Inflammopharmacol
DOI 10.1007/s10787-017-0384-6

Inflammopharmacology

ORIGINAL ARTICLE

Arctium minus crude extract presents antinociceptive effect in a mice acute gout attack model

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Received: 21 June 2017 / Accepted: 1 August 2017

Abstract Gout is a disorder that triggers a severe inflammatory reaction which generates episodes of intense pain and discomfort to the patient. *Arctium minus* (Hill) Bernh. (Asteraceae) is known as "burdock" and displays anti-inflammatory, antinociceptive, against rheumatic pain and radical-scavenging activities. Species of the genus *Arctium* have been used in assistant therapy of gout and other inflammatory processes. We investigated the antinociceptive and anti-edematogenic effects of the crude extract of *A. minus* seeds in an acute gout attack model induced by intra-articular injection of monosodium urate (MSU) crystals in adult male Swiss mice (25–30 g). The crude extract of *A. minus* (100 mg/kg, p.o.) reduced the mechanical allodynia induced by the injection of MSU (1.25 mg/site, i.a.) from 4 until 8 h after its administration. *A. minus* seeds crude extract prevented mechanical allodynia at doses of 30 and 100 mg/kg, but not 10 mg/kg. Allopurinol (10 μg/mL) and *A. minus* crude extract (10–300 μg/mL) inhibited the xanthine oxidase activity in vitro. The *A. minus* seeds crude extract did not cause adverse effects since did not change the toxicological parameters evaluated. *A. minus* crude extract can be used as an assistant therapy of gout pain, supporting its traditional use, without causing adverse effects.

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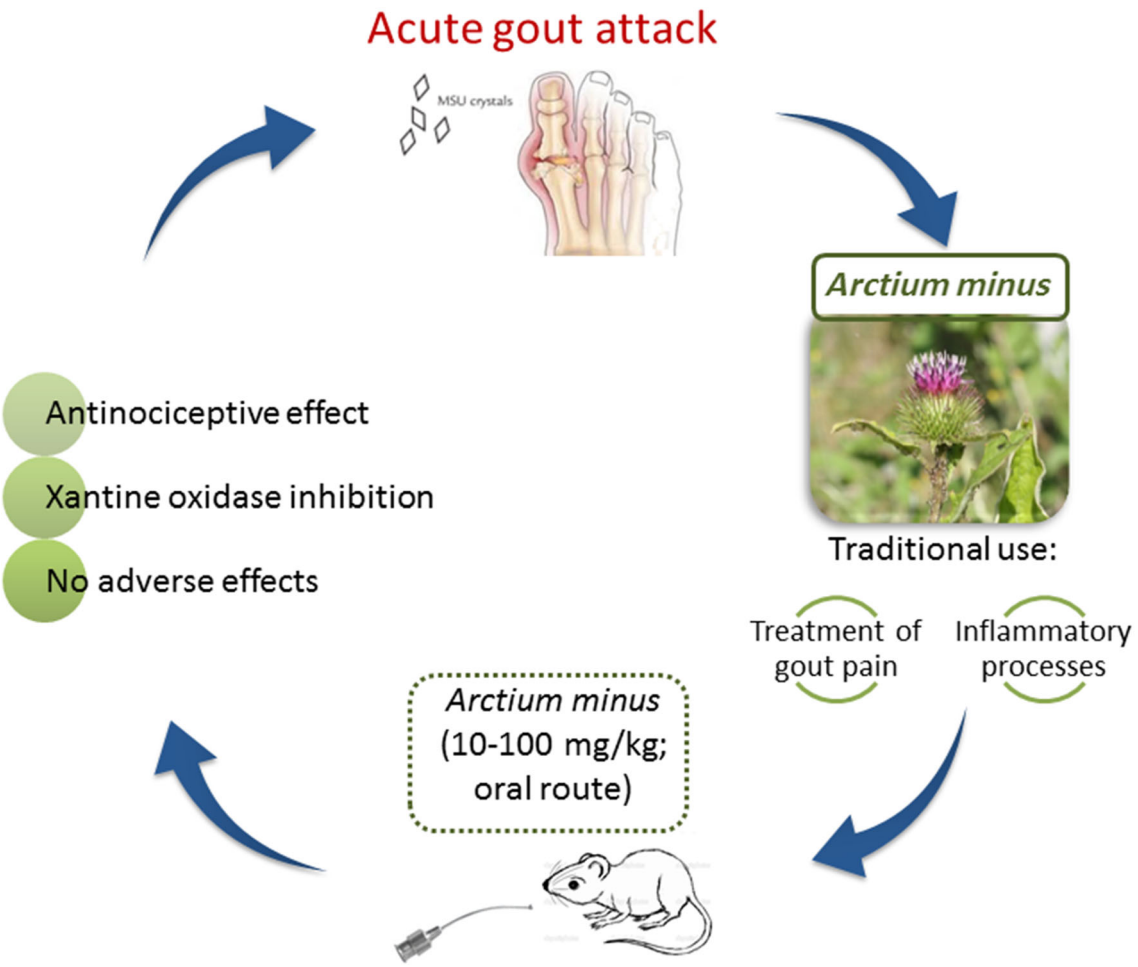
Published online: 11 August 2017

Graphical Abstract



Keywords Joint · MSU · Xanthine oxidase · Antioxidant · Pain · Burdock

Abbreviations

MSU	Monosodium urate
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
NSAIDs	Non-steroidal anti-inflammatory drugs
DPPH	1,1-Diphenyl-2-picrylhydrazyl
PWT	Paw withdrawal threshold

Introduction

The gout is an arthritis form characterized by an intense inflammatory reaction caused by the deposition of monosodium urate (MSU) crystals in tissues such as the joints, usually in the big toe or ankle (Choi et al. 2005). The MSU deposition occurs due to an excess of circulating uric acid (greater than 6.8–7.0 mg/dL) by under-excretion or overproduction, which can be easily determined in most patients and it is a predictive factor for the development of gout (Khanna et al. 2012; Zamudio-Cuevas et al. 2015). Previous disease, mechanical trauma, low temperatures, and underlying osteoarthritis make the joint susceptible to MSU crystals, whose repetitive accumulation forms tophi deposition (called tophaceous gout) (Roddy et al. 2007). The MSU crystals are originate from uric acid, an end-product of purine metabolism by the action of the xanthine oxidase enzyme (Richette and Bardin 2010).

The non-soluble MSU crystals, formed when urate concentrations exceed its solubility limit, trigger the formation of the interleukin-1β, which will activate their receptors. This activation induces the production of chemokines and cytokines and the recruitment of inflammatory cells, mainly neutrophils, which plays a crucial role in inflammatory hypernociception (Richette and Bardin

2010; Cronstein and Esserman 2013; Martinon et al. 2006). Thus, an acute gout attack induces the development of swelling, erythema, and temperature elevation of affected joint, with severe pain perception, which is a significant experience of patients with acute gouty arthritis and responsible for the reduction of their quality of life (Richette and Bardin 2010; Terkeltaub 2010).

Among the first-line agents for the treatment of acute attacks of gout are non-steroidal anti-inflammatory drugs (NSAIDs) or colchicine, an alkaloid derived from the autumn crocus (*Colchicum autumnale*). However, the use of NSAIDs is associated with gastrointestinal toxicity and cardiovascular risk (Cronstein and Esserman 2013; Zhang et al. 2006), while higher dosages of colchicine can cause blood dyscrasias, neuromuscular toxicity, and gastrointestinal adverse effects (Cronstein and Esserman 2013; Salvo et al. 2011). Allopurinol, a xanthine oxidase inhibitor, is the main agent used to reduce the hyperuricemia, although it also can cause adverse reactions including pruritus and rash (Khanna et al. 2012; Stamp et al. 2015).

The search for new analgesic molecules that combine high efficacy without inducing adverse effects is the main objective of the research in the field of pain and analgesia (Cocco et al. 2003). Natural products have been used in the process of discovery of new drugs with therapeutic relevance, including analgesic molecules (Koehn and Carter 2005; Calixto et al. 2000). *Arctium minus* (Hill) Bernh. is a plant which belongs to the family Asteraceae, known as "burdock" or "lesser burdock", and is a species native to Asia minor that has spread throughout North America and Asia (Erdemoglu et al. 2009; Robbins 2013). The *A. minus* leaves are used against rheumatic pain, fever, and sunstroke in Turkish folk medicine. Furthermore, *A. minus* displays anti-inflammatory, antinociceptive, and radical-scavenging activities (Erdemoglu et al. 2009; Fujita et al. 1995). Species from *Arctium* genus has been used as an assistant therapy for the treatment of gout, hypertension, arteriosclerosis, and other inflammatory diseases (Chan et al. 2011).

Since natural products have been biologically evaluated for their therapeutic potential, we verified the effect of the oral treatment with preparations from the *A. minus* seed crude extract on antinociceptive and anti-edematogenic effects in a model of acute gout attack induced by intra-articular injections of MSU.

Materials and methods

Plant collection and extraction

Flowers of *A. minus* were collected in Maximiliano de Almeida (The Rio Grande do Sul State of Brazil) in January (2015), and a dried voucher specimen is preserved in the herbarium of the Department of Biology at Federal University of Santa Maria (register number, SMBD 16046). The seeds (50 g) were removed carefully from flowers and placed to macerate in 70% ethanol for a week, with a daily shake-up. After filtration, the solvent was renewed each week during 1 month. The hydroalcoholic extract was evaporated under reduced pressure to remove the ethanol; the remaining aqueous was dried (temperature below 40 °C) to give the extract.

Drugs and reagents

Synthetic MSU crystals were prepared as described previously (Hoffmeister et al. 2011; Silva et al. 2013). First, 4 g of uric acid (Vetec, Brazil) was dissolved and heated in 800 mL of ultra-distilled H_2O (Milli-Q) adjusted to pH 8.9 with NaOH (9 mL, 0.5 N) at 60 °C, cooled overnight in a refrigerator, and then filtered and dried. The needle-like crystals were recovered and suspended in phosphate-buffered solution (PBS, 10.71 mM K_2HPO_4, 6.78 mM NaH_2PO_4, and 120.4 mM NaCl; pH 7.4). Polarized light microscopy was used to confirm that the crystals were rod-shaped and varied in length (12 ± 2 mm). The preparation was endotoxin-free, as determined by an amebocyte cell lysate assay (Sigma, St Louis, MO, USA). The crude extract of *A. minus* was diluted into Tween® 80 (polysorbate 80, 5%) and saline (0.9% NaCl, 95%) for oral administration (p.o.). Allopurinol was purchased from Sigma (USA), dissolved in saline and was also administered by oral route. Indomethacin was also purchased from local suppliers, but diluted in DMSO (10%), Tween® 80 (10%), and saline (0.9% NaCl, 80%), and it was also administered by oral route. In addition, xanthine oxidase (XO) from bovine milk and pterin was purchased from Sigma (USA).

Acetonitrile and methanol were of Chromasolv LC–MS grade and supplied by Sigma-Aldrich (St. Louis, MO, United States). Acetic acid was obtained from Sigma-Aldrich (St. Louis, USA).

The standards (+)-catechin, 3-acetyl coumarin, 3,6-dihydroxyflavone, 4-hydroxycoumarin, 6-hydroxycoumarin, apigenin, chlorogenic acid, chrysin, fisetin, galangin, gallic acid, kaempferol, luteolin, myricetin, *p*-coumaric acid, quercetin, quercitrin, *trans*-resveratrol, rosmarinic acid, rutin, *trans*-cinnamic acid, and vanillic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Caffeic and ferulic acids were obtained from Fluka Analytical (Buchs, Switzerland). All the standards were of analytical grade with a minimum of 95% purity and were used as received. Stock solutions of the phenolic standards (1000 mg L^{-1}) were prepared by dissolution of appropriate amounts of substances in LC–MS grade methanol.

The triterpenoids such as arjunic acid, maslinic acid, betulinic acid, ursolic acid, erythrodiol, lupeol, β-amyrin, α-amyrin, friedelin, sitosterol, stigmasterol, and campesterol were purchased from Fluka and Sigma-Aldrich (St. Louis, MO, United States).

Arctium minus analyses

UHLC-MS Chromatograph

An Agilent 1260 Infinity UHLC–MS chromatograph (Santa Clara, CA, United States) with automatic injection and an Agilent 6430 triple quadrupole mass spectrometer was used. High-purity nitrogen (99.999%) obtained from Linde (Munich, Germany) was used as the gas for inducing collision at ESI source and as the drying gas. A Zorbax SB-C18 Rapid Resolution HD column (2.1 × 50 mm, 1.8 µm, Agilent) was used at a temperature of 40 °C. Ultrapure water was obtained from a Milli-Q Synergy UV (Merck Millipore, Darmstadt, Germany) system.

Instrumentation, reagents, and solution

The standard solutions of each triterpenic compound as well the mixtures were prepared in acetonitrile as follows: 1000 mg L^{-1} for maslinic acid, arjunic acid, β-amyrin, α-amyrin, and erythrodiol; 985 mg L^{-1} for ursolic acid; 125 mg L^{-1} for campesterol; 196 mg L^{-1} for betulinic acid; 378 mg L^{-1} for sitosterol; 365 mg L^{-1} for lupeol; 481 mg L^{-1} for stigmasterol; and 500 mg L^{-1} for friedelin.

All the solutions were stored in ambar glass recipients at −30 °C until their use. Working solutions of the studied phenolic and terpenic compounds were prepared by dilution of the stock solutions in the respective solvents.

Determination of phenolic compounds

Around 60 mg of this powdered extract was resuspended in 3 mL of methanol, sonicated until complete dissolution, and diluted with 12 mL of ultrapure water and next acetic acid was added to a final proportion of 0.1% (v/v). Then, the extract was filtered through hydrophilic PTFE membranes with a 0.2 µm pore size. The final extracts obtained were submitted to a cleanup step using solid-phase extraction (SPE). The Strata C18-E cartridges (Phenomenex, Torrance, USA), 500 mg, 3 mL, were conditioned with 6 mL of MeOH:0.2% CH_3COOH (1:1, v/v) and equilibrated with 6 mL of 0.1% CH_3COOH (v/v) in water. A fixed volume of 2 mL of the obtained extract with a final MeOH:H_2O:CH_3-COOH composition of 20:80:0.1 (v/v) was percolated with a 2 mL min^{-1} flow rate, followed by washing with 2 mL of 0.1% CH_3COOH. Finally, the cartridge was eluted with 2 mL of MeOH. Just before the chromatographic analysis, the eluate obtained from the SPE procedure was diluted in an MeOH:0.2% CH_3COOH (1:1, v/v) solution. The standard addition method was used to quantify the samples. The calibration curves were built with seven equally spaced concentration levels, in addition to a blank extract. The data points of the calibration curve were determined in triplicate. The phenolic compounds were determined by the method previously developed by Silveira et al. (2016). This method uses a gradient elution composed of 0.1% acetic acid in water (A) and acetonitrile (B) as the mobile phase at a constant flow rate (0.8 mL min^{-1}) according to the following elution program: 8.0% B (0.00–0.10 min); 8.0–25.8% B (0.10–3.45 min); 25.8–54.0% B (3.45–6.90 min); 54.0–100.0% B (6.90–7.00 min); and 100.0% B (7.00–9.00 min). The injection volume was 5 µL, and the injected aliquots were acidified to a final concentration of 0.1% acetic acid (v/v).

Determination of triterpenic compounds

Approximately 50 mg of the dried extract was resuspended in 5 mL of 50% acetonitrile in H_2O (v/v) and vortexed for 30 s followed by 10 min sonication. This 1% extract was diluted 20 times in acetonitrile, filtered through a 0.2 µm PTFE filter, and injected into the chromatographic system. The triterpenic compounds were determined by the method previously developed by Silveira et al. (2016) in which each compound was individually injected into the mass spectrometer to obtain its spectrum and also to optimize the fragmentor and the energy of the collision cell. This method uses APPI ion source and toluene as a dopant. The determination of triterpenes was performed using a gradient elution that consisted initially of 70% acetonitrile/water over 4.5 min. The flow rate was 0.6 mL min^{-1} during the first 4.5 min and was increased to up to 0.8 mL min^{-1} in 5 min, and remained at this rate until the end of the run. The injection volume was 5 µL. A post-run period of 4 min was adopted to re-equilibrate the system.

Antioxidant capacity

The antioxidant capacity of the *A. minus* extract was evaluated according to the spectrophotometric assay described by Piana et al. (2016). The extract was evaluated at six ethanol dilutions (7.81, 15.62, 31.25, 62.50, 125, and 250 µg/mL). Each dilution was mixed with 1.0 mL of DPPH 0.3 mM in ethanol solution. After 30 min, the absorption was assessed at 518 nm. Ascorbic acid was used as positive control, and a solution of DPPH (1 mL, 0.3 mM) in ethanol (2.5 mL) was used as negative control. The assay was performed in triplicate, and the evaluation of the antioxidant capacity followed the equation:

$$\% \text{ inhibition} = 100 - \frac{(\text{ABS}_{\text{sample}} - \text{ABS}_{\text{blank}}) \times 100}{\text{Abs}_{\text{control}}},$$

where $\text{Abs}_{\text{sample}}$ is absorbance of each fraction, $\text{Abs}_{\text{blank}}$ is absorbance of fractions without adding the DPPH, and $\text{Abs}_{\text{control}}$ is absorbance the solution of ethanol in DPPH.

The scavenging concentration required to inhibit 50% of the DPPH in the assay medium was expressed in SC_{50} (μg/mL).

Animals

The experiments were conducted using adult male Swiss mice (25–30) maintained under controlled temperature (22 ± 1 °C) in light/dark cycle of 12 h, with free access to food and tap water. The experiments were performed in accordance with national and international legislation (Guidelines of Brazilian Council of Animal Experimentation—CONCEA—and of U.S. Public Health Service's Policy on Humane care and Use of Laboratory Animals—PHS Policy). All animal experiments also complied with the ARRIVE guidelines and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). The protocols were approved by Ethics Committee for Animal Research of the Federal University of Santa Maria (process number 1946180116/2016). The number of animals and noxious stimulus intensities used was the minimum necessary to demonstrate consistent treatment effects.

Induction of acute gout by MSU crystals injection

An arthritic gout model was induced by intra-articular injection of MSU crystals according to Silva et al. (2016) to study the effect of *A. minus* on acute gout attack. An injection of MSU suspension (1.25 mg/site; 20 μL) or vehicle (saline 0.9%; 20 μL) was performed into the tibial–tarsal joint (ankle) left in animals anesthetized with isoflurane. For verification of the analgesic and anti-edematogenic effect of *A. minus*, the animals were treated orally with vehicle (95% saline 0.9% + 5% Tween 80; 10 mL/kg, p.o.) or the crude extract of *A. minus* (10, 30, or 100 mg/kg, p.o.). The edema and nociceptive parameters were evaluated until when necessary to verify the consistent effects of treatment with *A. minus.*

Nociceptive and inflammatory parameters

Mechanical allodynia The mechanical allodynia measure was evaluated using the paradigm of up and down (Oliveira et al. 2016). The mice were acclimated first in transparent glass boxes individually (7 × 9 × 11 cm) on an elevated platform wire mesh to allow access to the plantar surface of the hind paws. Von Frey calibrated filaments of increasing stiffness (0.02–10.0 g) were applied to the plantar surface of the left hind leg of mice. The absence of a paw withdrawal after 5 s from the application of the filament led to the use of a filament with increasing stiffness. Paw lifting against the stimulus applied indicated a positive response to paw withdrawal and led to the use of a filament of a lesser stiffness. This paradigm continued until a total of six measurements or even four consecutive negative or positive responses. The mechanical paw withdrawal threshold (PWT) was calculated as described by Dixon (1980), expressed in grams (g) and evaluated before and several times after injection of MSU or treatments. A significant decrease in PWT compared to baseline values was considered as mechanical allodynia.

The basal mechanical threshold (baseline 1) of the animals was first evaluated with von Frey filaments. Then, one group of animals received the crude extract of *A. minus* (100 mg/kg, p.o.) or vehicle (10 mL/kg, p.o.) by the oral route. One hour after oral treatment, the intra-articular injection of MSU crystals was performed. The mechanical threshold was assessed from 1 up to 24 h after MSU injection (time–response curve). To calculate the 50% effective dose (ED_{50}) of oral treatment with *A. minus* extract was performed a dose–response curve (10, 30, or 100 mg/kg, p.o) at 8 h after MSU injection.

MSU-induced spontaneous nociception The spontaneous nociception behavior was observed immediately before starting the measurements of mechanical allodynia, and it was evaluated through of guarding behavior of the hind paw (Silva et al. 2016). According to the position of the hind paw at the time of the observation, a score of 0, 1, 2, or 3 was assigned. The score 1 was considered when the paw pressure was slightly reduced, with the paw completely on the floor, but the toes are not spread; score 2 was considered when the paw pressure was moderately reduced, with only some parts of the paw slightly touching the floor, and finally, the score 3 was used when the paw was completely removed from the floor.

Edema measurement The formation of edema was evaluated as an increase in the injected ankle thickness [in millimeters (mm)], and it was measured using a digital caliper before and at several times after MSU injection (Hoffmeister et al. 2011). The edema evaluation was observed after the measurements of the mechanical allodynia. The results were expressed as the difference

between the value of the ankle thickness after the injection of MSU and the baseline measurement.

Activity of xanthine oxidase

The activity of xanthine oxidase enzyme was determined in according to Silva et al. (2016). Xanthine oxidase (0.5 U/mL) was incubated with its substrate, pterin (20 mM) and *A. minus* crude extract (10–300 µg/mL), allopurinol (10 µg/mL) or vehicle, boiled, and centrifuged. The supernatant was diluted in sodium acetate buffer (0.1 M, pH 5.5). The xanthine oxidase activity was assessed using a spectrofluorometer, and the fluorescence was monitored at an excitation wavelength of 347 nm and an emission wavelength of 405 nm.

Adverse effect evaluation

We evaluated the possible adverse effects caused by *A. minus* seed crude extract at 8 h after its administration at the dose of 100 mg/kg (p.o.) and in other group of animals at 14 days after its single administration at the dose of 2000 mg/kg as preconized by the OECD Guideline 423 (2001). Indomethacin (100 mg/kg, p.o.) was used as a positive control.

Open-field and rotarod tests

To check the possible adverse effects (sedation, hyperactivity, and loss of motor coordination) of the *A. minus* seed crude extract, the animals were subjected to evaluation of forced and spontaneous locomotor activity (Trevisan et al. 2012). These tests were evaluated at 8 h or 14 days after the oral treatment with *A. minus* crude extract (100 or 2000 mg/kg, p.o., respectively), indomethacin (100 mg/kg, p.o.) or vehicle (10 mL/kg). For the forced locomotor activity was used the rotarod test, and the animals were trained to remain in the revolving cylinder (3.7 cm in diameter, 8 rpm) for 60 s without falling, 1 day before the treatment. On the test day, the animals were evaluated for 4 min, and the number of falls during this period was monitored. The spontaneous locomotor activity was evaluate using the open field test, consisting of glass box measuring 40 × 60 × 50 cm, wherein the floor of the box was divided into 12 equal squares. Thus, the number of areas crossed with all paws, and the number of rearing was counted during 5 min.

Body temperature

The body temperature of the animals was evaluated with a digital thermometer (Oliveira et al. 2009) and recorded before of oral administration (baseline 1) of the *A. minus* crude extract (100 or 2000 mg/kg, p.o.) or vehicle (10 mL/kg, p.o.) as well as at 8 h and 14 days after treatments. The variation in body temperature of the animals about basal temperature was assessed.

Biochemical markers

To evaluate the possible biochemical changes caused by the *A. minus* crude extract, the animals were treated with the crude extract (100 or 2000 mg/kg, p.o.), indomethacin (100 mg/kg, p.o.), or vehicle (10 mL/kg, p.o.). After 8 h or 14 days of the oral treatments, the animals were euthanized, and the serum was collected for the biochemical analysis. The AST (aspartate aminotransferase) and ALT (alanine aminotransferase) activities and urea, creatinine, and glucose levels were evaluated spectrophotometrically according to the standard procedures provided by commercially available kits (Labtest, Red Lake, MG, Brazil).

Gastric lesion assessment

For the gastric lesion test, the animals received oral administration of the *A. minus* seed crude extract (100 or 2000 mg/kg, p.o.), indomethacin (100 mg/kg, p.o.), or vehicle (10 mL/kg, p.o.). After 8 h or 14 days of treatment, they were euthanized and their stomachs removed. The quantification of the lesions was evaluated with the aid of a magnifying glass and was carried out by assigning a score according to the number and size of lesions on a scale from 0 up to 6 points (Oliveira et al. 2009).

Statistical analyses

The results are expressed as the mean ± standard error of the mean (SEM), except for the inhibitory doses (ID_{50}) or inhibitory concentration (IC_{50}) values, which were expressed as geometric means accompanied by their respective 95% confidence limits. Gastric lesion and spontaneous nociception scores were reported as medians followed by their 25th and 75th percentiles. All data were analyzed by Student's *t* test, one-way or two-way analyses of variance (ANOVA) followed by Bonferroni's post hoc test, Tukey's post hoc, Kruskal–Wallis test, or Student Newman–Keuls test when appropriate, using the GraphPad Prism 6.0 software (San Diego California, USA). $P < 0.05$ was considered as statistically significant. No statistical methods were used to predetermine group sizes, but our group sizes are similar to those reported in previous publications in the field.

To evaluate the phytochemical composition was used a calibration curve, and the experimental values were expressed as mean ± SEM ($n = 3$). Antioxidant activity values of SC_{50} (scavenging concentration required for to inhibit the DPPH radical or decrease carbonyl protein levels in 50%) were obtained by linear regression and expressed as mean ± SEM.

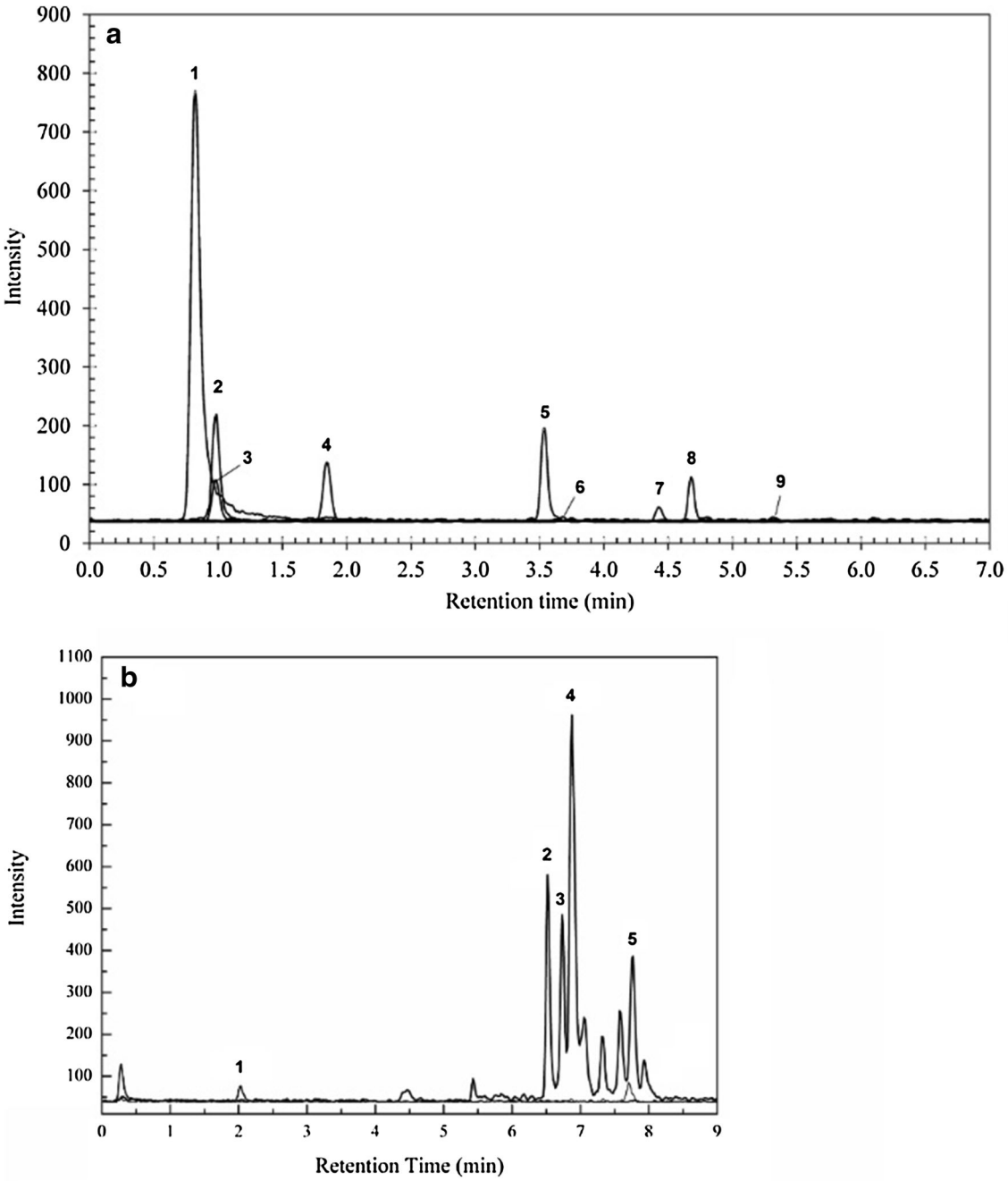


Fig. 1 Extracted ion chromatograms obtained from the LC–MS/MS analysis of the crude hydroethanolic extract of *A. minus*; **a** phenolic compounds: (*1*) chlorogenic acid, (*2*) vanillic acid, (*3*) caffeic acid, (*4*) ρ-coumaric acid, (*5*) ferulic acid, (*6*) quercitrin, (*7*) *trans*-cinnamic acid, (*8*) luteolin, and (*9*) apigenin; **b** triterpenic compounds: (*1*) ursolic acid, (*2*) lupeol, (*3*) β-amyrin, (*4*) α-amyrin, and (*5*) sitosterol. LC–MS/MS and sample conditions as described in "Materials and methods" section (Silveira et al. 2016)

Results

Presence of phenolic and triterpenic compounds in the *A. minus*

Chromatograms in Fig. 1a, b show the majority phenolic and triterpenic compounds found in crude extracts: chlorogenic acid, vanillic acid, caffeic acid, ρ-coumaric acid, ferulic acid, *trans*-cinnamic acid, quercitrin, luteolin, apigenin, ursolic acid, lupeol, sitosterol, α-amyrin, and β-amyrin. As can be seen in Table 1, chlorogenic, caffeic, and vanillic acids were found to be the majority phenolic compounds within the screened compounds by the LC–MS/MS method. Furthermore, α-amyrin, β-amyrin, ursolic acid, lupeol, and sitosterol were the more abundant triterpenic compounds found as active markers within this

Table 1 Phenolic and triterpenic chemical markers identified and quantified in extracts of *A. minus* by LC–MS/MS

Chemical compound	Concentration in crude extract (mg/kg)
Chlorogenic acid	727.3 ± 8.20
Vanillic acid	115.2 ± 7.00
Caffeic acid	225.0 ± 5.10
ρ-Coumaric acid	24.8 ± 0.90
Ferulic acid	11.4 ± 1.90
trans-Cinnamic acid	11.4 ± 2.10
Quercitrin	0.8 ± 0.30
Luteolin	29.6 ± 0.20
Apigenin	2.3 ± 0.40
Ursolic acid	162.21 ± 3.73
Lupeol	158.21 ± 3.62
Sitosterol	143.29 ± 4.59
α-Amyrin	331.35 ± 2.92
β-Amyrin	1465.26 ± 6.87

screened class. Furthermore, the majority compounds which were found in *A. minus* crude extract are structurally represented in Fig. 2.

Antioxidant capacity of *A. minus*

The *A. minus* seed crude extract presented an interesting antioxidant capacity, evaluated by DPPH test, when compared to positive control (ascorbic acid). The SC_{50} value of the *A. minus* seed crude extract was 49.17 ± 1.83 μg/ml, while the SC_{50} value of the ascorbic acid was 16.05 ± 2.72 μg/mL.

Effects of the *A. minus* crude extract on MSU-induced nociception and edema

The intra-articular injection of MSU-induced mechanical allodynia characterized by a reduction in the PWT that started from 4 h up to 8 h and disappeared 24 h after MSU injection when compared to the baseline (B). However, the intra-articular injection of the vehicle did not reduce the PWT when compared to the baseline (B) (Fig. 3a). The oral treatment with *A. minus* crude extract (100 mg/kg, p.o.) was able to prevent the mechanical allodynia induced by MSU from 4 to 8 h after its administration when compared to the oral vehicle-treated group, with a maximum inhibition of 76.2 ± 9% at 8 h (Fig. 3a). Therefore, the time of 8 h was selected (10–100 mg/kg, p.o.) to perform a dose-response analysis. Thus, we observed that the dose of 30 and 100 mg/kg of the *A. minus* crude extract was effective in preventing the mechanical allodynia with a maximum inhibition of 78.7 ± 7% (100 mg/kg, p.o). In contrast, the lower dose (10 mg/kg, p.o.) did not alter the MSU-induced mechanical allodynia when compared to the oral vehicle-treated group. The ID_{50} value (and the 95% confidence intervals) calculated was 265 (50–1399) mg/kg (Fig. 3b).

We also carried out a dose–response analysis (10–100 mg/kg, p.o.) to assess the effect of *A. minus* crude extract on edema and spontaneous nociception at 8 h after MSU injection. The animals that received an intra-articular injection of MSU crystals (1.25 mg/site) showed an increase in the score of spontaneous nociception when compared to the vehicle intra-articular injected group (Fig. 4a). The injection of MSU crystals was also able to induce an increase in ankle thickness indicating the development of edema when compared to the vehicle intra-articular injected group (Fig. 4b). However, *A. minus* crude extract neither alters the spontaneous nociception nor alters the MSU-induced edema formation in any of the doses tested when compared to the oral vehicle treated plus MSU intra-articular injected group (Fig. 4a, b).

In vitro xanthine oxidase assay

To verify if the *A. minus* crude extract exerts its antinociceptive effect through inhibition of xanthine oxidase enzyme, we evaluated the xanthine oxidase activity using pterin as a substrate in vitro. The allopurinol (10 μg/mL; used as a positive control) inhibited the xanthine oxidase activity by 30 ± 1%. Furthermore, the *A. minus* crude extract inhibited the xanthine oxidase activity in a concentration-dependent manner. The concentration of 300 μg/mL showed an inhibition of 26 ± 1%, similar to allopurinol, while the doses of 100 and 10 μg/mL showed inhibitions of 13 ± 1 and 8 ± 1%, respectively (Fig. 5). The IC_{50} value (and the 95% confidence intervals) calculated was 116.6 (88.86–153.1) μg/mL.

Adverse effects assessment caused by *A. minus* treatment

Open-field and rotarod tests

The treatment with *A. minus* crude extract (100 or 2000 mg/kg, p.o.) or indomethacin (100 mg/kg, p.o., used as a control) neither altered the number of crossings or rearing responses in the open-field test, nor the number of falls in the rotarod test at 8 h or 14 days after administrations, respectively, when compared with the vehicle group (Table 2). Thus, the *A. minus* crude extract caused no change in the spontaneous or forced locomotor activity of the animals, respectively.

Body temperature

Arctium minus crude extract (100 or 2000 mg/kg, p.o.) did not alter the body temperature of the animals at 8 h or

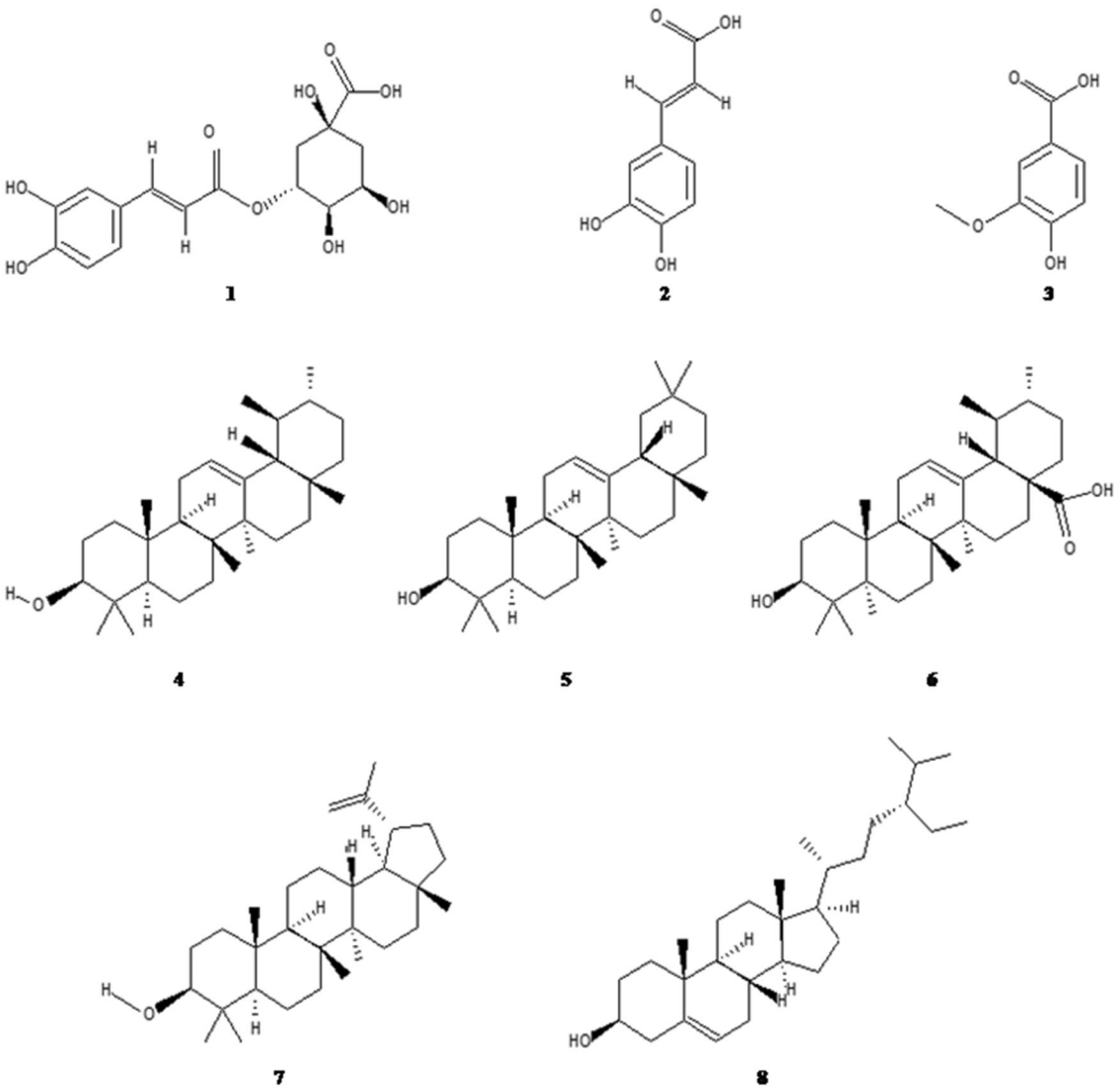


Fig. 2 Chemical structures of majority compounds found in the *A. minus* seed crude extract. Phenolic acids: chlorogenic acid (**1**), caffeic acid (**2**), vanillic acid (**3**), triterpenes: α-amyrin (**4**), β-amyrin (**5**), ursolic acid (**6**), lupeol (**7**), and sitosterol (**8**)

14 days after administrations, respectively, when compared with vehicle-treated animals (data not shown).

Biochemical markers

The hepatic alteration indicator serum activity (AST and ALT) and renal alteration indicator serum level (urea and creatinine) were not altered by treatment with the crude extract from *A. minus* seeds (100 mg/kg, p.o.) or (2000 mg/kg, p.o.) at 8 h or 14 days after administrations, respectively, when compared to vehicle-treated animals (Table 2). The indomethacin (100 mg/kg, p.o., used as a positive control) increased the ALT levels when compared with the vehicle-treated group at 14 days after treatment. Moreover, the *A. minus* crude extract treated group did not alter the glucose serum level. The serum levels of glucose at 8 h after treatments were 115 ± 6.8 and 114.9 ± 4.2 mg/dL to the vehicle or *A. minus* (100 mg/kg)-treated groups, respectively. Furthermore, after 14 days of treatments, serum levels of glucose of 104.1 ± 9.6 mg/dL to the vehicle and 98.72 ± 3.6 mg/dL to the *A. minus* (2000 mg/kg)-treated group were verified.

Gastric lesion assessment

The treatment with the crude extract from *A. minus* seeds at the doses of 100 or 2000 mg/kg did not induce gastric lesions when compared with vehicle-treated animals. The previous treatment (8 h or 14 days) with indomethacin (100 mg/kg, p.o.) induced gastric lesions when compared with vehicle-treated animals. The score of stomach lesions observed after 8 h of treatments was 1 (0.75–1.75) to the

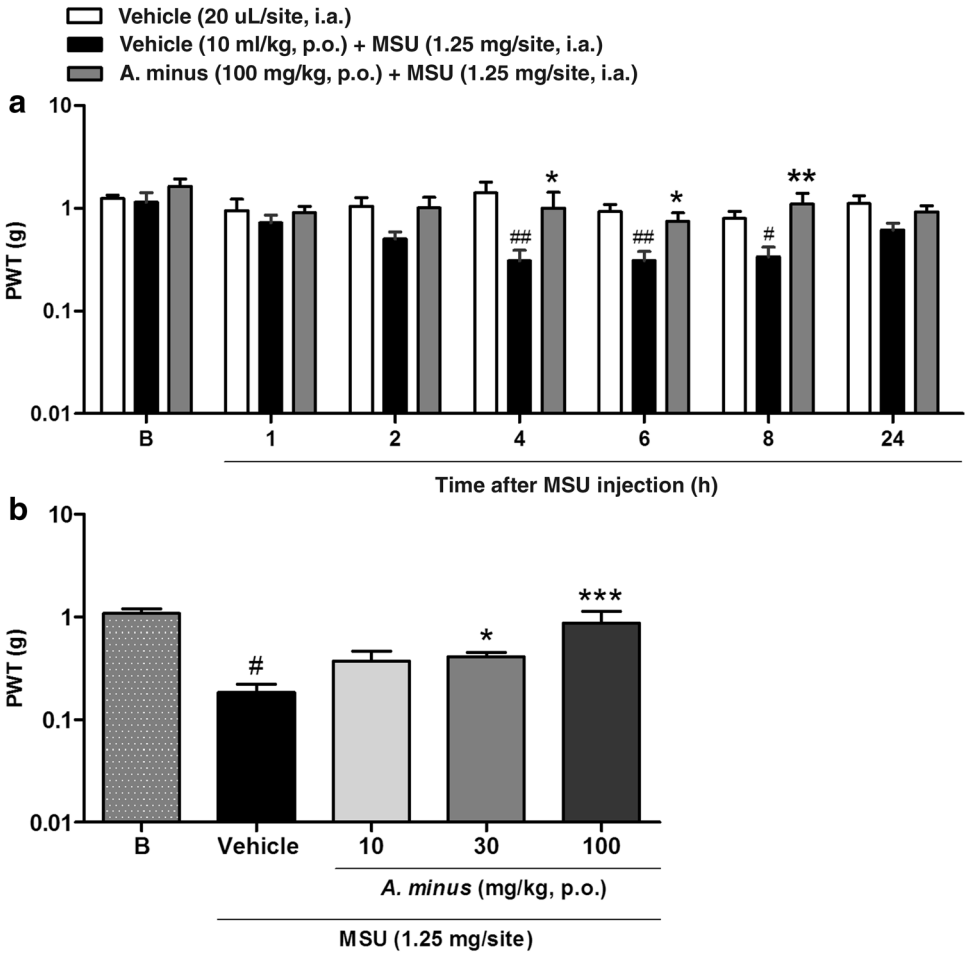


Fig. 3 Effect of the oral administration of the *A. minus* crude extract on the mechanical allodynia induced by intra-articular MSU injection (1.25 mg/site). Time–response (**a**) and dose–response (**b**) curves of the anti-allodynic effect of *A. minus*. The dose–response curve was performed at 8 h after the MSU injection. Data are expressed as the mean + SEM ($n = 5$–9 per group). *B* denotes baseline threshold before MSU injection. $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ when compared with the baseline; one-way ANOVA followed by Tukey's post hoc test. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ when compared with the oral vehicle-treated group plus MSU intra-articular injected; two-way ANOVA followed by Bonferroni's post hoc test

vehicle-treated group, 1 (1–4) to the *A. minus* crude extract (100 mg/kg)-treated group, and 4.5 (3.25–6.75) to the group treated with indomethacin (100 mg/kg, p.o.). The score of stomach lesions observed after 14 days of treatments was 1 (0.75–1.75) to the vehicle-treated group, 1 (1–4) to the *A. minus* crude extract (2000 mg/kg)-treated group, and 4 (3.75–6.25) to the group treated with indomethacin (100 mg/kg, p.o.). Gastric lesions score was reported as median followed by 25–75% quartiles.

Discussion

Gout is caused by deposition of MSU crystals mainly in joints, which triggers excruciating pain that can be reported as the most important clinical symptom in patients affected by this disease. Pain experienced by patients can be a consequence of inflammatory process induced by crystal deposition, which prompts the release of pro-nociceptive mediators (Terkeltaub 2010; Ramonda et al. 2015). In the folk medicine, *A. minus* is empirically used as anti-inflammatory, antinociceptive, and in the rheumatic pain treatment (Erdemoglu et al. 2009; Fujita et al. 1995). Here, we observed that the *A. minus* seeds crude extract prevented the mechanical allodynia, but it was ineffective in reducing the spontaneous nociception and edema induced by MSU injections. Thus, *A. minus* crude extract seems to not have anti-edematogenic activity on the MSU-induced gout model. However, the antinociceptive effect observed is an interesting finding, since the pain is the most frequent and unpleasant sign of acute gout attacks (Martinon et al. 2006; Terkeltaub 2010). This antinociceptive effect found can be attributed to the active constituent presents in the *A. minus*.

Active constituents are considered unique components that contribute to the therapeutic effects of herbal medicine, including primary and secondary metabolites. Thus, the identification and quantitation of constituents that occur in majority quantities are a very important step in phytochemical studies to correlate therapeutic effects with potentially active chemical structures. Phenolic and triterpenic compounds are secondary metabolites, which belong to a group of naturally occurring compounds, showing a broad spectrum of biological activities (Weng and Yen 2012). Thus, within the possible active markers that can be determined in *A. minus* extracts, this study focused on the

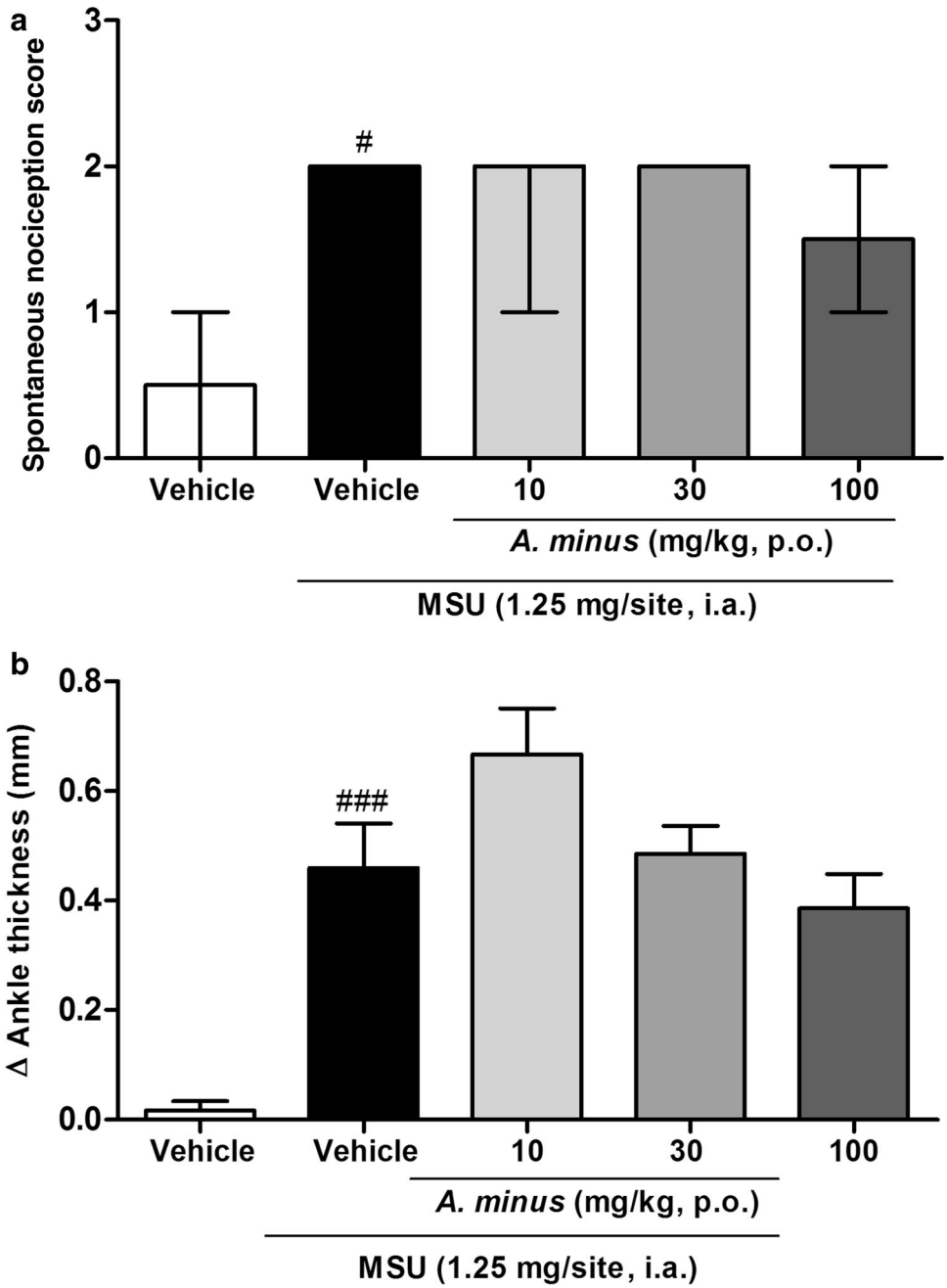


Fig. 4 Dose–response curve of the *A. minus* crude extract effect on the score of spontaneous nociception (**a**) and edema (**b**) induced by intra-articular MSU injection (1.25 mg/site) at 8 h after the MSU injection. ($n = 5$–8 per group). Data are expressed as the median and interquartile ranges for score; $^{\#}P < 0.05$ when compared with the vehicle intra-articular injected group; one-way ANOVA followed by Kruskal–Wallis test. Data are expressed as the mean + SEM for edema; $^{\#\#\#}P < 0.001$ when compared with the vehicle intra-articular injected group; one-way ANOVA followed by Bonferroni's post hoc test

possible phenolic and triterpenic antioxidants extracted in a hydroethanolic extract. Among the 14 chemical markers identified in the crude extract, one can see that α- and β-amyrin and chlorogenic acid are the more concentrated compounds and can be related to the biological activity of the *A. minus*. Furthermore, other compounds found in a smaller proportion (ursolic acid, lupeol, sitosterol, caffeic acid, and vanillic acid) can also contribute to the antinociceptive effect of the *A. minus*.

Previous studies demonstrated that α- and β-amyrin found in the *A. lappa* could be responsible for its biological activity, for example, in the treatment for erysipelas, abscess, carbuncles, and sores (But et al. 1997). *A. lappa* is also used to treat various chronic skin affections, rheumatism, and gout and it is also claimed to have diuretic and blood purifier properties (Lorenzi and Matos 2002; De Smet et al. 1993).

Terpenes such as α- and β-amyrin or plant extracts containing α- and β-amyrin exhibit a wide range of biological effects, including antinociceptive activity. The previous study demonstrated that α- and β-amyrin exhibit antinociceptive effect in screening tests for new analgesics and persistent nociception models (arthritic and neuropathic pain) besides present anti-inflammatory activity (Otuki et al.

Table 2 Effect of the crude extract of *A. minus* or indomethacin on spontaneous or forced locomotor activity, on AST and ALT activities and urea and creatinine levels 8 h or 14 days after treatments

	8 h after treatments			14 days after treatments		
	Vehicle (10 mL/kg, p.o.)	*A. minus* (100 mg/kg, p.o.)	Indomethacin (100 mg/kg, p.o.)	Vehicle (10 mL/kg, p.o.)	*A. minus* (2000 mg/kg, p.o.)	Indomethacin (100 mg/kg, p.o.)
Number of crossing	44.83 ± 5	36.83 ± 6	36.33 ± 3	51.67 ± 9	62.8 ± 8	46.75 ± 16
Number of rearing	21.83 ± 3	16.33 ± 4	18.5 ± 2	27.83 ± 4	33.4 ± 4	23.5 ± 11
Number of falls	0 ± 0	0.5 ± 0.3	0.16 ± 0.1	2.5 ± 0.4	1.6 ± 0.5	0.8 ± 0.5
AST activity (U/L)	15.36 ± 2	22.8 ± 4	19.35 ± 2	20.8 ± 6.1	17.2 ± 3.9	17.35 ± 2.3
ALT activity (U/L)	12.44 ± 3	16.44 ± 5	19.21 ± 4	22.4 ± 4	35.3 ± 4.3	36.48 ± 3*
Urea (mg/dL)	51.00 ± 7	34.42 ± 6	33.04 ± 4	26.6 ± 2.2	29.59 ± 5	33.6 ± 6.3
Creatinine (mg/dL)	2.33 ± 0.24	1.75 ± 0.25	1.58 ± 0.35	1.82 ± 0.1	2.34 ± 0.7	1.33 ± 0.3

Effect of the crude extract of *A. minus* (100–2000 mg/kg, p.o.), indomethacin (100 mg/kg, p.o.), or vehicle (10 mL/kg, p.o.) on spontaneous (open-field test) or forced (rotarod test) locomotor activity, on AST and ALT activities and urea and creatinine levels 8 h or 14 days after treatments. The data are presented as mean ± SEM ($n = 4$–6 per group)

* $P < 0.05$ when compared with the vehicle-treated group; one-way ANOVA followed by Tukey's test

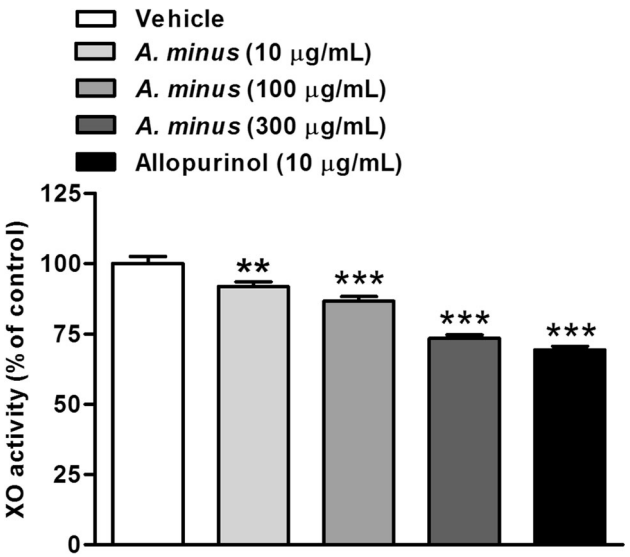


Fig. 5 Effect of different concentrations of the crude extract of *A. minus* (10, 100, and 300 μg/mL), allopurinol (10 μg/mL), or vehicle on xanthine oxidase activity in vitro. Data are expressed as the mean + SEM ($n = 5$ per group). $^{**}P < 0.01$ and $^{***}P < 0.001$ when compared with the vehicle group; one-way ANOVA followed by the Student–Newman–Keuls' test

2005; Lima-Júnior et al. 2006). This antinociceptive effect of α- and β-amyrin seems to involve PKA and PKC pathways, cannabinoids receptors, as well as opioid receptor and transient receptor potential vanilloid 1 (TRPV1) (Lima-Júnior et al. 2006; Chicca et al. 2012, Simão da Silva et al. 2011). Lima-Júnior et al. (2006) showed that the mixture α, β-amyrin may function as a non-selective TRPV1 antagonist, and this finding can be relevant on gout, since the MSU can produce nociception by activating TRPV1 receptor (Hoffmeister et al. 2011; 2013). In addition to α- and β-amyrin, other terpenes such as ursolic acid, lupeol, and sitosterol can also contribute to the antinociceptive effect of the *A. minus*, since these compounds have analgesic action in pre-clinical pain models, including inhibition TRPV1 channels involved in arthritic pain, in the case of ursolic acid (Abdul-Wahab et al. 2012; Alonso-Castro et al. 2016; De lima et al. 2013, Rodrigues et al. 2012; Verano et al. 2013).

In addition to terpenes, *A. minus* presents high amounts of phenolic compounds which also exhibit antinociceptive activity. The phenolic compound which was found in higher concentration in the *A. minus* was the chlorogenic acid. Literature data show that chlorogenic acid presents antinociceptive effects in screening tests for new analgesics and neuropathic pain models (Bagdas et al. 2013; Qu et al. 2014; Hara et al. 2014). Thus, chlorogenic acid can be a responsible, at least in part, by antinociceptive effect promoted by *A. minus*. Other phenolic compounds found in the *A. minus* crude extract were caffeic and vanillic acids. Both also possess antinociceptive properties (Morucci et al. 2012).

In addition to the analgesic activity, phenolic and flavonoid compounds also present antioxidant activity, which may be related to the analgesic action of the *A. minus*. It has been reported that *A. minus* is a rich source of natural antioxidant compound (Erdemoglu et al. 2009). In addition, the burdock has high phenolic compound levels in seed

extracts (Ferracane et al. 2010). These information are consistent with our results, since the phytochemical screening revealed that the *A. minus* seed extract contains high quantity of phenolic compounds and a great amount of flavonoids which seem to be responsible for their antioxidant capacity as verified by DPPH test.

The precipitation of urate crystals can be an indirect result of hyperuricemia, which has been considered the most important factor for the development of gout (Zamudio-Cuevas et al. 2015; Busso and So 2010; Zhu et al. 2011). Allopurinol is the first xanthine oxidase inhibitor discovered, and it has been the most frequently used drug at hyperuricemia control (Nuki and Simkin 2006; Rundles et al. 1963). Previous studies have shown that the xanthine oxidase inhibition and hypouricemic activities of some medicinal plants may be attributed to the presence of flavonoids and other phenolic compounds, due to their anti-inflammatory and antioxidant activities (Umamaheswari et al. 2007; Irondi et al. 2016). The detection of some secondary metabolites as flavonoids and triterpenes may be related to anti-hyperuricemic effects (Lima et al. 2015). Recent studies demonstrated that β-amyrin (Ferraz-Filha et al. 2016; Lin et al. 2011), chlorogenic acid (Meng et al. 2014; Chen et al. 2011), and caffeic acid (Chen et al. 2011) are capable of inhibiting xanthine oxidase. On this study, *A. minus* crude extract was effective in inhibiting xanthine oxidase activity in vitro, an ability that can be associated with substances contained in this extract with different potential to inhibit this enzyme as the β-amyrin, chlorogenic, and caffeic acids. Thus, the antinociceptive effect of *A. minus* may have occurred by xanthine oxidase inhibition. This finding suggests that the *A. minus* could be useful to treat gout attacks acting in a similar way to allopurinol.

Although effective in the treatment of acute attacks of gout, the use of NSAIDs and colchicine is limited by adverse effects, such as gastrointestinal toxicity (Cronstein and Esserman 2013; Zhang et al. 2006; Salvo et al. 2011). Herein, we observed that oral treatment with *A. minus* did not cause gastric damage. Moreover, we also did not find any alteration in the activity of enzymes AST and ALT or alteration in the urea and creatinine contents, which indicate liver or renal injury, respectively. The locomotor activity and the body temperature of mice also were not altered. Taken together, these results indicate that the *Arctium minus* crude extract presents low toxicity in mice.

Conclusion

The present data indicate that *Arctium minus* seed crude extract presents antinociceptive effect on the MSU crystal-induced gout arthritis model and that this effect may be attributed to xanthine oxidase enzyme inhibition. These results support the use of this plant to the treatment of acute gout attack.

Acknowledgements The authors would like to thank Professor Juliano Ferreira for donated reagents. This study was supported by the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul—FAPERGS (process number 16/2551-0000281-9) and by the Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq. We thank CNPq and CAPES for their fellowship support.

Author contributions All authors had full access to all the data in the study and take responsibility for the integrity and accuracy of the data analysis. Study concept and design: SPMF, IB, and SMO. Acquisition of data: SPMF, IB, CC, MP, HF, LAG, LMC, and SMO. Analysis and interpretation of data: SPMF, IB, CC, MP, HF, LAG, LMC, and SMO. Drafting of the manuscript: SPMF, IB, MP, and SMO. Study supervision: SMO.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical approval All procedures performed were in accordance with international and national and were approved by Ethics Committee for Animal Research of the Federal University of Santa Maria (process number 1946180116/2016).

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