

Supercritical Extracts from *Arctium lappa* as a Potential Inhibitor for the Activation of Complement System



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ABSTRACT

Arctium lappa is a perennial species of the Asteraceae family originally from Europe and Asia. Considered a weed species in the southern region of Brazil, it is popularly used as a natural anti-inflammatory. The complement system is an important component of the innate immune response. However, its exacerbated activation can lead to harmful conditions like auto-immune and inflammatory disorders. Plants that inhibit the activation of complement can be a promising tool in the treatment of inflammatory diseases. Here, we evaluated the effect of *A. lappa* leaves extracts on the activation of the classical and alternative pathways of complement system. Two extracts were obtained under supercritical conditions using scCO₂ with ethanol as cosolvent, at 313.15K, 15 MPa (E1) and 25 MPa (E2). Classical and alternative activation were evaluated using complement fixation test. Different concentrations of *A. lappa* extracts E1 and E2 showed an inhibitory effect on both complement pathways, and heparin was used as control. The IC₅₀ of E1, E2, and heparin were 28.26, 20.12 and 92.54 µg/mL for classical and 26.12, 27.70, 27.78 µg/mL for the alternative pathway. Results demonstrate that *A. lappa* is a promising complementary treatment for diseases associated with complement activation.

Introduction

The plant *Arctium lappa* L., popularly known as Burdock, originally from Europe and Asia, is a perennial species of the Asteraceae family [1]. Burdock grows in all Brazilian territory with impressive spread. Considered a weed species in the southern region, it is popularly used for the treatment of skin injuries as well as depurative and anti-inflammatory [1, 2]. It is also consumed as infusion, decoction, or externally used as a plaster [3]. Moreover, Burdock has been used in traditional Chinese medicine and in alternative ther-

apies in Europe, Asia, and North and South America [1, 2]. Several parts of this medicinal herb, including, roots, leaves, seeds, and fruits, have different scientifically proven biological activities such as hypolipidemic [4], anti-inflammatory [5, 6], antifungal [6], and antitumor properties [7]. In addition, Holetz *et al.* demonstrated that the extracts of *A. lappa* from plants collected in southern Brazil presented bactericidal activity against Gram-positive and Gram-negative bacteria [2]. The major active compounds isolated from *A. lappa* include tannin, arctigenin, arctiin, beta-eudesmol, caffeic

acid, chlorogenic acid, inulin, trachelogenin 4, sitosterol-beta-D-glucopyran-oxide, lappaol, and diartigenin [1].

The complement system is one of the main effector branches of the innate immune response. It is constituted by more than 35 soluble or cell membrane proteins that can be activated by 3 pathways: classical, alternative, and lectin [8]. While the classical pathway is activated primarily by the recognition of immune complexes [9], the alternative starts with the spontaneous hydrolysis of the component C3. In the lectin pathway, collectins or ficolins recognize microorganisms' carbohydrates, such as mannose and acetylated compounds, inducing its activation [9, 10]. Once complement becomes activated, all pathways culminate with the formation of C3 and C5 convertases, leading to the production of anaphylatoxins and the membrane attack complex (MAC) [8]. As a consequence of complement activation several biological processes take place, including opsonization, phagocytosis, inflammation, leukocyte chemotaxis, release of histamine from mast cells, active leukocyte oxygen species, vasoconstriction, increased vessel permeability, platelet aggregation, and cytolysis, among others [8, 11].

It is well known that complement plays a pivotal role in host defense against pathogens. However, when its activation becomes exacerbated or uncontrolled it can lead to harmful reactions, such as those seen in autoimmune and inflammatory diseases [12]. Therefore, the modulation or inhibition of complement activation is an important target for therapies in the treatment of inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, atypical uremic hemolytic syndrome, Alzheimer's disease, atherosclerosis, paroxysmal nocturnal hemoglobinuria, and psoriasis, among others [13]. Despite the variety of natural products with diverse biological activities, including antiviral, analgesic, antitumor, antiallergic, anti-inflammatory, and immunomodulatory [14], natural compounds with action on complement activation are scarce [13]. Since the activation or imbalance of the complement system may be associated with several inflammatory disorders, the investigation of vegetal species that can act as inhibitors of complement is of clinical relevance.

Previous studies have identified bioactive compounds of *A. lappa* extracted by conventional extraction techniques, such as low pressure methods (Soxhlet), ultrasound, and microwave [15–17]. However, these techniques require the use of high temperatures for long extraction times, which can lead to the loss of thermosensitive compounds. In addition, there is a search for techniques considered unconventional that use lower amounts of solvents, causing less environmental impact and still presenting good yields. Thus, supercritical extraction process is the most appropriate technology to obtain extracts with low residual solvent content and low final toxic potential [18], being a great alternative as presented in previous studies [19, 20]. Considering that the extracts of *A. lappa* leaves obtained from the mixture of supercritical carbon dioxide (scCO₂) as solvent with ethanol as cosolvent showed higher efficiency, the best conditions of overall extraction yield were obtained at 313.15 K and both pressures 15 and 25 MPa, and due to the great antioxidant effect that has already presented [19], these same extracts were chosen to evaluate complement modulation. Therefore, as natural products may represent a complementary approach for the treatment of inflammatory diseases associated with exacerbated complement activity, our aim is to evaluate the effect of

supercritical extracts from *A. lappa* leaves on the activation of the classical and alternative pathways of complement.

Results

The extracts E1 and E2 of *A. lappa* showed inhibitory effect on the activation of both classical and alternative pathways when compared to the positive control normal human serum (NHS), as shown in ► **Fig. 1**. The extract E1 (at concentration 183 µg/mL) inhibited 36.1 and 49.1 % of hemolysis by classical and alternative pathway, respectively. On the other hand, the extract E2 (at concentration 150 µg/mL) inhibited 34.2 and 49.9 % of hemolysis by classical and alternative pathways, respectively. The inhibitory effect of *A. lappa* extracts on the classical and alternative pathways was also observed at lower concentration, such as 22.88 µg/mL and 18.75 µg/mL for E1 and E2, respectively. The effect of both extracts and heparin on the complement activation was compared with the positive control of 50 % of hemolysis.

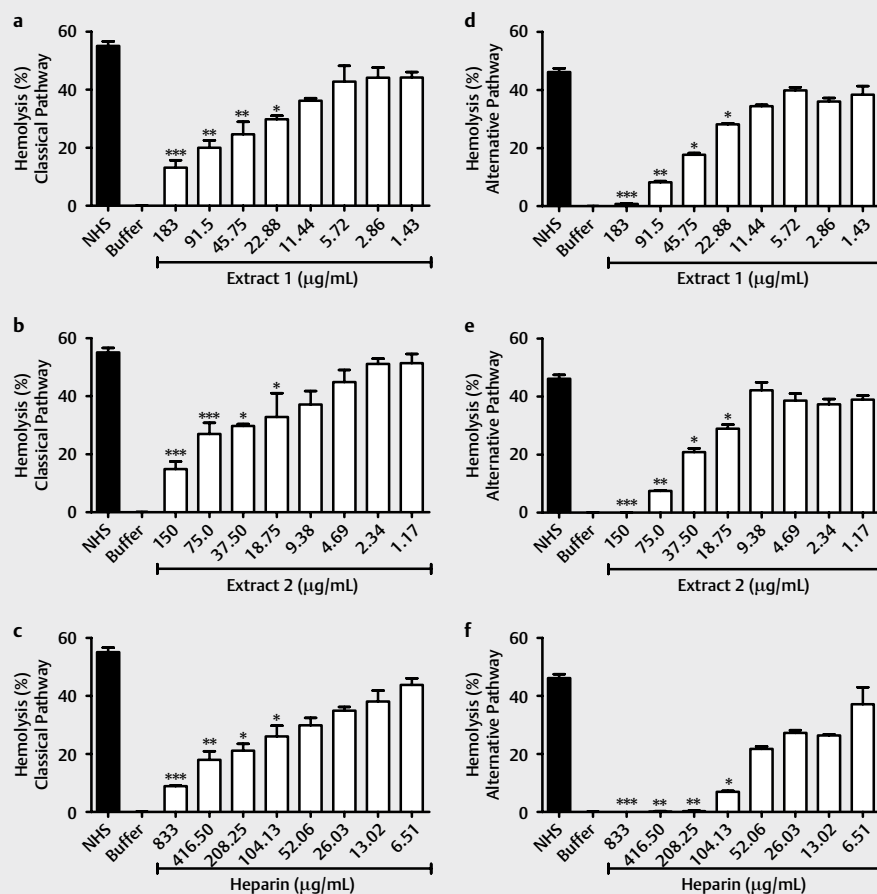
In addition, the half maximal inhibitory concentration (IC₅₀) was 28.26 µg/mL (confidence interval, CI: 22.70–35.17; R²=0.92) for extract E1, 20.12 µg/mL (CI: 13.51–29.95; R²=0.79) for extract E2, and 92.54 µg/mL (CI: 73.29–116.8; R²=0.92) for heparin on the activation of the classical pathway. Likewise, the IC₅₀ was 26.12 µg/mL (CI: 21.34–31.96; R²=0.93) for extract E1, 27.70 µg/mL (CI: 22.56–34.03 R²=0.92) for extract E2, and 27.78 µg/mL (CI: 22.42–34.42; R²=0.94) for heparin on the alternative pathway. These results indicate a dose-dependent inhibitory effect of the extracts E1 and E2 of *A. lappa* on both classical and alternative pathways of complement activation.

Discussion

Supercritical extracts from *A. lappa* leaves were able to inhibit complement activation by both classical and alternative pathways using functional assays (► **Fig. 2**). In addition, this inhibitory effect was observed at different concentrations of *A. lappa* extracts E1 and E2. Thus, our results showed a dose-dependent inhibitory effect for both extracts on the activation of classical and alternative pathways.

Although several biological properties of *A. lappa* have been described [1], our study is the first to describe its action as complement inhibitor. This immunomodulatory activity of *A. lappa* extracts corroborates with the anti-inflammatory actions previously reported such as activation of antioxidant enzymes and scavenging of free radicals [19, 21], inhibition of inducible nitric oxide synthase (iNOS) pathway [22], suppression of pro-inflammatory cytokine expression [22], inhibition of the nuclear factor-kappa B [23], and inhibition of NOD-like receptor pyrin domain-containing-3 (NLRP3) inflammasome activation [5].

It is noteworthy that the inhibitory effect of *A. lappa* extracts was 13 % (extract E1) to 15.7 % (extract E2) higher on the alternative when compared to classical pathway. This difference may be due to the inhibitory effect of the extracts on the initiating molecules, the ions involved (Ca and Mg), or preventing the formation of C3 and C5 convertases, which are specific for each pathway. Thus, the same extract may be more or less potent on different pathways. Furthermore, although the 3 complement pathways are triggered by different signals, the alternative pathway acts as an

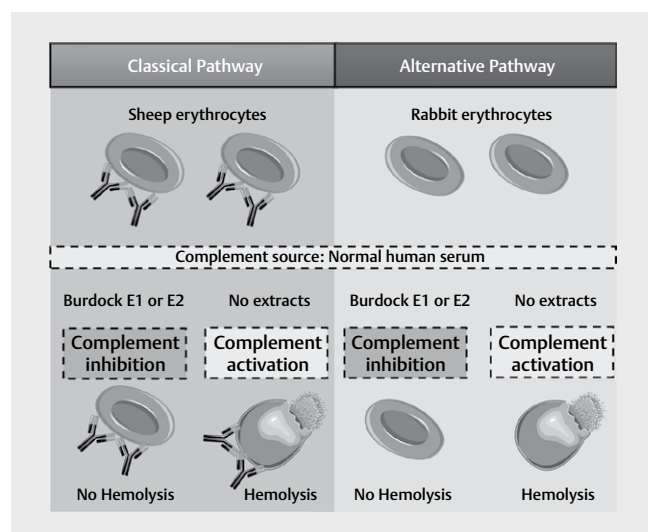


► **Fig. 1** Classical and alternative pathways activation are suppressed by supercritical extracts of *A. lappa*. Activation of classical and alternative pathways is represented from **a–c** and **d–f**, respectively. The final concentrations of both extracts evaluated and of the heparin control are represented in the x-axis. All extracts dilutions were compared with the positive control (SHN diluted to represents 50% of hemolysis) by Kruskal-Wallis with Dunn's multiple comparison as post-test (* * * $p < 0.001$, * $p < 0.01$, and * $p < 0.05$). The negative control (buffer) represents 0% hemolysis. In addition, heparin dilutions were used as control of inhibitory effect on complement activation (**c** and **f**).

amplification loop of all pathways, corresponding to 80–90% of C5 activation and MAC formation when classical pathway is activated [24, 25]. In addition, IC_{50} for the extracts E1 (28.26 $\mu\text{g/mL}$; 26.12 $\mu\text{g/mL}$) and E2 (20.12 $\mu\text{g/mL}$; 27.70 $\mu\text{g/mL}$) were lower or similar to than those found to heparin (92.54 $\mu\text{g/mL}$; 27.78 $\mu\text{g/mL}$) on the activation of the classical and alternative pathway, respectively. These same extracts presented antioxidant activity with an IC_{50} of 424 $\mu\text{g/mL}$ and 383 $\mu\text{g/mL}$, for E1 and E2 [19]. These concentrations are at least 16 times higher than the concentrations found in our study, indicating a potent action of *A. lappa* extracts on complement.

Considering the similar results for the extracts E1 and E2 of *A. lappa* on complement activation assays, the different pressures and times of extraction of both samples do not appear to have affected the inhibitory properties of the extracted compounds. The predominant compounds with biological activity shared by extract E1 and E2 were the triterpenoids lupeol acetate and amyirin acetate and the diterpene phytol. These components have been described as anti-inflammatory substances [26, 27]. Lupeol acetate identified in *Cariniana domestica* (Mart.) Miers family *Lecythidaceae* and *Bride-*

lia retusa (Spreng) family *Euphorbiaceae* presented anti-inflammatory effect in skin inflammation and in adjuvant-induced arthritis models [28, 29]. One of the anti-inflammatory mechanisms of lupeol acetate in arthritis models was the suppression of pro-inflammatory cytokines expression and inhibition of osteoclastogenesis [30]. Furthermore, lupeol acetate and amyirin acetate, detected as main components in extracts of *Cnidioscolus chayamansa* (Mc Vaugh) family *Euphorbiaceae* leaves, presented hepatoprotective and anti-inflammatory activities in chronic models of liver damage [26]. Amyirin acetate isolated from *Alstonia boonei* De Wild family *Apocynaceae* showed significant inhibition in paw and ear edema models. It also reduced total leucocyte migration and neutrophil infiltration *in vivo* tests [31]. Our results corroborate the findings of Jin *et al.* [32], who found that amyirin acetate isolated from the leaves and twigs of *Acer okamotoanum* Nakai family *Aceraceae* exhibited a significant inhibitory effect on activation of the classical pathway. The diterpene phytol was identified as the most abundant compound of *Cleome rutidosperma* DC family *Cleomaceae* aerial essential oil and exhibited antimicrobial activity against pathogenic bacteria and fungus strain [33]. Finally, diterpene phytol was shown



► **Fig. 2** Inhibitory effect of *A. lappa* extracts on functional complement assays. For the hemolytic assays, different dilutions of each extract (E1 and E2) were incubated with normal human serum (as source of complement) and sensitized SE or RE for classical and alternative pathways assays, respectively. Both extracts were able to inhibit the different pathways of complement activation.

also to decrease autoimmune response in both acute and chronic phases of experimental arthritis [27], as well as phytol attenuates the inflammatory response in paw edema model by inhibiting neutrophil migration, reducing inflammatory cytokines levels and oxidative stress [34].

It is important to consider that extracts composition can be influenced by extraction process conditions. Some compounds are more sensitive than others and, depending on the time of exposition to high temperatures or pressures, can be degraded. This may justify the lack of arctigenin in our extracts, a compound identified in fractions of *A. lappa* with an important anti-inflammatory role [35–37]. However, the anti-inflammatory activity observed with E1 and E2 extracts of *A. lappa* may be due to other components, such as lupeol, amyrin, and phytol [31, 34, 38], which were also found in extracts from *A. lappa* leaves obtained by pressurized liquid extraction using aqueous ethanol [20].

The present study also demonstrated that supercritical extraction using CO₂ as solvent results in extracts with important biological activities, as observed on complement activation. Moreover, the number of studies on plant compounds obtained by supercritical extraction has increased significantly in the last years, mainly due to their high applicability in health, herbal medicines, cosmetics, and nutraceutical foods [39]. The solvent CO₂ present some advantages as safety, availability, low cost, nontoxicity in low concentrations, nonflammable, and noncarcinogenic. The pressure and temperatures used in supercritical extractions are relatively low and safe [40]. Nevertheless, the solubility of CO₂ is limited to the nonpolar organic materials, the addition of a small amount of organic solvent, such as ethanol, may increase the solubility of polar compounds [41]. In addition, supercritical extraction is a field of interest for the pharmaceutical industry to exploit new plant species with potential medicinal use [42].

One limitation in our study was the initial concentration from both extracts, since supercritical extraction method resulted in approximately 0.8 grams (3.5 %) from the whole leaf weight (22.8 g) used in the beginning of the extraction process. Thus, although there is a high demand for the substitution of synthetic substances for natural resources, sustainability is based on the abundance and availability of raw materials. As future perspectives, the effects of *A. lappa* extracts on the activation of the lectin pathway should be also considered, as well as the identification of the components responsible for the inhibitory effect on complement.

Considering that activation of the complement plays a central role in the maintenance of inflammation, the search for substances that modulate its activation is of great concern since only Eculizumab and C1 esterase-inhibitor are options approved by the U.S. Food and Drug Administration for complement inhibition [43]. Thus, our results contribute for the better understanding of the pharmacological and medicinal properties of *A. lappa* extracts highlighting its application as a complementary treatment for inflammatory diseases associated with complement activation in future clinical studies.

Materials and Methods

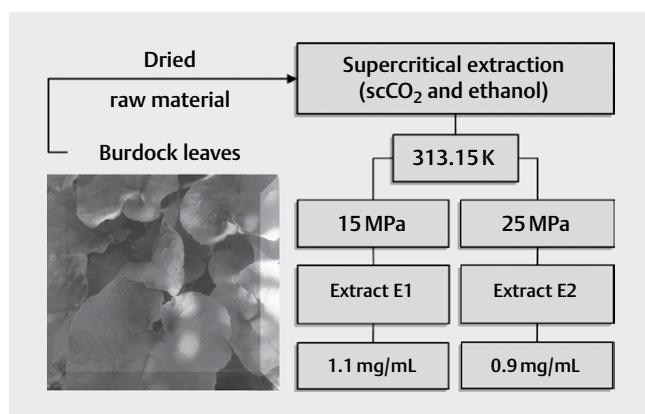
This work was approved by the Human Research Ethics Committee of the Federal University of Paraná (n. 1.703.531, approved in August 30, 2016).

Supercritical extracts

The raw material used in this study comprised of the aerial parts of *A. lappa*. All samples used belong to a single lot harvested in July 2016 from a local property in Ivaiporã (State of Paraná, Southern, Brazil) [19]. The raw material (*A. lappa* leaves) presented a residual moisture of 5.97 ± 0.02 wt %, average particle diameter of $(1.3 \pm 0.4)10^{-3}$ m, and real particle density of 1480 kg m^{-3} , as described in a previous study by Souza *et al.* [19]. The extracts of *A. lappa* leaves were obtained by supercritical extraction using a bench-scale supercritical extraction unit, where CO₂ was used as solvent and ethanol as cosolvent. Two extraction conditions were performed, the extract 1 (E1) was obtained under 15 MPa in 50 min, the extract 2 (E2) under 25 MPa in 75 min (► **Fig. 3**). The temperature was fixed at 313.15 K for both extracts. The extracts were analyzed by gas chromatography-mass spectrometry (Agilent 7890A/5975C GC-MS system), and the phytochemical compounds profile were identified by Souza *et al.* [19] as shown in ► **Table 1**. We used the same samples described and evaluated by Souza *et al.* [19] to perform the complement assays.

Complement assays

Classical and alternative complement pathways activity were assessed by complement fixation tests adapted from Alban *et al.* [44]. HEPES buffer solution (HEPES 10 mM, 150 mM NaCl, pH 7.4) (HB) supplemented with 0.11 mM CaCl₂ and 0.5 mM MgCl₂ (HBC), or 7 mM MgCl₂ and 10 mM EGTA pH 7.4 (HBA) were used to evaluate the activation of classical and alternative pathways. The extracts E1 and E2 were dissolved in the minimum amount of solvent (HB with an addition of 20 % DMSO) capable of promoting their complete solubilization. The final concentration obtained for the extracts E1 and



► **Fig. 3** Supercritical extracts from *A. lappa* leaves. The extracts of *A. lappa* leaves were obtained by supercritical extraction using CO₂ as solvent and ethanol as cosolvent. Two extraction conditions were performed: the extract 1 (E1) was obtained under 15 MPa in 50 min and the extract 2 (E2) under 25 MPa in 75 min. The temperature was fixed at 313.15 K for both extracts. The extracts were dissolved in the minimum amount of solvent capable of promoting their complete solubilization.

► **Table 1** Chemical composition of *A. lappa* leaves extracts.

Compounds	Relative (%)	
	Extract 1	Extract 2
Pyranone	0.45	0.28
Decanoic acid	0.62	nd
β -Eudesmol	0.26	0.26
Methyl palmitate	0.45	0.43
1-(3-Methyl-cyclopent-2-enyl)-cyclohexene	1.38	1.50
Hexadecanoic acid ethyl ester	0.78	0.74
α -Phytol	4.12	4.00
9-Octadecenoic acid methyl ester	0.59	0.57
Methyl stearate	1.84	1.78
Linolenic acid ethyl ester	0.21	0.21
9-12-Octadecadienoic acid ethyl ester	1.58	1.15
3-Ethyl-5-(2'ethyl)octadecane	nd	0.98
Palmitic acid β -monoglyceride	0.49	0.37
Diisooctyl phthalate	26.108	29.42
Methyl-7,10,13,16-docosatetraenoate	2.01	1.54
Squalene	3.17	0.75
Vitamin E	1.00	1.02
Stigmasterol	0.397	0.41
λ -sitosterol	1.6	1.57
β -amyirin	1.30	1.29
α -amyirin	0.66	0.40
9-19-Cyclolanostane-3,7-diol	nd	0.42
Stigmast-5-em-3-yl acetate	nd	nd
Amyirin acetate	13.69	15.19
Lupeol acetate	17.37	18.23

Source: adapted from Souza *et al.* [16]; nd: not detected.

E2 were 1.1 mg/mL and 0.9 mg/mL, respectively. Different dilutions of heparin were used as negative control of hemolysis [45].

Normal human serum

A pool of NHS was prepared from freshly collected peripheral blood obtained from 10 healthy volunteers at the Clinical Analysis Laboratory of Federal University of Paraná and used as complement source. The blood was maintained under refrigeration (275.15–281.15 K) until total coagulation (15–30 min), centrifuged (1440 g, 20 min, 285.15 K), and the supernatant collected, pooled, and stored at 193.15 K until further use.

Preparation of sheep and rabbit erythrocytes suspensions

In order to analyze the activation of classical and alternative pathways, sheep (classical) and rabbit (alternative) red blood cells samples were commercially purchased (Kalifarma). Both erythrocytes were centrifuged (1500 g, 5 min), the supernatant removed, and the red blood cells washed 3 times with saline (0.9 % NaCl). Sheep erythrocytes (SE) were also washed once with HBC and rabbit erythrocytes (RE) with HBA. After the last centrifugation, SE were separated and resuspended in HBC buffer (2.4 % vol./vol. cell suspension) and sensitized with equal volume of anti-sheep erythrocyte antibodies (1:2000, Kalifarma) for 30 min at 310.15 K. Thus, suspensions of SE (1.2 % vol./vol.) and RE (2.4 % vol./vol.) were used for the classical and alternative hemolytic assays, respectively.

Classical and alternative pathways hemolysis assays

For the hemolytic assays, 20 μ L of the different dilutions of each extract (E1 and E2) were incubated with 50 μ L of NHS (equivalent to 50 % of hemolysis) and 50 μ L of sensitized SE or RE for classical and alternative pathways assays, respectively, for 30 min at 310.15 K. In addition, the following controls were included in the classical and alternative hemolysis assays: (a) 70 μ L of ultra-pure water and 50 μ L of SE or RE previously sensitized, corresponding to 100 % hemolysis, (b) 20 μ L of HBC or HBA, 50 μ L of NHS and 50 μ L of SE or RE, corresponding 0 % hemolysis, and (c) 20 μ L of HBC or HBA, 50 μ L of NHS and 50 μ L of SE or RE, corresponding to 50 % hemolysis. Samples and controls were centrifuged at 1500 g for 5 min (Centrifuge 5430, Eppendorf). The supernatant was transferred to a 96-well flat bottom plate and the absorbance read at 405 nm in a spectrophotometer (Biotek Instruments ELx800). All the extracts were previously tested for hemolytic activity in the same concentrations used in all experiments. None of them presented detectable hemolysis, being both considered nonhemolytic. All the experiments were executed in triplicate. The results were expressed as the percentage of hemolysis obtained compared with control samples adapted from Alban *et al.* [44].

Statistical analysis

All experiments were performed in triplicate and the results expressed as mean and standard deviation. Data were analyzed by Kruskal Wallis with Dunn's multiple comparison as post-test (** * $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$) and the IC₅₀ was calculated using GraphPad Prism 5 software.

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Conflict of Interest

The authors declare no conflict of interest.

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