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Antinociceptive and antiedematogenic effect of pecan (*Carya illinoensis*) nut shell extract in mice: a possible beneficial use for a by-product of the nut industry

Abstract

Background: Interest in pecan (*Carya illinoensis*) nut shells, a by-product of the nut industry, has increased due to its anti-inflammatory and antioxidant activities. The goal of this study was to evaluate the antinociceptive and antiedematogenic activity and the mechanisms of the pecan shell aqueous extract (AE).

Methods: First, we performed fingerprinting of *C. illinoensis* AE. The antinociceptive and antiedematogenic effects of AE intragastric (i.g.) administration in mice (male Swiss mice 20–30 g) were evaluated using the acetic acid test or after subcutaneous (s.c.) paw injection of diverse transient receptor potential ankyrin 1 (TRPA1) agonists, including hydrogen peroxide (H_2O_2), allyl isothiocyanate, or cinnamaldehyde. We also observed AE antinociceptive and antiedematogenic effects after carrageenan s.c. paw injection and measured H_2O_2 production. Moreover, we observed the development of adverse effects after AE i.g. treatment.

Results: The high-performance liquid chromatography fingerprinting of AE showed the presence of rutin. AE or rutin i.g. treatment produced antinociception in the acetic acid test and reduced the nociception and edema mediated by H_2O_2 s.c. hind paw injection or nociception induced by other TRPA1 agonists. Moreover, AE or rutin reduced the hyperalgesia, edema, and H_2O_2 production induced by carrageenan s.c. paw injection. No motor, gastric, or toxicological alterations were observed after AE administration.

Conclusions: Collectively, the present results show that AE and its constituent rutin produced antinociceptive and antiedematogenic action in models of acute and persistent inflammatory nociception and it seems to be related to the inhibition of TRPA1 receptor activation.

Keywords: antioxidant; inflammation; nociception; rutin; transient receptor potential ankyrin 1 (TRPA1) channel.

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Introduction

Pain is a main symptom in diverse inflammatory diseases, including osteoarthritis and rheumatoid arthritis; however, its treatment is a complicated issue and many times poorly managed [1]. There are increasing evidences showing that the endogenous production of oxidant substances is related to the induction and maintenance of inflammatory pain [2]. In this view, the overproduction of oxidant substances is capable of activating nonselective cation channels expressed in a subset of sensory fiber nociceptors, including the transient receptor potential ankyrin 1 (TRPA1) [3, 4]. TRPA1 is a relevant detector of noxious stimuli leading to neurogenic inflammation and hypersensitivity [5]. Therefore, the search for new alternative treatments with antioxidant potential to inflammatory pain treatment is a relevant issue.

The pecan tree [*Carya illinoensis* (Wangenh.) C. Koch] is a member of the Juglandaceae family, along with walnuts (*Juglans* sp.) [6]. This plant is native to the southern United States and northern Mexico and is currently cultivated for edible nut production in the United States, Mexico, and in the South of Brazil [7]. The kernel of pecan nut is a relevant product in the pecan industry, although this process causes the production of a great amount (40%–50%) of shells, a brown by-product [8]. Pecan shells have been pointed as a good source of antioxidant compounds [6, 9]. In fact, the antioxidant activity of pecan shells was related with a wide range of pharmacological properties [10–12].

Thus, the goal of the present study was to evaluate the possible antinociceptive effects and some of the mechanisms underlying the action of the aqueous extract (AE) of *C. illinoensis* shells on inflammatory and algogen models of pain in mice.

Materials and methods

Plant material and preparation of AE of *C. illinoensis* shells

The unprocessed material (shells) was kindly donated by a pecan processing company, which also processes the nut shells for the preparation of marketable tea. This product is currently sold in supermarkets, with permission of the Ministry of Agriculture of Brazil. Briefly, for the preparation of *C. illinoensis* AE, the shells were left overnight at 35°C in a hot air oven and then fine powdered. The AE of the shells was freshly prepared by infusion (1:40 w/v, 90°C), filtered using filter paper, and cooled to room temperature. During this procedure, the extract was protected from light [11]. The criteria for the selection of the dose of pecan shells AE were based on previous studies conducted in our laboratory, and the initial range of dose was based on previous studies [11, 12].

Drugs

If not otherwise indicated, reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). However, methanol, acetic acid, and gallic acid were purchased from Merck (Darmstadt, Germany).

Fingerprinting of *C. illinoensis* AE

High-performance liquid chromatography (HPLC)-diode array detection (DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan) equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector (DAD) UV-VIS detector, and LC solution 1.22 SP1 software. Reverse phase chromatographic analyses were carried out under gradient conditions using C18 column (4.6 mm×150 mm) packed with 5-µm-diameter particles; the water mobile phase contained 2% acetic acid (A) and methanol (B), and the composition gradient was 5% of B until 2 min and then changed to obtain 25%, 40%, 50%, 60%, and 70% B at 10, 20, 30, 40, and 50 min, respectively, following the method described before with slight modifications [13]. The AE of *C. illinoensis* was analyzed dissolved in ethanol at a concentration of 10 mg/mL. Identification of compounds was performed by comparing their retention time and UV absorption spectrum with commercial standards. The flow rate was 0.6 mL/min, injection volume was 40 µL, and wavelength was 254 nm for gallic acid, 280 nm for catechin, 327 nm for ellagic acid, and 365 nm for rutin. All the samples and mobile phases were filtered through a 0.45-µm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standard references were prepared in the HPLC mobile phase at a concentration range of 0.006–0.250 mg/mL for gallic acid and catechin and 0.031–0.250 mg/mL for ellagic acid and rutin. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200–500 nm). Calibration curve was computed as follows: for gallic acid, Y=12,407x+1059.8 (r=0.9993); for catechin, Y=11,355x–1047.1 (r=0.9968); for ellagic acid, Y=13,581x+1273 (r=0.9994); and for rutin, Y=12,646x–1175.9 (r=0.9987). All chromatography operations were carried out at ambient temperature and in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves as defined by Sabir et al. [13]. LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

Animals

Male Swiss mice (20–30 g) bred in-house were used in all experiments. Animals were kept in a controlled environment (22±2°C) with a 12-h light/dark cycle (lights on 6:00 a.m. to 6 p.m.) and fed

standard lab chow and tap water ad libitum. All experiments were carried out between 8:00 a.m. and 5:00 p.m. Animal care and experiments were conducted in accordance with the ethical principles for animal research (Scientific procedures) Act (UK, 1986) and were authorized by the Ethics Committee of the Federal University of Santa Maria (process number 124/2011) and are in accordance with current ethical guidelines for the investigation of experimental pain in conscious animals [14]. Experimenters were blinded to treatment conditions.

Evaluation of AE antinociceptive and antiedematogenic effects

Acetic acid-induced writhing response

The number of abdominal writhing induced by the intraperitoneal (i.p.) injection of acetic acid (0.6%, 10 mL/kg) was cumulatively counted over a period of 20 min and was considered as indicative of nociception as described before [15]. First, animals were pretreated with different doses of AE (100, 300, or 1000 mg/kg, intragastric route [i.g.]), rutin (10 mg/kg, i.g., used as a positive control and found in the extract) [16], indomethacin (100 mg/kg, i.g., used as a positive control to the anti-inflammatory effect) [15], or vehicle (distilled H_2O, 10 mL/kg, i.g.) 1 h prior to the i.p. injection of acetic acid. The control group received the same volume of 0.9% NaCl (10 mL/kg, i.p.); however, it did not evoke nociceptive behavior (data not shown).

Mechanical hyperalgesia and paw edema mediated by carrageenan

Animals were subcutaneously injected (s.c.) under the plantar surface of the right hind paw with 20 µL of carrageenan (300 µg/paw in saline, 0.9% NaCl), which was performed 30 min after the administration of AE (100, 300, or 1000 mg/kg, i.g.), rutin (10 mg/kg, i.g.), indomethacin (100 mg/kg, i.g.), or vehicle (10 mL/kg, i.g.) as described before [17]. The 50% mechanical paw withdrawal threshold response was measured using von Frey filaments in the "Up-and-Down" paradigm as described previously [18]. Hind paw edema formation was described as Δpaw thickness=test paw thickness–basal paw thickness; paw thickness was measured using a caliper (Amatools, Piracicaba, São Paulo, Brazil) [18].

Measurement of the hind paw skin content of hydrogen peroxide in the carrageenan inflammatory pain model

Initially, animals received a s.c. paw injection of carrageenan (300 µg/paw, 20 µL) or vehicle (saline, 20 µL) 30 min after the administration of AE (300 mg/kg, i.g.), rutin (10 mg/kg, i.g.), or vehicle (10 mL/kg, i.g.). After 60 min, the hydrogen peroxide (H_2O_2) content in the plantar skin surface of the right hind was assessed as described before [19]. Results were expressed as microgram of H_2O_2 per gram of protein, on the basis of a standard curve of HRPO-mediated oxidation of phenol red by H_2O_2.

H_2O_2 neutralization in vitro

We also investigated if AE directly neutralizes H_2O_2. This technique is based on the monitoring of the absorbance of H_2O_2 spectrophotometrically at 285 nm [20]. Different concentrations of AE, rutin (used as positive control), or its vehicle were incubated with 20 mM of H_2O_2 in the dark for 30 min. A reduction in the absorbance of H_2O_2 was considered as a consumption of the total H_2O_2 present in the reaction, indicating H_2O_2 neutralization. The results were expressed as percentage of neutralization in comparison with control.

H_2O_2-induced spontaneous nociception, mechanical hyperalgesia, and edema

Briefly, animals were pretreated with AE (100, 300, or 1000 mg/kg, i.g.), rutin (10 mg/kg, i.g.), HC-030031 (300 mg/kg, i.g.; a selective TRPA1 antagonist) [21], or vehicle (10 mL/kg, i.g.) 1 h before the s.c. paw injection of H_2O_2 (2 µmol/paw prepared in phosphate buffered saline [PBS], composed of 137 mmol/L NaCl, 2.7 mmol/L KCl, and 10 mmol/L phosphate buffer, 20 µL) into the right hind paw as described before [4]. Spontaneous nociception was observed for 5 min after injection, and mechanical hyperalgesia and edema formation were evaluated 20 min after the s.c. injection of H_2O_2.

Algogen-induced spontaneous nociception

The antinociceptive effect of the AE of *C. illinoensis* was further evaluated in other models of spontaneous nociception induced by agonists of the TRPA1 receptor (allyl isothiocyanate [AITC] and cinnamaldehyde) as previously described [22]. For that purpose, animals were pretreated with AE (30, 100, 300, or 1000 mg/kg, i.g.), rutin (10 mg/kg, i.g.), HC-030031 (300 mg/kg, i.g.), or vehicle (10 mL/kg, i.g.) 1 h prior to the s.c. paw injection of the algogenic substances. After the acclimatization period, AITC (10 nmol/paw prepared in 0.05% DMSO in PBS) or cinnamaldehyde (100 nmol/paw prepared in 0.05% DMSO in PBS) was s.c. injected in the paw in different groups of animals. Immediately, mice were placed into glass cylinders of 20-cm diameter and the amount of time spent licking or biting the injected paw was timed with a chronometer observed for 5 min. Treatment with their vehicle solutions did not evoke nociception behavior (data not shown).

In addition, we also s.c. coinjected H_2O_2 (2 µmol/paw) or AITC (10 nmol/paw) with AE (2 µg/paw) or vehicle into the hind paw after 30 min of incubation. Then, after injection, the amount of time spent licking or biting the injected paw was timed with a chronometer observed for 5 min.

Measurement of motor performance

To assess the possible nonspecific muscle relaxant or sedative effects of AE, we examined spontaneous motor coordination using the open-field test and forced motor activity using the rotarod test as described before [18]. On the day of the experiment, animals were preinjected with AE (300 or 1000 mg/kg, i.g.) or vehicle (10 mL/kg, i.g.) 1 h prior to the tests.

Adverse effects evaluation

Mice were fasted for 18 h (water ad libitum) prior to treatment. Four hours after treatment (AE 300 or 1000 mg/kg or vehicle, i.g.), mice were euthanized by pentobarbital injection (50 mg/kg, i.p.); the blood was collected for alanine aminotransferase (ALT) and aspartate aminotransferase (AST), indicators of hepatic injury, and creatinine and urea levels, indicators of renal lesion; and the stomachs were removed for gastric tolerability evaluation, as described before [15]. Blood samples were collected from hepatic vein and centrifuged at 2500×*g* for 15 min for serum separation. All measurements were performed using standard methods on a Cobas MIRA (Roche Diagnostics, Basel, Switzerland) automated analyzer. Indomethacin (100 mg/kg, i.g.) was used as positive control for gastric tolerability test. The quantification of gastric mucosal lesions was scored according to their number and size in a scale from 0 to 8 points, as described before [15].

Statistical analysis

The results are presented as mean±SEM, except the inhibitory dose 50% (ID_{50}) or inhibitory concentration 50% (IC_{50}) values (i.e., the dose or concentration of AE reducing the nociceptive responses by 50% relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits, as well the results of the chemical composition of AE, which are expressed as mean±SD of three determinations. The ID_{50} or IC_{50} and its 95% confidence values were determined by nonlinear regression analyses with a sigmoid dose-response equation using the GraphPad Software 5.0 (GraphPad Software, Inc., San Diego, CA, USA) and are reported as geometric means accompanied by their respective 95% confidence limits. The percentages of maximal inhibition (I_{max}) are reported as the mean±SEM of inhibition obtained in each individual experiment in relation to the control values. Data were analyzed using Student's t-test or one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. Nonparametric Kruskal-Wallis followed by Dunn's test was used to analyze gastric lesion scores. p-Values <0.05 were considered as indicative of significance.

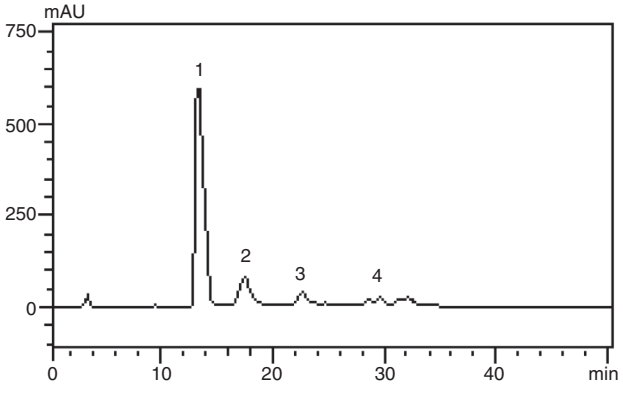

Figure 1 Representative HPLC profile of *C. illinoensis* shell AE.
Detection UV was at 254 nm for gallic acid, 280 nm for catechin, and 365 nm for rutin. Gallic acid, peak 1; catechin, peak 2; ellagic acid, peak 3; and rutin, peak 4.

Results

HPLC analysis of AE

HPLC fingerprinting of *C. illinoensis* AE (10 mg/mL) revealed the presence of gallic acid (tR=13.87 min; peak 1), catechin (tR=17.63 min; peak 2), ellagic acid (tR=22.38 min; peak 3), and rutin (tR=29.75 min; peak 4) (Figure 1 and Table 1).

AE produced an antinociceptive effect in the abdominal constriction test induced by acetic acid

Figure 2 shows that AE (300 and 1000 mg/kg), given by i.g. route 1 h before the test, produced dose-related inhibition of acetic acid-mediated abdominal constrictions in mice.

Table 1 Composition of *C. illinoensis* shell AE.

Compounds	*C. illinoensis* AE	
	mg/g	%
Gallic acid	167.09±0.07	16.70
Catechin	50.26±0.02	5.02
Rutin	13.84±0.11	1.38

Results are expressed as mean±SD of three determinations.

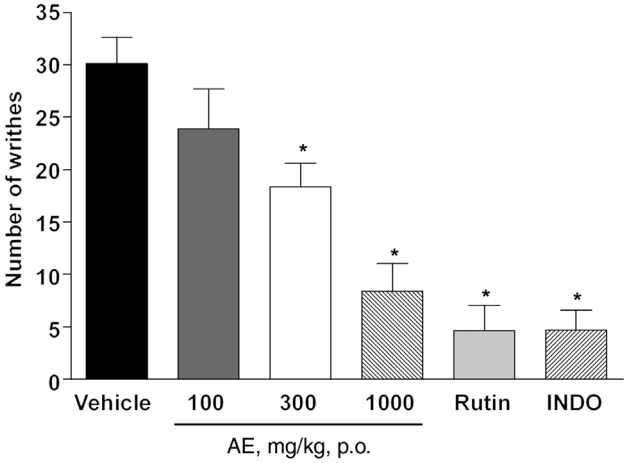

Figure 2 Antinociceptive effect of *C. illinoensis* AE against acetic acid-induced writhing response in mice.
AE (100, 300, or 1000 mg/kg, i.g.), rutin (10 mg/kg, i.g.), indomethacin (INDO, 100 mg/kg, i.g.), or vehicle (10 mL/kg) was administered 1 h prior to the test. Each point represents the mean±SEM for eight to nine animals. Asterisks denote the significance levels: *p<0.05 when compared with the vehicle pretreated group (one-way ANOVA followed by Bonferroni's post hoc test).

The calculated mean ID_{50} value was 477 (267–853) mg/kg, and I_{max} value was 71%±9% at a dose of 1000 mg/kg. The flavonoid rutin, used as positive control and founded in the extract, and indomethacin (a noted anti-inflammatory compound) possessed an antinociceptive action in this test (85%±8% and 87%±5% inhibition, respectively).

AE presented antihyperalgesic and antiedematogenic effects in carrageenan-induced paw inflammation

The AE (300 and 1000 mg/kg, i.g.) pretreatment inhibited carrageenan-mediated hyperalgesia and hind paw edema; the I_{max} values were 100% and 30%±4% (1000 mg/kg, i.g.), respectively (Figure 3A and C). The antihyperalgesic and antiedematogenic effects of AE (300 mg/kg, i.g.) and rutin (10 mg/kg, i.g., 100% and 35%±6% inhibitions at 1 h for mechanical hyperalgesia and edema formation, respectively) were observed from 0.5 to 2 h after the s.c. injection of carrageenan in mice (Figure 3B and D). In addition, the administration of indomethacin (100 mg/kg, i.g.) reduced mechanical hyperalgesia (100% inhibition at 1 h) and edema (53%±4% inhibition at 1 h) from 0.5 to 4 h after treatment. The administration of AE (100, 300, and 1000 mg/kg, i.g.), rutin (10 mg/kg, i.g.), or indomethacin (100 mg/kg, i.g.) did not alter the mechanical thresholds of naïve animals (data not shown).

Evaluation of *C. illinoensis* AE antioxidant effect ex vivo and in vitro

We observed the capacity of AE (300 mg/kg, i.g.) or rutin (10 mg/kg, i.g.) to reduce the H_2O_2 content in paw skin of mice treated with carrageenan (300 µg/paw) 1 h after s.c. injection of carrageenan (98%±2% and 99%±1% inhibition for AE and rutin, respectively), when compared with animals injected with carrageenan and pretreated with vehicle (Figure 3E). Moreover, we also observed that AE was capable of directly neutralizing the H_2O_2 in vitro, with an IC_{50} of 8 (6–12) µg/mL (Figure 3F); rutin presented an IC_{50} of 24 (20–27) µg/mL.

AE possessed antinociceptive and antiedematogenic effects in H_2O_2-elicited nociception, mechanical hyperalgesia, and edema

AE (100, 300, and 1000 mg/kg, i.g.) caused a dose-related inhibition of the spontaneous nociception produced by H_2O_2 (2 µmol/paw), with a mean ID_{50} value of 286 (185–443) mg/kg and I_{max} of 62%±5% at a dose of 1000 mg/kg (Figure 4A). The AE (300 and 1000 mg/kg, i.g.) administration produced a dose-dependent antihyperalgesic effect observed 20 min after H_2O_2 s.c. injection, with a mean ID_{50} value of 294 (229–376) mg/kg and I_{max} value of 100% at a dose of 1000 mg/kg (Figure 4B). The edema formation was also dose dependently reduced by i.g. administration of AE (300 and 1000 mg/kg) in H_2O_2-induced paw edema, with a mean ID_{50} value of 420 (254–694) mg/kg and I_{max} of 68%±9% at a dose of 1000 mg/kg (Figure 4C). Rutin (10 mg/kg, i.g.) and HC-030031 (300 mg/kg, i.g.) administration also decreased spontaneous nociception (67%±4% and 86%±3% inhibition, respectively), mechanical hyperalgesia (100% and 100% inhibition, respectively), and edema formation (42%±7% and 63±5% inhibition, respectively) (Figure 4).

Antinociceptive action of AE in algogen s.c. injection mediated spontaneous nociception

AE administration showed an antinociceptive effect in the cinnamaldehyde (100 nmol/paw)-induced nociceptive behavior test; the I_{max} was 68%±4% at a dose of 1000 mg/kg (Figure 4D). The results of Figure 4E demonstrate that AE given by the i.g. route (300 and 1000 mg/kg) dose dependently inhibited AITC (10 nmol/paw)-induced nociception; the calculated mean ID_{50} value was 617 (365–1045) mg/kg, and the I_{max} was 58%±8%. Rutin and HC-030031 possessed an antinociceptive action in both tests, with 55%±6% or 85%±2% and 33%±7% or 67%±5% inhibitions for cinnamaldehyde- or AITC-induced nociception, respectively.

Moreover, when H_2O_2 was coinjected with AE after incubation, we observed a large reduction in H_2O_2-induced spontaneous nociception (Figure 4F). However, the coinjection of AITC plus AE after incubation only partially reduced the AITC-elicited nociception (Figure 4F).

Side effect investigation

Animals given AE (300 mg/kg, i.g.) did not show altered spontaneous locomotor activity in the open-field test or forced locomotor activity in the rotarod test when compared with animals that received vehicle (control group) (Table 2).

We also investigated the possible capacity of AE to cause gastric lesions. Animals administered AE by the i.g. route (300 or 1000 mg/kg) did not show induced

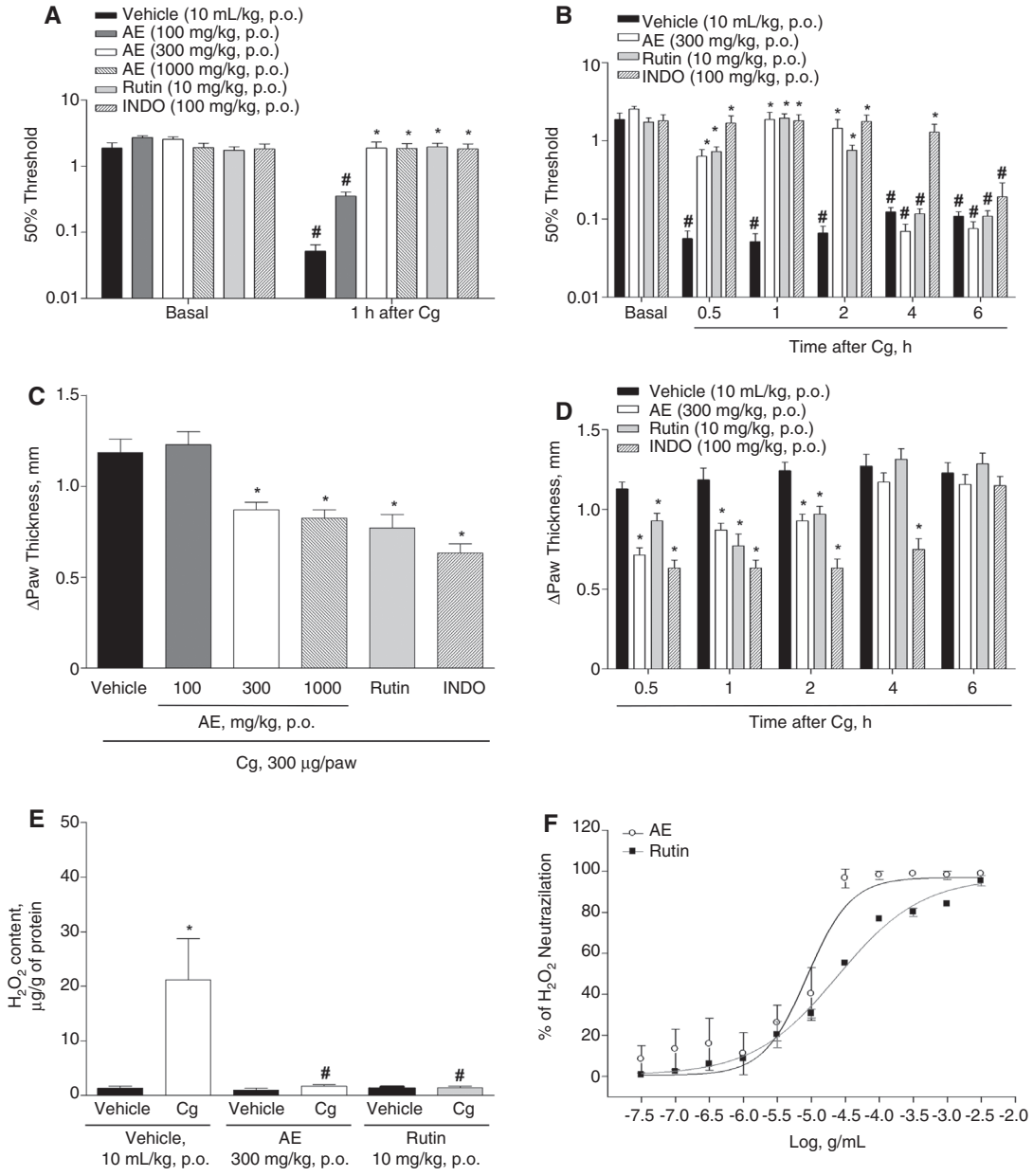

Figure 3 Antihyperalgesic and antiedematogenic effects of *C. illinoensis* AE in carrageenan (Cg)-induced inflammatory nociception model. (A, C) AE (100, 300, or 1000 mg/kg, i.g.), rutin (10 mg/kg, i.g.), or vehicle (10 mL/kg) was administered 0.5 h prior to the s.c. injection of carrageenan (300 μg/paw, 20 μL) for the dose-response curves (measures 1 h after s.c. carrageenan injection). (B, D) AE (300 mg/kg, i.g.), rutin (10 mg/kg, i.g.), indomethacin (INDO, 100 mg/kg, i.g.), or vehicle (10 mL/kg) was administered 0.5 h before the s.c. injection of carrageenan (300 μg/paw) for time-response curves (measures at 0.5–6 h after s.c. carrageenan injection). Basal values were observed prior to carrageenan injection. (E) *C. illinoensis* AE or rutin treatment significantly reduced the Cg-mediated endogenous H_2O_2 production. (F) AE directly neutralized the H_2O_2 in vitro, with an IC_{50} of 8 (6–12) μg/mL. Rutin presented an IC_{50} of 24 (20–27) μg/mL. Data are expressed as mean±SEM of mechanical painful hypersensitivity, expressed as 50% mechanical paw withdrawal threshold (g) or variation in paw thickness. Each point represents the mean±SEM for seven to eight animals or four to five samples. Asterisks denote the significance levels: *$p<0.05$ when compared with the vehicle pretreated group or #$p<0.05$ when compared with basal values or the vehicle/Cg-treated group (two-way ANOVA followed by Bonferroni's post hoc test in A, B, and D or one-way ANOVA followed by Bonferroni's post hoc test in C and E). The IC_{50} values were calculated using nonlinear regression (in F).

ulcerogenic activity when compared with the vehicle-treated group, whereas indomethacin treatment (100 mg/kg, i.g.) provoked the induction of gastric lesions (median [25th–75th percentile] lesion scores of 0 [0–1], 3 [2–4.5], 0 [0–1], and 0 [0–1] for vehicle, indomethacin, and AE 300 or 1000 mg/kg, respectively).

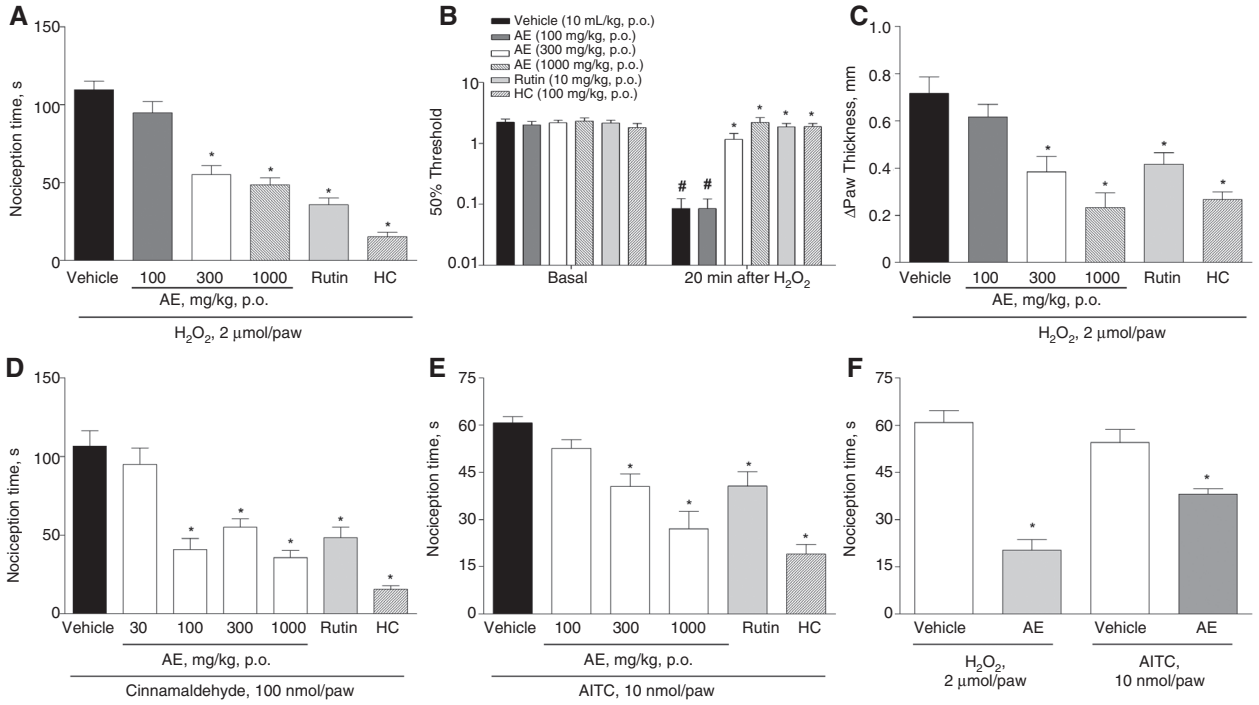

Figure 4 *C. illinoensis* AE reduced the spontaneous nociception (A), mechanical hyperalgesia (B), and edema formation (C) induced by s.c. injection of H_2O_2 (2 µmol/paw).
AE (100, 300, or 1000 mg/kg), rutin (10 mg/kg), HC-030031 (HC, 300 mg/kg), or vehicle (10 mL/kg) was administered by i.g. route 1 h prior to the nociceptive test. Nociception time was measured for 5 min after algogen injection. Mechanical hyperalgesia and edema formation were assessed 20 min after s.c. injection of H_2O_2 (2 µmol/paw). The antinociceptive action of *C. illinoensis* AE or rutin against cinnamaldehyde (100 nmol/ paw) (D) or AITC (10 nmol/paw) (E) mediated spontaneous nociception in mice is shown. AE (2 µg/paw) reduced the spontaneous nociception induced by H_2O_2 (2 µmol/paw) or AITC (10 nmol/paw) when coinjected subcutaneously after incubation (30 min). Each point represents the mean±SEM for seven to nine animals. Asterisks denote the significance levels: *$p<0.05$ when compared with the vehicle pretreated group or $^{\#}p<0.05$ when compared with basal values observed prior to H_2O_2 paw injection (one-way ANOVA followed by Bonferroni's post hoc test in A, C, D, and E or two-way ANOVA followed by Bonferroni's post hoc test in B).

Finally, we assessed if AE (300 mg/kg, i.g.) single administration altered the hepatic (ALT and AST enzymatic activity) or renal (urea and creatinine content) damage parameters in the serum samples 4 h after its treatment or with vehicle. The treatment with AE (300 or 1000 mg/kg) did not alter any damage factor evaluated when compared with the vehicle (Table 3).

Table 2 Effect of *C. illinoensis* shell AE (300 or 1000 mg/kg, p.o.) or vehicle (10 mL/kg, p.o.) on spontaneous and forced locomotor activity in mice, observed 1 h after treatment.

Treatment (p.o.)	Open field		Rotarod	
	Crossing	Rearing	First fall	No. fall
Vehicle	82±5	52±3	214±19	0.5±0.3
AE 300 mg/kg	83±10	45±4	183±38	0.6±0.5
AE 1000 mg/kg	80±7	49±3	216±11	0.4±0.2

No significant differences were observed between groups (Student's t-test). Results are expressed as mean±SEM (n=6 for vehicle or 7 for AE-treated group).

Discussion

The nut shells of *C. illinoensis* are a considerable by-product of the nut industry and are currently used in tea form for the treatment of diverse pathologies, some related to inflammation and pain [8, 12, 23]. However, until now, its analgesic effect has not been evaluated. The current study demonstrates that the i.g. administration of *C. illinoensis* AE induced a relevant inhibition of nociception and edema mediated by inflammatory or chemical stimulus in mice. In addition, we showed that AE reduced the H_2O_2 content after carrageenan s.c. paw injection and also possesses the capacity to neutralize H_2O_2 in vitro. Moreover, AE administration reduced the nociception induced by TRPA1 agonists. The antinociceptive effect of AE i.g. treatment was

Table 3 Effect of *C. illinoensis* shell AE (300 or 1000 mg/kg, p.o.) or vehicle (10 mL/kg, p.o.) on AST and ALT enzymatic activity or urea and creatinine levels in the serum of mice observed 4 h after treatment.

Treatment (p.o.)	AST, U/L	ALT, U/L	Urea, mg/dL	Creatinine, mg/dL
Vehicle	170.2±9.8	58.7±1.3	66.6±10.2	0.13±0.01
AE 300 mg/kg	173.8±12.2	67.7±4.1	74.9±5.2	0.10±0.01
AE 1000 mg/kg	181.9±8.3	57.9±4.7	49.9±2.0	0.15±0.03

No significant differences were observed between groups (Student's t-test). Results are expressed as mean±SEM (n=6 for vehicle and 7 for AE-treated group).

not related to any motor or gastric parameter and did not alter any hepatic or renal damage parameter.

The acetic acid-induced writhing test is a well-established nociceptive test that is mediated by the activation of nonselective cation channels expressed at peripheral nociceptive fibers, including transient receptor potential vanilloid 1, TRPA1, and glutamate receptors [24–26]. In this test, administrated AE by the i.g. route produced marked and dose-related reduction in the number of abdominal constrictions in mice. However, despite the test's reliability, it can sometimes produce false-positive results [27]. Therefore, we verified our results using carrageenan-mediated inflammatory nociception.

Carrageenan-mediated paw mechanical hypersensitivity and edema are a relevant model of inflammatory pain [28]. Pretreatment with AE consistently inhibited carrageenan-induced persistent mechanical hyperalgesia in the mouse paw. Interestingly, we also found that AE administration partially decreased the edema induced by carrageenan in mice. During inflammation, oxidant species are produced and may modulate the activity of some nociceptors, such as TRPA1 [3, 4]. Indeed, carrageenan-mediated paw edema and mechanical hyperalgesia are dependent on the production of H_2O_2 and the activation of TRPA1 receptor [4, 29]. Accordingly, we showed the capacity of AE to decrease not only inflammatory hyperalgesia but also H_2O_2 formation after the s.c. injection of carrageenan in mice.

It has been shown that the s.c. paw injection of H_2O_2 in mice induced nociception dependent on TRPA1 activation [3, 4]. In this study, we observed the capacity of AE to diminish the spontaneous nociception, mechanical hyperalgesia, and edema formation elicited by H_2O_2 s.c. injection in mice. Moreover, the administration of AE significantly reduced the electrophilic TRPA1 agonists cinnamaldehyde or AITC-mediated spontaneous nociception. This result might indicate that AE possibly exerted an antinociceptive action by a mechanism involving the neutralization of oxidant substances that may activate the TRPA1 receptor. Moreover, we have preliminary results indicating that gallic acid could act as a TRPA1 antagonist (data not shown). Since AE contains this compound, the antinociceptive effect produced by AE may be a combination of its antioxidant effect and the antagonism of TRPA1 by gallic acid.

Neutralization of oxidant substances may be due to the presence of phenolic acids, flavonoids, and condensed tannins (gallic acid, rutin, and catechin, respectively), which could have contributed to the capacity of AE to neutralize the H_2O_2 in vitro. Prior studies have also indicated the high antioxidant potential of AE in vivo and in vitro [10–12, 30]. Moreover, it has shown the beneficial use of AE for the treatment of oxidative damage and inflammation related to cigarette smoke exposure, a mechanism mediated in part through TRPA1 activation by crotonaldehyde and acrolein [5, 12]. In this study, we observed that the s.c. coinjection of AE and H_2O_2 or AITC into the hind paw after incubation largely reduced the H_2O_2-induced nociception and partially inhibited AITC-triggered nociception. Thus, we could assume that AE effect could be produced by its antioxidant effect and also by TRPA1 antagonism.

The search for new, safe, low-cost, and effective treatments for pain of inflammatory origins is an important issue. AE treatment has not elicited any alteration in motor function, gastric mucosa integrity, and renal or hepatic function, all adverse effects commonly associated with the main class of analgesics used nowadays [31]. Chronic inflammatory diseases are frequently associated with severe pain, which leads to reduced quality of life and distress [1, 32]. Of note, recent studies also have shown the safety of AE treatment, even after chronic administration in rodents [10, 12, 30].

Collectively, we have shown that AE produces antinociceptive and antiedematogenic effects when assessed in models of either algogenic-induced spontaneous nociception or persistent inflammatory pain. Thus, the present study confirms the popular use of this by-product of the nut industry, which represents a reliable source of interest for the development of new and safe analgesic drugs for inflammatory pain treatment.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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