



# Antinociceptive and anti-inflammatory activity of the siaresinolic acid, a triterpene isolated from the leaves of *Sabicea grisea* Cham. & Schltdl. var. *grisea*

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Received: 2 July 2014 / Accepted: 25 December 2014 / Published online: 24 January 2015  
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**Abstract** In the present study, siaresinolic acid (siaresinol, SA) was isolated from the leaves of *Sabicea grisea* and studied to evaluate its antinociceptive and anti-inflammatory activity. The antinociceptive effect of SA was investigated in mice using different animal models to study pain. In the acetic acid-induced writhing test, intraperitoneal (i.p.) injection of SA (0.1, 1, and 10 mg/kg, i.p.) 1 h before a pain stimulus significantly reduced the nociceptive response (by 42.3, 68.2, and 70.9 %, respectively). Pretreatment with glibenclamide, but not with yohimbine, metoclopramide, ketanserin, or naloxone, restored the antinociceptive effect induced by SA in the writhing test, suggesting that the K<sup>+</sup>ATP channel pathway might be involved in its mechanism of action. In the formalin test, SA (1 mg/kg, i.p.) decreased licking time in the second phase only, thereby indicating an anti-inflammatory effect. In the hot plate test, there was no significant difference in nociceptive behavior. In the rota-rod test, it was verified that a high dose of SA (10 mg/kg, i.p.) did not affect the

locomotor activity of mice. In the pleurisy model, induced by carrageenan, treatment with SA inhibited important events involved in inflammatory responses, namely leukocyte influx, plasma leakage, and increased inflammatory mediators (TNF- $\alpha$ , IL-1 $\beta$ , and chemokine CXCL1), in the pleural exudate. Additionally, SA itself was not cytotoxic when evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in macrophages cultured for 24 h at concentrations ranging from 1 to 200  $\mu$ g/mL. These results suggest, for the first time, that SA attenuates nociceptive behavior through mechanisms involving receptors for ATP-dependent potassium channels, in addition to suppressing acute inflammatory responses.

**Keywords** *Sabicea grisea* · Rubiaceae · Siaresinolic acid · Siaresinol · Nociception · Inflammation

## Introduction

The Rubiaceae family contains species of great economic importance, which are utilized as food, for ornamental purposes, and in the pharmaceutical industry. This family contains about 650 genera with 13,000 species, which are distributed mainly in tropical and subtropical regions [1–3]. The plants belonging to the genus *Sabicea*, a member of this family, grow in Africa and South America and consist of approximately 146 species. Some of these species are used in traditional medicine to treat various diseases, including epilepsy, stomach disorders, fever, and malaria [4–6]. Their wide use in traditional medicine has encouraged phytochemical investigation and pharmacological study of their active components [7]. In this regard, the observed effects of the *Sabicea* species-derived medicines on fever and malaria suggest that their chemical

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components may be effective for the treatment of pain and inflammation.

Previous studies have identified the presence of active components in the leaves of *Sabicea grisea* Cham. & Schltdl. var. *grisea* [8], but its phytochemical profile has not yet been completely characterized. However, it is known that plants of the Rubiaceae family produce a wide variety of secondary metabolites, including triterpenoids [9–12].

A growing interest has been focused on triterpenoids over the past decade due to their beneficial effects on human health [13, 14]. Triterpenoids have been reported to exhibit a wide range of biological activities, including cytotoxic [15], antimalarial [16], antinociceptive [17], and anti-inflammatory [18] effects. Siarésinolic acid (SA), also known as siarésinol, is a triterpene derivative of oleanolic acid and exhibits antidiabetic activity [19], in addition to exerting antiproliferative effects on human leukemia cells and inhibiting their differentiation [20]. In our study, this compound was isolated from the chloroform fraction of an ethanol extract from *S. grisea* var. *grisea* leaves. To date, there have been no previous reports on the antinociceptive and anti-inflammatory properties of this triterpene. Thus, this study was conducted to evaluate the effects of SA in nociception and acute inflammation using experimental animal models.

## Materials and methods

### Animals

Male Swiss mice weighing 18–22 g were obtained from the breeding colonies of the Universidade Federal de Alagoas. Animals were allowed free access to food and water and maintained at  $22 \pm 2$  °C with a controlled 12 h light–dark cycle at the Instituto de Ciências Biológicas e da Saúde animal housing facility. Experiments were performed during the light phase of the cycle. The animals were allowed to adapt to the laboratory for at least 2 h before testing and used only once. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals by the Brazilian Society of Laboratory Animal Science (SBCAL). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Alagoas (Comissão de Ética no Uso de Animais—CEUA, license no. 014512/2011-65). All efforts were made to minimize the suffering of the animals.

### Plant material

Leaves of *S. grisea* var. *grisea* were collected in May 2008, at Horto Municipal de Maceió, Alagoas State, Brazil, and

identified by Rosângela P. Lyra Lemos of the Instituto do Meio Ambiente do Estado de Alagoas, where a voucher specimen was deposited (MAC-11356).

### Reagents

The drugs used were formalin and acetic acid (both from Vetec, Rio de Janeiro, RJ, Brazil), indomethacin, yohimbine, metoclopramide, glibenclamide,  $\lambda$ -carrageenan, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), and Roswell Park Memorial Institute (RPMI-1640) medium (Sigma Chemical Co., St. Louis, MO, USA). Immediately before use, SA was dissolved in 200  $\mu$ L of absolute ethanol and 3,800  $\mu$ L of saline (NaCl, 0.9 %) to give a concentration of 2 mg/mL, which was diluted to achieve the required concentrations for subsequent experiments. Other drugs and reagents used were of analytical grade.

### Isolation and characterization of SA

Air-dried and powdered leaves of *S. grisea* var. *grisea* (515 g) were exhaustively extracted with 90 % ethanol at room temperature and the solvent evaporated under vacuum (71.8 g). This extract was suspended in methanol–water (MeOH–H<sub>2</sub>O) solution (3:2) and successively extracted with hexane, chloroform, and ethyl acetate (EtOAc). The chloroform fraction (4.4 g), which gave positive results in a preliminary antinociceptive assay, afforded SA (38 mg) as an amorphous solid after successive chromatographic fractionations over silica gel using a gradient of EtOAc in hexane and Sephadex LH-20 with MeOH. The melting point was measured using an MQAPF-302 apparatus. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 400, operating with either <sup>1</sup>H and <sup>13</sup>C at 400 and 100 MHz, respectively, in CDCl<sub>3</sub>/CD<sub>3</sub>OD solutions. The <sup>1</sup>H and <sup>13</sup>C chemical shifts were referenced to the residual solvent peak at  $\delta_{\text{H}}$  3.32 and  $\delta_{\text{C}}$  49.1 for CD<sub>3</sub>OD or at  $\delta_{\text{H}}$  7.27 and  $\delta_{\text{C}}$  77.0 for CDCl<sub>3</sub>, respectively. Infrared (IR) spectra were obtained on a Perkin-Elmer model 1750 FTIR spectrometer. Silica gel (70–230 mesh, Merck) and Sephadex LH-20 (Pharmacia) were used for column chromatography separations, while analytical thin layer chromatography (TLC; 0.5 mm thickness) was carried out on the silica gel plates (60 F<sub>254</sub>, Merck). The structure of SA was identified by interpretation of its spectroscopic data and comparison with those reported in the published literature [21, 22]. The percentage purity of SA used in the biological experiments was determined as 95 %.

### Acetic acid-induced writhing

The writhing test was carried out in mice according to the method previously described by de Barros et al. [23].

Abdominal writhing was caused by intraperitoneal (i.p.) injection of 0.1 mL of 0.6 % acetic acid/10 g body weight. Control mice received an equivalent volume of saline (NaCl, 0.9 %). Animals were administered i.p. SA (0.01, 0.1, 1, and 10 mg/kg) 1 h before stimulation with acetic acid. Five minutes after the acetic acid injection, the number of times that each animal displayed abdominal constriction was counted for 10 consecutive minutes. The percentage of inhibition was determined for each experimental group using the following formula: inhibition (%) =  $100 - [(T \times 100)/C]$ , where *C* and *T* indicate non-treated (vehicle) and drug-treated, respectively.

To assess the possible mechanisms involved in the antinociceptive effect of SA, mice were pretreated subcutaneously (s.c.) or intraperitoneally (i.p.) with different antagonists, yohimbine (5 mg/kg, s.c.), metoclopramide (1 mg/kg, s.c.), glibenclamide (5 mg/kg, s.c.), ketanserine (0.3 mg/kg, i.p.), and naloxone (5 mg/kg, i.p.), in the acetic acid animal model. After 15 min, the animals were administered SA (1 mg/kg, i.p.).

#### Formalin-induced nociception

The procedure described by de Souza Ferro et al. [24] was used, with slight modifications. Mice were injected in the subplantar area of the right hind paw with formalin (2.5 %, 20  $\mu$ L). The duration of paw licking, a nociception index, was measured from 0 to 5 min (neurogenic phase) and from 15 to 30 min (inflammatory phase) after formalin administration. Animals were treated with SA (1 mg/kg, i.p.) 1 h before intraplantar injection of the stimulus. Indomethacin (10 mg/kg, i.p.) was used as the standard comparator drug, and control animals were treated with sterile saline (vehicle).

#### Hot plate test

Animals were placed individually on a hot plate metallic surface (Insight®, Brazil; model EFF-361) maintained at  $54 \pm 1$  °C, and the time between placement of the animal on the hot plate and occurrence of either licking of the hind paws, shaking, or jumping off the surface was recorded in terms of the reaction time (s). The reaction time was measured 1 h following treatment with saline (i.p.), SA (1 mg/kg, i.p.), or morphine (5 mg/kg, i.p.), with a cut-off time of 20 s. Each experimental group contained at least six animals.

#### Rota-rod apparatus

To investigate if the treatments could influence the motor activity of the animals and, consequently, impair assessment of nociceptive behavior in experimental models, the

motor activity of the animals was evaluated on a rota-rod apparatus, according to a procedure proposed by de Oliveira et al. [8]. The animals were trained on the rota-rod apparatus (Insight®, Brazil; model EFF-412) for 2 days before the experiment. On the day of the experiment, mice were treated with SA (10 mg/kg, i.p.) or diazepam (10 mg/kg, i.p.) and tested on the rota-rod apparatus, starting 1 h after drug administration. After treatment, the motor performance time (s) was recorded with a stopwatch for up to 240 s.

#### Carrageenan-induced pleurisy in mice

Pleurisy was induced in mice by intrapleural administration of 100  $\mu$ L of 1 % (w/v) carrageenan suspension in sterile saline solution [8]. A specially adapted 13  $\times$  5 needle was introduced into the right side of the thoracic cavity for injection of the carrageenan solution, and an equal volume of saline was injected into the controls. The animals were sacrificed in a carbon dioxide (CO<sub>2</sub>) gas chamber 4 h after the carrageenan injection, and the thoracic cavity was opened and washed with 1 mL of PBS containing ethylenediaminetetraacetic acid (EDTA; 10 mM). Pleural wash samples were diluted in Turk fluid (2 % acetic acid) to measure total leukocytes, using Neubauer chambers. Cytospin preparations of the samples were stained with May–Grünwald–Giemsa to obtain the differential leukocyte count, which was performed under the immersion objective of a light microscope. Subsequently, groups of animals were pretreated (1 h before pleurisy induction) with SA (0.01, 0.1, and 1 mg/kg, i.p.) and 4 h later, the same inflammatory parameters as above were evaluated.

#### Cytokine/chemokine analysis

Cytokines were measured using a commercially available MagPix Milliplex kit (Merck-Millipore). The immune kit comprised analyte-specific components for simultaneous measurement of the following cytokines from mice: tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 beta (IL-1 $\beta$ ), and chemokine (C-X-C motif) ligand 1 (CXCL1). A minimum of 100 positive beads for each cytokine was acquired with a Luminex MagPix instrument (Luminex Corporation, Austin, TX, USA). All samples were run in duplicate and analyzed on the same day to minimize day-to-day variation. Manufacturer-supplied controls were used to monitor the coefficients of variation, which were <10 %.

#### The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay

The cytotoxic effect of SA on macrophages was determined using the MTT assay method according to

Guimarães et al. [25]. Murine peritoneal macrophages ( $2.5 \times 10^5$  cells) were treated with SA at concentrations ranging from 1 to 200  $\mu\text{g}/\text{mL}$  and further cultured in RPMI-1640 media supplemented with 10 % fetal bovine serum (FBS) for 24 h. Thereafter, the medium was replaced with fresh RPMI containing 5  $\text{mg}/\text{mL}$  of MTT. After an additional 4-h incubation period at 37 °C, the supernatant was discharged and DMSO solution (150  $\mu\text{L}/\text{well}$ ) added to each culture plate. After 15 min of incubation at room temperature, the absorbance of solubilized MTT formazan product was spectrophotometrically measured at 540 nm. Five individual wells were assayed per treatment, and the percentage of viability was determined in relation to the controls [(absorbance of treated cells/absorbance of untreated cells)  $\times$  100].

### Statistical analysis

Data are reported as mean  $\pm$  standard error of the mean (SEM) and were analyzed using the GraphPad Prism® software, version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical differences between the groups were determined using the Student's *t* test or via two-way analysis of variance followed by the Student–Neuman–Keuls test. *p*-Values  $<0.05$  were considered statistically significant. All statistical analyses were done using GraphPad Prism® software, version 5.0.

## Results

### Isolation and identification of SA

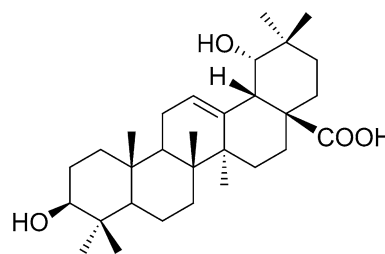
SA was obtained as an amorphous solid, melting point 279–281 °C (literature value: 275–278 °C), and gave a positive response to the Liebermann–Burchard test, suggesting a triterpenoid nature [26]. Its IR spectrum showed absorption bands characteristic of hydroxyl ( $3425\text{ cm}^{-1}$ ) and carboxyl ( $1687\text{ cm}^{-1}$ ) groups and a double bond ( $1608\text{ cm}^{-1}$ ). A detailed analysis of the NMR spectral data of this compound revealed features of an olean-12-ene type triterpene containing a hydroxyl group at C-3 ( $\delta_{\text{H}}$  3.15 [dd,  $J = 10.2$  and  $5.0$  Hz];  $\delta_{\text{C}}$  79.21 [CH]), an  $\alpha$ -hydroxyl group at C-19 ( $\delta$  3.27 [d,  $J = 3.5$  Hz];  $\delta_{\text{C}}$  82.02 [CH]) and a carboxyl group at C-28 ( $\delta_{\text{C}}$  181.75 [C]) [21]. The  $^{13}\text{C}$  NMR spectrum revealed 30 carbon signals to a triterpenoid skeleton. Assignments for all hydrogen and carbon resonances were achieved by a combination of distortionless enhancement by polarization transfer (DEPT), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple-bond correlation (HMBC) experiments. The  $\Delta^{12}$  functionality was deduced from the resonance of the  $\text{sp}^2$  carbons C-12 ( $\delta_{\text{H}}$  5.35 [s] and  $\delta_{\text{C}}$  124.97 [CH];  $\delta_{\text{C}}$

143.48 [C, C-13]), as well as a methine hydrogen ( $\delta_{\text{H}}$  3.05 [sl, H-18];  $\delta_{\text{C}}$  44.30 [C-18]) and signals for seven tertiary methyl groups ( $\delta_{\text{H}}$  0.71 [correlated in the HSQC spectrum with  $\delta_{\text{C}}$  17.23], 0.74 [ $\delta_{\text{C}}$  15.88], 0.87 [ $\delta_{\text{C}}$  28.31], 0.92 [ $\delta_{\text{C}}$  15.46], 0.93 [ $\delta_{\text{C}}$  25.02 and 28.33], and 1.23 [ $\delta_{\text{C}}$  24.85]). The HMBC experiment exhibited correlations particularly between H-19 ( $\delta$  3.27) and methyl groups at C-20 ( $\delta$  28.31 [C-29] and  $\delta$  25.02 [C-30]). These results and comparison with those reported in the literature [21, 22] supported the elucidation of the structure of SA (Fig. 1).

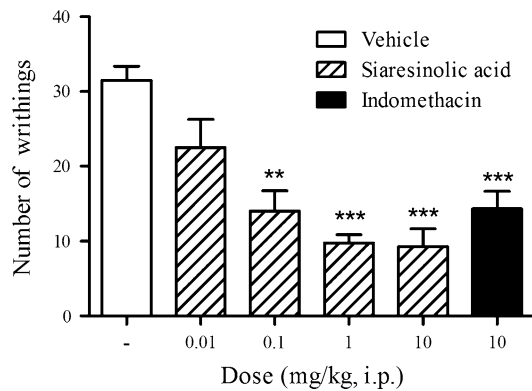
### Acetic acid-induced writhing

An acetic acid-induced writhing test was used to detect possible antinociceptive effects. In this model, the animals were pretreated with SA (0.01, 0.1, 1, and 100  $\text{mg}/\text{kg}$ , i.p.) and nociceptive behavior was evaluated 1 h post-acetic acid injection. The administration of this acid caused stereotypical behavior, characterized by abdominal contractions. At 0.01  $\text{mg}/\text{kg}$ , SA produced no alteration in nociceptive behavior, but pretreatment with SA at 0.1, 1, and 10  $\text{mg}/\text{kg}$  reduced the number of abdominal writhes by 42.3, 58.2, and 70.9 %, respectively. Indomethacin (10  $\text{mg}/\text{kg}$ , i.p.), used as the positive control, also significantly inhibited abdominal writhing (by 57.3 %; Fig. 2).

In another set of experiments, a lower SA dose (1  $\text{mg}/\text{kg}$ , i.p.) was able to induce a maximum effect in the writhing test. The possible mechanism of action involved in the antinociceptive response of SA was evaluated by subcutaneously treating animals with an  $\alpha 2$ -adrenergic antagonist (yohimbine; 5  $\text{mg}/\text{kg}$ ), serotonergic and dopaminergic receptor antagonists (metoclopramide; 1  $\text{mg}/\text{kg}$ ), and a selective  $\text{K}^+$ ATP channel antagonist (glibenclamide; 5  $\text{mg}/\text{kg}$ ) 15 min before the administration of SA. As shown in Fig. 3, pretreatment with metoclopramide or yohimbine produced no significant changes in SA-induced antinociception (1  $\text{mg}/\text{kg}$ ), while pretreatment with glibenclamide resulted in full impediment of the antinociceptive effect of SA. Additionally, ketanserin ( $22.0 \pm 0.7$  writhes) and naloxone ( $22.7 \pm 2.2$  writhes) were unable to reverse the antinociceptive effects of SA ( $28.0 \pm 2.0$  writhes) compared to saline-treated mice ( $40.2 \pm 3.3$



**Fig. 1** Chemical structure of siaresinolic acid (SA)

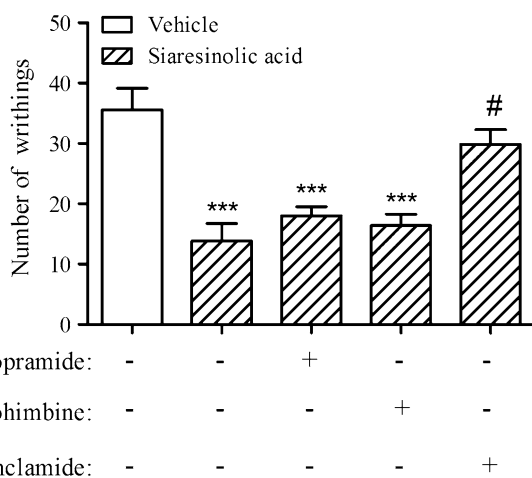


**Fig. 2** Effects of SA on acetic acid-induced writhing. Mice were treated with SA (0.01, 0.1, 1, and 10 mg/kg) or vehicle (5 % ethanol in saline; control group) by the intraperitoneal (i.p.) route 1 h before acetic acid 0.8 % (injected at time zero). Indomethacin (10 mg/kg, i.p.) was the positive control. Data are expressed as mean  $\pm$  standard error of the mean (SEM);  $n = 6$  mice per group. \*\* $p < 0.01$  and \*\*\* $p < 0.001$  indicate statistically significant differences compared to the vehicle-treated group

writhes). Individually, glibenclamide ( $31.0 \pm 1.4$  writhes), metoclopramide ( $33.0 \pm 2.7$  writhes), yohimbine ( $31.7 \pm 1.7$  writhes), ketanserin ( $37.3 \pm 3.1$  writhes), and naloxone ( $39.8 \pm 2.4$  writhes) did not alter the nociceptive behaviors induced by acetic acid ( $31.5 \pm 1.8$  writhes).

#### Formalin-induced nociception

As shown in Table 1, intraplantar injection of 2.5 % formalin evoked a characteristic biphasic licking response. The duration of licking for the first phase (0–5 min,



**Fig. 3** Effect of metoclopramide (1 mg/kg, i.p.), yohimbine (5 mg/kg, i.p.), and glibenclamide (5 mg/kg, i.p.) against the antinociceptive action of SA (1 mg/kg) in acetic-induced writhing in mice. Data are expressed as mean  $\pm$  SEM;  $n = 6$  mice per group. \*\*\* $p < 0.001$  and # $p < 0.01$  indicate statistically significant differences compared to the vehicle-treated group and the SA-treated group, respectively. Under the graph, the signs + and – indicate the presence or absence of the respective treatment

neurogenic) was  $49.4 \pm 1.1$  s and for the second phase (15–30 min, inflammatory) it was  $234.3 \pm 9.2$  s in the control groups treated with the vehicle. Pretreatment of animals for 1 h with SA (1 mg/kg, i.p.) had no significant effect on the licking activity duration in the first phase ( $57.6 \pm 3.5$  s), while in the second phase, the SA-treated mice showed a significant reduction ( $186.0 \pm 22.6$  s) in the licking activity induced by nociceptive stimuli. The reference drug indomethacin (10 mg/kg) significantly inhibited pain only in the second phase ( $98.7 \pm 15.1$  s).

#### Hot plate test

Intraperitoneal administration of SA (1 mg/kg) was not effective in blocking the nociceptive response to thermal stimuli ( $8.8 \pm 0.8$  s) when compared to the saline-treated group ( $7.0 \pm 0.8$  s). On the other hand, morphine (5 mg/kg, i.p.; positive control) significantly increased the response latency ( $18.9 \pm 1.4$  s).

#### Evaluation of motor activity

In the rota-rod test, mice in the vehicle group remained on the rota-rod apparatus for  $205.3 \pm 37.9$  s in a period of 1 h. This performance was not affected by the administration of SA (1 mg/kg, i.p.), in which case the animals remained on the rota-rod for  $226.0 \pm 45.6$  s, whereas diazepam (10 mg/kg, i.p.) pretreatment significantly changed the motor response of the animals, decreasing the time spent on the rota-rod by 68 % ( $66.2 \pm 15.3$  s).

#### Carrageenan-induced pleurisy

The intrapleural injection of carrageenan produced an acute inflammation within 4 h characterized by plasma leakage and considerable leukocyte migration, represented by neutrophils and mononuclear cells (Fig. 4a–d). The administration of SA (0.01, 0.1, and 1 mg/kg, i.p.) 1 h prior to carrageenan significantly decreased the total cell count and number of neutrophils, but not the mononuclear cell count (Fig. 4a, b, d). The same doses of SA also reduced plasma leakage (Fig. 4c).

A substantial increase in TNF- $\alpha$ , IL-1 $\beta$ , and CXCL1 production was found in pleural exudates collected from mice 4 h after carrageenan injection (Table 2). At a dose of 1 mg/kg, SA significantly reduced the levels of TNF- $\alpha$ , IL-1 $\beta$ , and CXCL1 with inhibitions of 82.3, 95.7, and 89.3 %, respectively (Table 2).

#### Evaluation of cytotoxicity

Its direct effect on cell viability in vitro revealed that SA (1, 10, 100, and 200  $\mu$ g/mL) did not cause significant

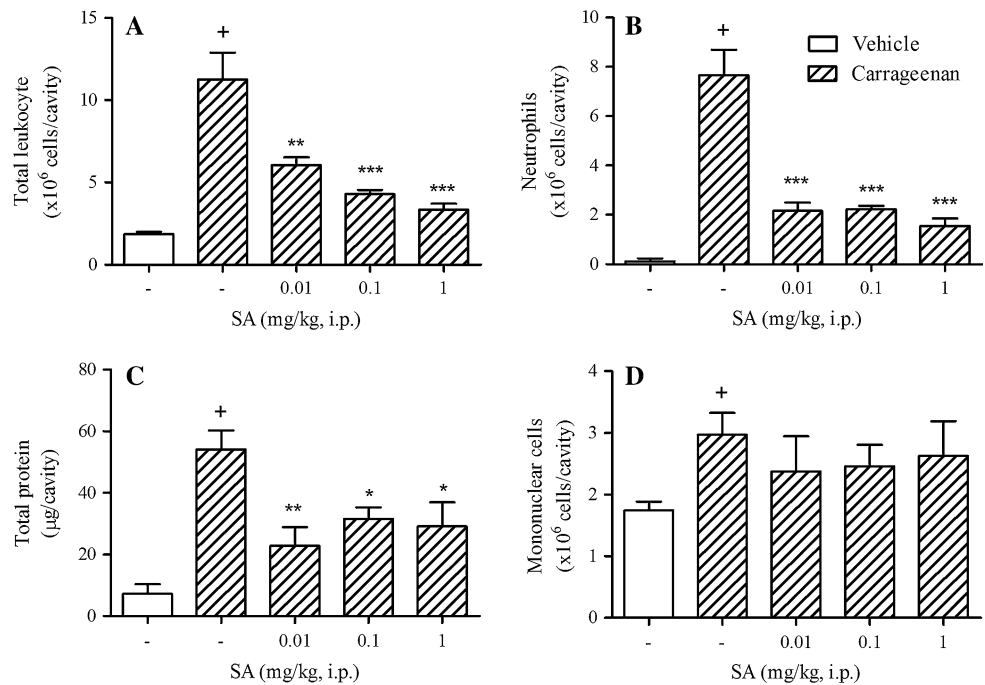
**Table 1** Effect of siaresinolic acid (SA) on formalin-induced nociception in mice

Groups (dose in mg/kg)	Linking time (s)	
	First phase (0–5 min)	Second phase (15–30 min)
Vehicle	49.4 ± 1.1	234.3 ± 9.2
SA (1 mg/kg)	57.6 ± 3.5	186.0 ± 22.6 <sup>+</sup>
Indomethacin (10 mg/kg)	48.5 ± 2.3	98.7 ± 15.1 <sup>+++</sup>

Treatments were administered (intraperitoneally, i.p.) 1 h before the formalin test. Each data represents the mean ± standard error of the mean (SEM); n = 6 mice per group

<sup>+</sup> p < 0.05 and <sup>+++</sup>p < 0.001 indicate statistically significant differences compared to the respective vehicle-treated group

**Fig. 4** Effects of treatment with SA on the inflammation induced by carrageenan in the mouse pleurisy model. Effects of SA (0.01, 0.1, and 1 mg/kg, i.p.) upon total leukocytes (a), neutrophils (b), total protein (c), and mononuclear cells (d). The open bars represent animals that were stimulated by vehicle (saline, control group). The hatched bars represent animals that were stimulated by carrageenan. Data are expressed as mean ± SEM; n = 6 mice per group. <sup>+++</sup>p < 0.001 indicate statistically significant differences compared to vehicle-treated groups. \*p < 0.05, \*\*p < 0.01, and <sup>\*\*\*</sup>p < 0.001 indicate statistically significant differences compared to carrageenan-stimulated groups



cytotoxic effects on murine peritoneal macrophages after a 24-h exposure (96.1 ± 0.2, 96.1 ± 0.2, 98.0 ± 0.4, 94.4 ± 0.2 % of RPMI control, respectively). This finding clearly indicates that SA treatment did not affect the mitochondrial reduction of MTT to formazan, resulting in no detection of any cytotoxic effect.

**Discussion**

In the present study, we demonstrated that systemic administration of SA, a pentacyclic triterpene isolated from *S. grisea* var. *grisea*, significantly inhibited nociceptive behavior and acute inflammatory responses in mice. The possible involvement of ATP-sensitive potassium channel receptors in SA-induced antinociception in mice was also demonstrated. Initially, the effects of SA on peripheral nociception were determined using the acetic acid-induced writhing model, which is frequently used to estimate both

**Table 2** Effect of SA on the protein levels of tumor necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β), and chemokine (C-X-C motif) ligand 1 (CXCL1) in the carrageenan-induced pleurisy in mice

Groups	TNF-α (pg/mL)	IL-1β (pg/mL)	CXCL1 (pg/mL)
Vehicle	19.5 ± 11.4	20.3 ± 1.0	36.0 ± 3.8
Cg	164.5 ± 50.9 <sup>++</sup>	350.8 ± 91.6 <sup>++</sup>	656.0 ± 55.6 <sup>+++</sup>
SA + Cg	9.3 ± 1.4 <sup>**</sup>	81.5 ± 7.3 <sup>**</sup>	61.7 ± 11.4 <sup>***</sup>

Each data represents the mean ± SEM; n = 6 mice per group. Vehicle = animals that received injection of vehicle (saline) in the cavity; Cg = carrageenan; SA = siaresinolic acid (1 mg/kg)

<sup>++</sup> p < 0.01 and <sup>+++</sup>p < 0.001 indicate statistically significant differences compared to vehicle-treated group

<sup>\*\*</sup>p < 0.01 and <sup>\*\*\*</sup>p < 0.001 indicate statistically significant differences compared to Cg group

the central and peripheral analgesic effects of drugs [27]. The acetic acid-induced writhing test has been associated with an increased level of prostaglandins and cytokines in peritoneal fluids [28]. These mediators induce abdominal

constrictions by activating and sensitizing peripheral chemosensitive nociceptors, which are largely associated with the development of inflammatory pain [29]. The administration of SA clearly showed a dose-related antinociceptive effect when assessed in the writhing test, with greater effectiveness than indomethacin [a nonsteroidal anti-inflammatory drug (NSAID) with a broad analgesic spectrum]. This analgesic potential might be related to the inhibition of prostanoids, suggesting that the antinociceptive effect of SA could be associated with an anti-inflammatory action. Additionally, different triterpenoids have been found to be antinociceptive and anti-inflammatory agents due to their ability to inhibit arachidonic acid metabolism [30, 31].

In the present study, an attempt was made to pharmacologically characterize and investigate the possible pathways involved in the analgesic effect of SA. The involvement of neurotransmitter systems, such as the dopaminergic and adrenergic systems, as well as ATP-gated potassium channels, were evaluated using the acetic acid-induced writhing test. The roles of these receptors in regulating the modulation of nociceptive processing have been demonstrated in several previous studies [32–34]. In the present study, metoclopramide, a D2-receptor antagonist, failed to alter the antinociceptive effect of SA. We also noted that yohimbine, an  $\alpha$ 2-adrenergic receptor antagonist, failed to interfere with the antinociception induced by SA. These observations seem to eliminate the involvement of the dopaminergic and adrenergic systems in the antinociceptive actions of SA. Other neurotransmission systems able to modulate pain threshold, such as opioid and serotonergic, are not involved in SA antinociception, since the opioid antagonist naloxone and the 5-HT(2A) antagonist ketanserin were unable to prevent the effect of SA. However, antinociception induced by SA was prevented by the administration of glibenclamide, a blocker of the K<sup>+</sup>ATP channel, suggesting that the antinociceptive effect of SA involves ATP-gated potassium channels. Previous reports showed that triterpenoids achieve their effects in pathological conditions by engaging ATP-sensitive potassium channels [35, 36]. Corroborating these data, Longhi-Balbinot and coworkers demonstrated that glibenclamide also reversed the antinociceptive action of the triterpene 3 $\beta$ ,6 $\beta$ ,16 $\beta$ -trihydroxylup-20(29)-ene in mice [37].

Another interesting finding in the present study was the demonstration that SA caused significant antinociception only in the second phase of the formalin test. The formalin test produces a distinct biphasic nociceptive response. A first phase (neurogenic pain), occurring within seconds of formalin injection, is elicited by direct chemical activation of nociceptive primary afferent fibers. A second phase (inflammatory pain), occurs as a result of ongoing activity

in the primary afferents and increased sensitivity of the dorsal horn neurons [28]. Central analgesic drugs, such as morphine, inhibited both phases equally, while peripherally acting drugs, such as NSAIDs, suppressed mainly the second phase [38]. Thus, the inhibition of nociception in the second phase in the formalin test suggests a probable anti-inflammatory action of SA, thus indicating that this triterpene acts peripherally, similarly to NSAIDs, and corroborating the results of the tests for abdominal contortions.

Seeking to dismiss a possible central involvement in the antinociceptive effect of SA, a hot plate assay was conducted. This model is known to evaluate the possible specific central actions by which analgesic effects are exerted through opioid agents acting on supraspinal and spinal receptors [39]. This test, however, is insensitive to NSAIDs such as cyclooxygenase inhibitors [28]. Our results showed that SA did not alter the latency time for reaction responses, indicating that the antinociceptive action of SA occurs via a peripheral rather than a centrally acting mechanism.

Because the antinociceptive effect of SA was evidenced by a change in the nociceptive behavior of mice, caution was taken to discard possible interfering variables. Thus, in order to determine if SA affected motor coordination, mice were exposed to its highest concentration tested and evaluated in the rota-rod test. Our data showed that SA did not alter the motor performance of mice in the rota-rod test, thereby validating the specificity of its antinociceptive effect.

The effects induced by SA on the acute inflammatory response induced by carrageenan were also evaluated. A pleurisy model has been used for decades to induce inflammation and study its mediators, along with the effectiveness of anti-inflammatory agents [40, 41]. Carrageenan is a phlogistic agent that, when injected into the pleural cavity, promotes a severe inflammation, resulting in an intense leukocyte migration and pleural exudate formation caused by protein extravasation. Our data supported a previous study showing that the injection of carrageenan into the pleural cavity of mice elicited an acute inflammatory response [40], characterized by infiltration and accumulation of fluid (edema) containing high levels of polymorphonuclear leukocytes (PMNs). Treatment of the animals with SA attenuated total leukocytes, polymorphonuclear cell counts, and the pleural volume of exudate after carrageenan challenge; a phenomena that may be related to the lower amount of inflammatory mediators at the inflammation site. In addition, macrophages are specialized cells, with a key role in innate and acquired defense process by releases of proinflammatory mediators. Thus, potential toxic effects on this cell can result in severe problems to the patient. Here, we observed that the

concentrations of SA used *in vitro* did not affect the reduction of MTT in murine macrophages.

In the present study, SA significantly attenuated the production of TNF- $\alpha$ , IL-1 $\beta$ , and CXCL1 in the pleural exudates of carrageenan-injected mice. These proinflammatory molecules are released into the pleural exudates of rodents in response to carrageenan [42]. In addition, these mediators can cause chemotaxis, attracting granulocytes and monocytes that, in turn, stimulate production of additional cytokines and other proinflammatory mediators [43]. There are several studies demonstrating that TNF- $\alpha$  and IL-1 $\beta$  are involved in the upregulation of adhesion molecules on the surface of leukocytes [44]. Moreover, CXCL1 is known as a neutrophil chemotactic factor, which promotes neutrophil chemotaxis and degranulation in rodents. Thus, as a result, the anti-inflammatory effect of SA may be due to suppression of cell recruitment and subsequent generation of proinflammatory mediators. Therefore, we cannot rule out the possibility that SA could produce its effects by inhibiting the synthesis of other proinflammatory mediators. However, this proposal still needs to be evaluated.

In summary, the results of the present study show for the first time that SA, isolated from *S. grisea* var. *grisea*, produces peripheral antinociception when assessed in models of nociception in mice. This antinociceptive action seems to be mediated by ATP-gated potassium channels, but not by pathways involving dopaminergic, adrenergic, serotonergic, and opioid receptors, or through motor impairment. In addition, SA showed significant anti-inflammatory effects on carrageenan-induced pleurisy in mice. Based on these results, this compound may hold great promise for treating painful conditions and inflammatory diseases. However, its molecular mechanism remains unknown. The mechanisms of action of SA on cell activation and other proinflammatory mediators will be explored in future studies.

**Acknowledgments** This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo à Pesquisa do Estado de Alagoas (FAPEAL) (Brazil).

**Conflict of interest** There is no conflict of interest among the authors.

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