the rotating rod was measured at 6 time points, which corresponded to the time points when the nociception was assessed.

Statistical Analysis

The obtained results are presented as the mean ± standard error of the mean. Data were calculated using two-way analysis of variance with repeated measures followed by Tukey's test for multiple comparisons. The Student's t-test was used for independent samples. $P < 0.05$ was considered statistically significant.

Results

Effects of the Extract of J. nigra Leaf on Thermally Induced Nociception in Rats

Injection of the vehicle had no effect on the tail-withdrawal latency time after exposure to a thermal nociceptive stimulus (warm water 55 ± .5°C). Compared to the control, pretreatment with the extract of *J. nigra* leaf induced a significant increase in thermal latency time (F = 29.509; $P = .000$), which significantly decreased during the observed time (F = 6.652; $P = .000$) (Figure 1). Plant extract at doses of 200 mg/kg ($P = .000$) and 330 mg/kg ($P = .000$) showed antinociceptive activity with a duration of about 180 min. The maximal antinociceptive effect were obtained 30 min after administration of 330 mg/kg plant extract and 90 min after administration of 200 mg/kg plant extract and was about 30% ($P=.000$) and 24% ($P = .015$), respectively (Figure 1). The latency time in rats that received 200 mg/kg (4.7 ± .8 s) and 330 mg/kg (4.2 ± .1 s) of extract were comparable ($P > .05$). At all time periods, there was a statistical significance ($P = .000$) of the difference in the thermal latency time of the two higher tested doses (220 and 330 mg/kg) in relation to the lowest tested dose (100 mg/kg) of the hydroethanolic extract. The maximal value of thermal latency in the group treated with 100 mg/kg of the extract was 2.8 ± .3 s. In the control group, the highest concentration of ethanol (50%) producing maximally reduced nociception was up to about 25% and contributed to the effect of the highest tested dose of the extract (Figure 1). Except for the 30 min time point, there was no significant difference ($P > .05$) in thermal latency between the highest tested dose of the extract (330 mg/kg) and the highest concentration of ethanol (50%).

Effects of the Extract of the J. nigra Leaf on Formalin-Induced Nociception in Rats

Figure 2 shows the time course of the formalin-induced nociceptive responses and hydroethanolic extract in the formalin test. Formalin injection into the rat paw induced characteristic nociceptive behavior during phase 1 and phase 2; the two phases were separated by an intermediate phase with slight nociceptive activity. Cumulatively, the total time spent in active

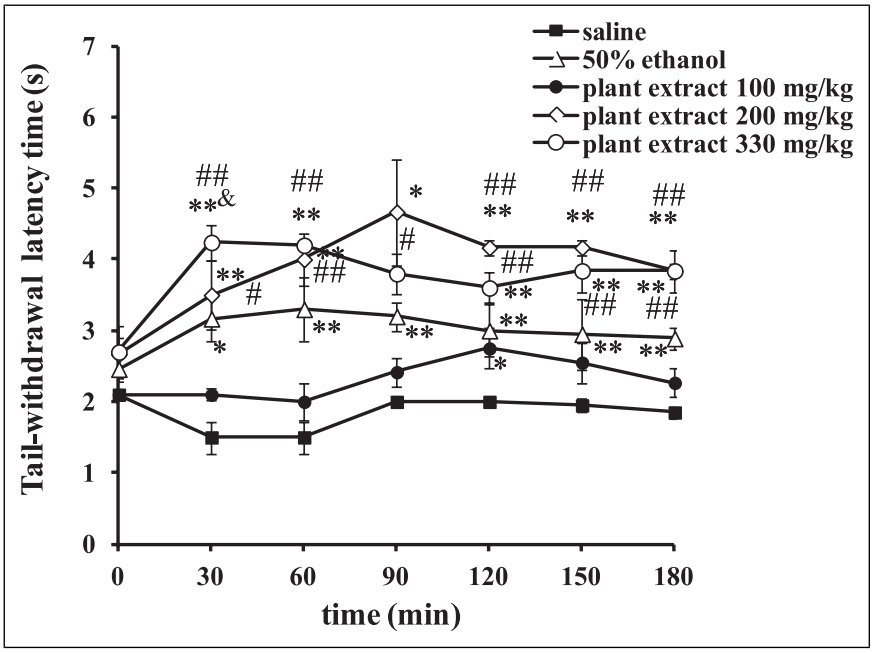

Figure 1. Effects of the hydroethanolic extract of *J. nigra* leaf on nociceptive activity in rats in the tail-immersion test. Each point represents the mean±SEM of the reaction time (n = 6); * – significantly different from saline (*$P < .05$; **$P < .01$); # – significantly different from 100 mg/kg plant extract (#$P < .05$; ##$P < .01$); & – significantly different from 50% ethanol (&$P < .05$); plant extract – hydroethanolic extract of *J. nigra* leaf; SEM – standard error of the mean.

nociception in phase 1 was 260 s, in phase 2 it was 1126 s, while in the intermediate phase it was 107 s (Figure 2B). In the control groups, an equivalent volume of saline did not produce any effect. In phases 1 and 2 of the test, an equivalent volume of 50% ethanol significantly ($P = .000$ and $P = .001$) decreased pain-related behavior by about 20% and significantly ($P = .000$) contributed to the antinociceptive effect of the highest tested dose of the extract (330 mg/kg) (Figure 2A and 2B).

Pretreatment with the hydroethanolic extract of *J. nigra* leaf (41.3–330 mg/kg) significantly inhibited the nociceptive response (F = 58.401; $P = .000$ for intervals) and its time course during the period of observation (F = 14.582; $P = .000$ for intervals; Figure 2A). In both phases of the test, the extract reduced nociception in comparison to the control group ($P = .001$) in a dose-dependent manner. In phase 1 of the test, rats treated with 41.3 and 120 mg/kg of the extract had similar ($P > .05$) and lower nociception scores compared to rats treated with 330 mg/kg of extract ($P = .013$ and $P = .046$, respectively; Figure 2B). In phase 2, there was a statistically significant difference in reduction of nociception between rats injected with different doses of hydroethanolic extract of *J. nigra* leaf ($P = .021$ for the dose of 41.3 mg/kg; $P = .000$ for the dose of 120 mg/kg; $P = .000$ for dose of 330 mg/kg; Figure 2B). Maximal reduction of formalin-induced nociceptive behavior in both phases of the test was achieved with the highest tested dose of extract (330 mg/kg) and was similar and about 75.6 ± 6.9% and 85.2 ± 4.3% in the neurogenic (phase 1) and inflammatory phase (phase 2) of the formalin test, respectively (Figure 2B). During the intermediate phase of the formalin test, the nociceptive response (21.0 ± 6.3-22.5 ± .8 s) in rats treated with different doses of the extract was comparable ($P > .05$) but significantly lower ($P = .029$ for the dose of 41.3 mg/kg; $P = .008$ for the dose of 120 mg/kg; $P = .007$ for the dose of 330 mg/kg) than the control (107.4 ± 32.9 s) (Figure 2B). Inhibition of nociception ranged from about 80–98%.

Effects of the Extract of the J. nigra Leaf on Motor Performance in Rats

In the rotarod test, only the extract at the highest tested dose (330 mg/kg) caused a significant reduction in time spent on the rotarod compared with the control group (Figure 3). The observed reduction of time spent on the rotarod was time-dependent; the peak effects occurred 30 min after drug administration (percent of inhibition was 24.4 ± 8.3), and the reduced effect was sustained for up to 180 min. No effect on locomotion was observed in rats administered with the extract at a dose of 200 mg/kg compared to the control. The highest concentration of ethanol solution (50%) caused a significant reduction in the time spent on the rotarod, with a maximal effect of about 40% (Figure 3). There was no significant ($P > .05$) difference between groups that received 50% ethanol and the highest dose of the extract (330 mg/kg).

Discussion

The present study demonstrated that the hydroethanolic extract of the *J. nigra* leaf has (i) an antinociceptive effect

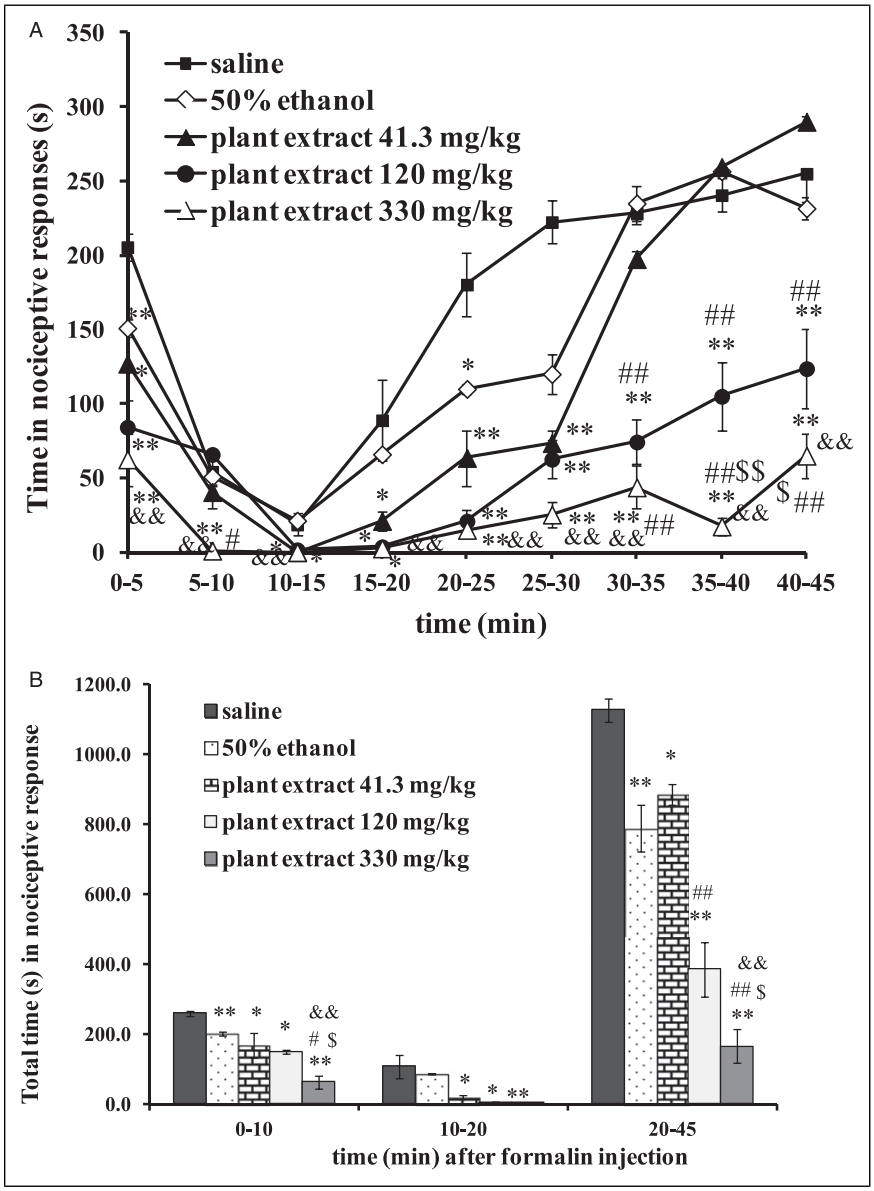

Figure 2. Effects of the hydroethanolic extract of *J. nigra leaf* on nociceptive activity in rats in the paw formalin test. Figure 2A – the time intervals of nociceptive behavior in 5-min measurements. Figure 2B – the total time spent in nociceptive behavior in phase 1 (0–10 min), the intermediate phase (10–20), phase 2 (20–45 min after formalin injection) of the test. Each point represents the mean±SEM of the reaction time (n = 6); * – significantly different from saline (*$P < .05$; **$P < .01$), # – significantly different from the 41.3 mg/kg plant extract (#$P < .05$; ##$P < .01$); $ – significantly different from the 120 mg/kg plant extract ($$P < .05$); & – significantly different from 50% ethanol (&$P < .05$; &&$P < .01$); plant extract – hydroethanolic extract of *J. nigra* leaf; SEM – standard error of the mean.

depending on the nature of pain, and (ii) an adversely limited antinociceptive effect. The significant increase in thermal latency indicates that the extract has low and limited antinociceptive activity in thermally induced acute nociceptive pain. The significant decrease in chemically induced pain-related behavior indicates that the extract at a dose that did not induce motor impairment has a low effect on acute nociceptive pain compared to the effect on inflammatory pain. Also, these results suggest that the extract had a greater effect on chemically induced inflammatory pain than on thermal stimuli-induced acute nociceptive pain in rats. Thus, higher doses were needed to reduce acute nociceptive–neurogenic pain ("pure nociception") than inflammatory pain, for which the extract was much more potent. Also, the antinociceptive effect was limited since the highest effective dose of the extract alone has some antinociceptive effect (about 20%), and the induced adverse effect such as motor impairment could affect the validity of the results of the antinociceptive tests. The highest concentration of ethanol solution (50%) partially contributed to these effects of the extract.

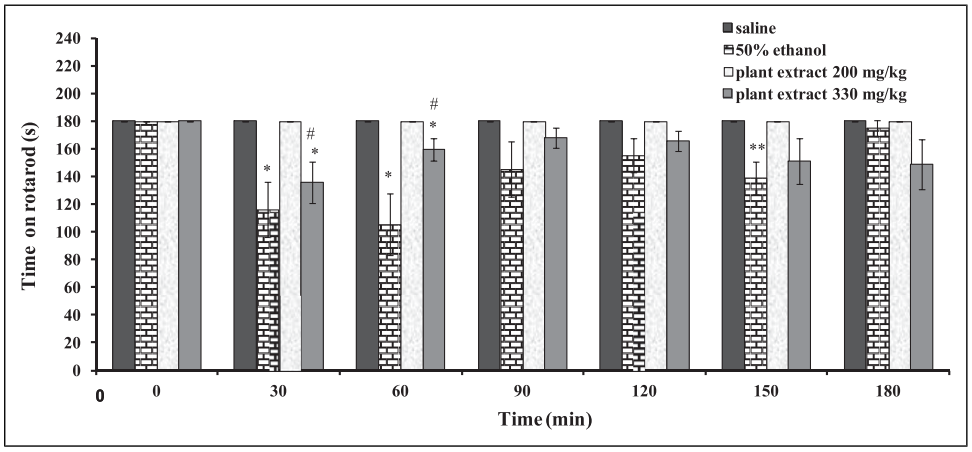

Figure 3. Effects of the hydroethanolic extract of *J. nigra* leaf on motor coordination in rats. Reduced time (s) spent on the rotarod indicates motor impairment. Bar charts denote the mean±SEM values (n = 6). Each point represents the mean±SEM of the reaction time (n = 6); * – significantly different from saline (*$P < .05$); # – significantly different from the 200 mg/kg plant extract (#$P < .05$); plant extract – hydroethanolic extract of *J. nigra* leaf; SEM – standard error of the mean.

In the present study, we examined for the first time the effect of *J. nigra* leaf extract in formalin and tail-immersion tests in rats. The results of this study have shown that the extract is more effective in both phases of the formalin test than in the tail-immersion test in rats. This discrepancy between phase 1 of the formalin test and tail-immersion test in testing so-called "pure nociception" is most probably due to the difference in origin of pain, that is, chemical vs thermal nociceptive stimuli. In the tail-immersion test transient, a thermal stimulus is used to produce pain. The tail-immersion test is an acute thermal and phasic pain model that is selectively attenuated by centrally-acting morphine-like drugs.[27] In accordance with this, our results may suggest that the extract at a dose that did not impair motor coordination probably does not have central mechanisms of action and does not affect thermal and phasic pain. Given that the alcohol solution in the highest tested dose of the extract achieves an effect like the extract, it can be concluded that in the thermally induced pain (tail-immersion test in hot water), the extract does not have an effect because the solvent (50% ethanol) contributes to the antinociceptive effect of the extract. In accordance with this, the displayed low activity in the tail-immersion test may indicate a non-opioid mechanism of action. This result is in accordance with the observation that an opioid antagonist does not affect the analgesic effect of the *J. nigra* leaf extract in the hot-plate test.[10] The formalin test is a chemical assay of injury-produced inflammatory pain and has continuous nociceptive stimulation.[28] The formalin test exemplifies both nociceptive – neurogenic (in phase 1) and inflammatory (in phase 2) pain. In accordance with this, the formalin test is a very useful model of pain since pain in clinical settings often has a mixed etiology, with nociceptive, inflammatory, and neuropathic components.[28] The intermediate phase of the formalin test is a period of low nociceptive activity or inactivity, which is a consequence of activated endogenous inhibitory mechanisms.[29-32] This suggests that the plant extract may produce an antinociceptive effect by potentiating inhibitory pain pathways.[29-32]

Our results agree with the results of previous studies that showed that the *J. nigra* leaf extract has an antinociceptive effect in other models of pain.[6,15,16] Nasiry et al[6] documented that the extract reduced nociception in diabetes mellitus with a similar efficacy in both preventive and curative protocols of administration. The antinociceptive effect of the *J. nigra* leaf extract was shown in the p-benzoquinone-[15] and acetic acid–induced[16] abdominal contraction tests and in the hot-plate test in mice.[16] Results obtained in all of these nociceptive tests indicate that *J. nigra* leaf extract has peripheral and central antinociceptive effects. The possible mechanisms of the analgesic effect of the extract have not been fully elucidated. Although the direct mechanism of antinociceptive action has not been investigated, the results of studies[6,15,16] indicate that the antinociceptive effect is achieved through non-opioid receptors and at least in part by reducing inflammation. However, the p-benzoquinone- and acetic acid-induced writhing models depend on prostanoids and different cytokines such as tumor necrosis factor alpha (TNFα), interleukin (IL) −1, −18, −33, and interferon γ.[33-35] It was shown that treatment with the extract has an antiinflammatory effect due to decreased cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression in diabetic rats.[6] Also, the extract exhibits an antiinflammatory effect *in vivo*, as observed in decreased carrageenan-[15] and xylene-induced paw[16] and ear swelling in mice, respectively.

One study used the extract at doses that are higher than the doses applied in our study,[15] with our results indicating that even at 8-fold lower doses the extract can impair motor coordination in rats. Additionally, we showed in the inflammatory model of pain that the plant extract had a high antinociceptive effect with about a 10-fold lower dose without

affecting motor coordination. Also, the highest tested dose of the *J. nigra* ethanolic leaf extract (330 mg/kg, i.p.) in this study was a 10-fold lower dose than the LD_{50} values for the extract in rats (3.3 g/kg, i.p.), and the maximum non-fatal dose was 2.93 g/kg.[16]

In the extract used in this study, we previously identified different flavonoids and elements, such as K, Mg, Ca, Zn, Mn, Sr, and I.[14] The extract examined herein is rich in hyperoside and quercitrin, flavonoids with valuable antioxidant activity. Quercitrin alone has a dose-dependent antinociceptive effect in the acetic acid–induced visceral pain model in mice,[36] suggesting that this flavonoid contributed to the antinociceptive activity of the plant extract.[36] The extract is rich in elements with high extraction coefficients in the following relative order: K>Mg>Zn.[14] Our previous results indicate that Mg administered alone in doses equivalent to doses for supplementation has a significant antinociceptive effect in inflammatory pain.[25,37] It is well known that Zn has antinociceptive and antiinflammatory effects in different models of pain and inflammation.[38] Both Mg and Zn decrease pain by modulating glutamate concentrations and the N-methyl-D-aspartate (NMDA) receptor.[39] It is well known that in acute nociceptive and inflammatory pain there are low and high levels of activated NMDA receptor, respectively,[40] which may explain, at least in part, the different efficacies of the extract in nociceptive and inflammatory pain.

In accordance with the antinociceptive effect of the extract and its compounds, future research should investigate the potential pharmacodynamic interactions between *J. nigra* leaf extract and standard analgesics. This research gives information about the selected doses of the extract, considering that it has the potential to damage motor coordination.

Conclusions

The hydroethanolic extract of *J. nigra* leaf in rats produces an antinociceptive effect in the formalin test, but not in the tail-immersion test in hot water. This suggests that the extract has an antinociceptive effect in chemically induced pain, while it has no effect in pain caused by thermal stimuli. The extract shows greater potency in inflammatory than in "pure" nociceptive models of pain. The impaired motor activity caused by the high concentration of ethanol as a solvent may limit the antinociceptive effect of high doses of the extract. The extract of *J. nigra* leaf is a potential adjuvant analgesic agent that can be used in diseases associated with inflammation or tissue injury.

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Declaration of Conflicting Interests

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References

1. Kültür S. Medicinal plants used in Kirklareli Province (Turkey). *J Ethnopharmacol*. 2007;111(2):341-364.
2. El Beyrouthy M, Arnold N, Delelis-Dusollier A, Dupont F. Plants used as remedies antirheumatic and antineuralgic in the traditional medicine of Lebanon. *J Ethnopharmacol*. 2008; 120(3):315-334.
3. Kunwar RM, Adhikari N. Ethnomedicine of Dolpa district, Nepal: The plants, their vernacular names and uses. *Lyonia*. 2005;8:43-49.
4. Foster S, Duke JA. *Medicinal Plants*. Boston: Houghton Mifflin; 1990:276-277.
5. Zhao MH, Jiang ZT, Liu T, Li R. Flavonoids in Juglans regia L. leaves and evaluation of in vitro antioxidant activity via intracellular and chemical methods. *Sci World J*. 2014;2014: 303878.
6. Nasiry D, Khalatbary AR, Ahmadvand H, Talebpour Amiri F, Akbari E. Protective effects of methanolic extract of Juglans regia L. leaf on streptozotocin-induced diabetic peripheral neuropathy in rats. *BMC Compl Alternative Med*. 2017;17(1): 476.
7. Carvalho M, Ferreira PJ, Mendes VS, et al. Human cancer cell antiproliferative and antioxidant activities of Juglans regia L. *Food Chem Toxicol*. 2010;48(1):441-447.
8. Rather MA, Dar BA, Dar MY, et al. Chemical composition, antioxidant and antibacterial activities of the leaf essential oil of Juglans regia L. and its constituents. *Phytomedicine*. 2012;19: 1185-1190.
9. Noumi E, Snoussi M, Hajlaoui H, Valentin E, Bakhrouf A. Antifungal properties of Salvadora persica and Juglans regia L. extracts against oral Candida strains. *Eur J Clin Microbiol Infect Dis*. 2010;29:81-88.
10. Nasiry D, Khalatbary AR, Ahmadvand H, Talebpour Amiri F, Juglans Regia L. Leaf extract attenuates diabetic nephropathy progression in experimental diabetes: An immunohistochemical study. *Iran J Med Sci*. 2019;44(1):44-52.
11. Hosseini S, Huseini HF, Larijani B, et al. The hypoglycemic effect of Juglans regia leaves aqueous extract in diabetic patients: A first human trial. *Daru*. 2014;22:19.
12. Hosseini S, Jamshidi L, Mehrzadi S, et al. Effects of Juglans regia L. leaf extract on hyperglycemia and lipid profiles in type two diabetic patients: A randomized double blind, placebo-controlled clinical trial. *J Ethnopharmacol*. 2014;152:451-456.

13. Pereira JA, Oliveira I, Sousa A, et al. Walnut (Juglans regia L.) leaves: phenolic compounds, antibacterial activity and antioxidant potential of different cultivars. *Food Chem Toxicol*. 2007; 45:2287-2295.
14. Rajković K, Vasić M, Drobac M, et al. Optimization of extraction yield and chemical characterization of optimal extract from Juglans nigra L. leaves. *Chem Eng Res Des*. 2020; 157:25-33.
15. Erdemoglu N, Küpeli E, Yeşilada E. Anti-inflammatory and antinociceptive activity assessment of plants used as remedy in Turkish folk medicine. *J Ethnopharmacol*. 2003;89:123-129.
16. Hosseinzadeh H, Zarei H, Taghiabadi E. Antinociceptive, anti-inflammatory and acute toxicity effects of juglans regia L. Leaves in mice. *Iranian Red Crescent Med J*. 2011;13:27-33.
17. Fatima H, Shahid M, Jamil A, Naveed M. Therapeutic potential of selected medicinal plants against carrageenan induced inflammation in rats. *Dose Response*. 2021;19(4):15593258211058028.
18. Rauf A, Abu-Izneid T, Rashid U, et al. Anti-inflammatory, antibacterial, toxicological profile, and in silico studies of dimeric naphthoquinones from diospyros lotus. *BioMed Res Int*. 2020;2020:1-10.
19. Grace PM, Gaudet AD, Staikopoulos V, et al. Nitroxidative signaling mechanisms in pathological pain. *Trends Neurosci*. 2016;39(12):862-879.
20. Al Shoyaib A, Archie SR, Karamyan VT. intraperitoneal route of drug administration: Should it be used in experimental animal studies? *Pharm Res (N Y)*. 2019;37(1):12.
21. Vucković S, Prostran M, Ivanović M, Ristović Z, Stojanović R. Antinociceptive activity of the novel fentanyl analogue isocarfentanil in rats. *Jpn J Pharmacol*. 2000;84(2):188-195.
22. Tjølsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. The formalin test: An evaluation of the method. *Pain*. 1992;51(1):5-17.
23. Jovanovic B, Juglans L. In: Josifovic M, ed. In Flora SR Srbije. Srpska akademija nauka i umetnosti, Beograd. (In Flora of SR Serbia. Serbian Academy of Sciences and Arts, Belgrade) 1970; Vol II.
24. Nasirinezhad F, Basir P, Raiati H, Asghari M. Comparing the analgesic effect of hydroalcoholic extract of green tea and sodium salicylate in the formalin test in rats. *J Med Physiol*. 2016; 1(1):31-35.
25. Srebro DP, Vučković SM, Dožić IS, et al. Magnesium sulfate reduces formalin-induced orofacial pain in rats with normal magnesium serum levels. *Pharmacol Rep*. 2018;70(1):81-86.
26. Im KS, Jung HJ, Kim JB, et al. The antinociceptive effect of acetaminophen in a rat model of neuropathic pain. *Kaohsiung J Med Sci*. 2012;28(5):251-258.
27. D'Amour FE, Smith DL. A method for determining loss of pain sensation. *J Pharmacol Exp Ther*. 1941;72:74–79.
28. Dubuisson D, Dennis SG. The formalin test: A quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain*. 1977;4:161-174.
29. Henry JL, Yashpal K, Pitcher GM, Coderre TJ. Physiological evidence that the 'interphase' in the formalin test is due to active inhibition. *Pain*. 1999;82(1):57-63.
30. Franklin KB, Abbott FV. Pentobarbital, diazepam, and ethanol abolish the interphase diminution of pain in the formalin test: Evidence for pain modulation by GABAA receptors. *Pharmacol Biochem Behav*. 1993;46(3):661-666.
31. Moslem AR, Amin B, Heidari-Oranjaghi N, Azhdari-Zarmehri H. Involvement of endogenous opioid system in swim stress-induced pain modulation during the interphase of the formalin test. *Basic Clin Neurosci*. 2019;10(4):305-312.
32. Savić Vujović K, Vučković S, Vasović D, et al. Involvement of serotonergic and opioidergic systems in the antinociceptive effect of ketamine-magnesium sulphate combination in formalin test in rats. *Pharmacol Rep*. 2019;71(6):1014-1019.
33. Verri WA Jr, Cunha TM, Magro DA, et al. Role of IL-18 in overt pain-like behaviour in mice. *Eur J Pharmacol*. 2008;588(2-3): 207-212.
34. Ribeiro RA, Vale ML, Thomazzi SM, et al. Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in mice. *Eur J Pharmacol*. 2000;387(1):111-118.
35. Magro DA, Hohmann MS, Mizokami SS, et al. An interleukin-33/ST2 signaling deficiency reduces overt pain-like behaviors in mice. *Braz J Med Biol Res*. 2013;46(7):601-606.
36. Gadotti VM, Schmeling LO, Machado C, et al. Antinociceptive action of the extract and the flavonoid quercitrin isolated from Bauhinia microstachya leaves. *J Pharm Pharmacol*. 2005; 57(10):1345-1351.
37. Srebro DP, Vučković S, Vujović KS, Prostran M. Anti-hyperalgesic effect of systemic magnesium sulfate in carrageenan-induced inflammatory pain in rats: Influence of the nitric oxide pathway. *Magnes Res*. 2014;27(2):77-85.
38. Liu H, Kang P, Liu Y, et al. Zinc oxide nanoparticles synthesised from the Vernonia amygdalina shows the anti-inflammatory and antinociceptive activities in the mice model. *Artif Cells Nanomed Biotechnol*. 2020;48(1):1068-1078.
39. Torabi M, Kesmati M, Galehdari H, Varzi HN, Pourreza N. MgO and ZnO nanoparticles anti-nociceptive effect modulated by glutamate level and NMDA receptor expression in the hippocampus of stressed and non-stressed rats. *Physiol Behav*. 2020; 214:112727.
40. Voscopoulos C, Lema M. When does acute pain become chronic? *Br J Anaesth*. 2010;105 (1):i69-i85.