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Effects of *Uncaria tomentosa* and *Uncaria guianensis* aqueous extract on enzyme activity and modulation of hemostasis

Efeitos do extrato aquoso de *Uncaria tomentosa* e *Uncaria guianensis* na atividade enzimática e modulação da hemostasia

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The species *Uncaria tomentosa* and *Uncaria guianensis* are medicinal plants rich in bioactive compounds with anti-inflammatory properties. The objectives of this study were to evaluate the potential of these herbal medicines in modulating phospholipases A2 and proteases (involved in hemostasis and inflammation), using *Bothrops moojeni* venom as a study model. To achieve this, the plant extracts were characterized for their phenolic composition by chromatography, and the action of the extracts was evaluated through plate inhibition assays. The phospholipase activity induced by the venom of *B. moojeni* was significantly inhibited by both extracts, ranging from 10 to 18% and 10 to 46% for the extracts *U. tomentosa* and *U. guianensis*, respectively. The greatest inhibitory action of hemolysis was observed for the extract of *U. guianensis*, when incubated with *B. moojeni* venom, with results between 14 and 60%. In the thermal hemolysis assay, the highest doses tested exerted a greater protective effect on erythrocyte membranes. The proteolytic activity was significantly inhibited, showing results from 10 to 27% for *U. tomentosa* and 10 to 40% for *U. guianensis*. For thrombolytic activity, significant inhibition was observed only at the highest dose of *U. guianensis* (53%) and controls containing only the extracts showed thrombolytic action. Both extracts were able to prolong the clotting time induced by *B. moojeni* venom. The results demonstrate that the extracts act as modulators on phospholipases A_2 and proteases. Thus, these herbal medicines are promising alternatives in the treatment of inflammatory diseases and hemostatic disorders.
Keywords: anti-inflammatories, hemostasis modulation, enzyme inhibitors.

As espécies *Uncaria tomentosa* e *Uncaria guianensis* são plantas medicinais ricas em compostos bioativos com propriedades anti-inflamatórias. Os objetivos desse estudo foram avaliar o potencial desses fitoterápicos em modular fosfolipases A_2 e proteases (envolvidas na hemostasia e inflamação), utilizando a peçonha de *Bothrops moojeni* como modelo de estudo. Para isso, o extrato das plantas foi caracterizado quanto a sua composição fenólica por cromatografia e a ação dos extratos foi avaliada em testes de inibição em placa. A atividade fosfolipásica induzida pela peçonha de *B. moojeni* foi significativamente inibida por ambos os extratos, variando de 10 a 18% e 10 a 46% para os extratos de *U. tomentosa* e *U. guianensis*, respectivamente. A maior ação inibitória da hemólise foi observada para o extrato de *U. guianensis*, quando incubado com a peçonha de *B. moojeni*, com resultados entre 14 e 60%. No ensaio de hemólise térmica, as doses mais elevadas testadas exerceram maior efeito protetor nas membranas eritrocitárias. A atividade proteolítica foi significativamente inibida, apresentando resultados de 10 a 27% para *U. tomentosa* e 10 a 40% para *U. guianensis*. Para a atividade trombolítica, foi observada inibição significativa apenas na dose mais elevada de *U. guianensis* (53%) e os controles contendo apenas os extratos apresentaram ação trombolítica. Ambos os extratos foram capazes de prolongar o tempo de coagulação induzido pela peçonha de *B. moojeni*. Os resultados demonstram que os extratos atuam como moduladores de fosfolipases A_2 e proteases. Assim, esses fitoterápicos são alternativas promissoras no tratamento de doenças inflamatórias e distúrbios hemostáticos.
Palavras-chave: anti-inflamatórios, modulação da hemostasia, inibidores enzimáticos.

1. INTRODUCTION

Phytotherapics are obtained from medicinal plants and represent a viable alternative in the treatment of various pathologies [1]. Currently, they are the focus of studies, as they are a promising source of compounds with biological activity. Their use has advantages such as relatively low cost, accessibility, and generally fewer side effects. In this sense, the search for therapeutic agents with fewer adverse effects has drawn attention to medicinal plants with anti-inflammatory properties [2, 3].

The species *Uncaria tomentosa* (Willd.) DC. and *Uncaria guianensis* (Aubl.) Gmel. belong to the botanical family Rubiaceae. They are popularly known as cat's claw and are commonly found in tropical forests of South and Central America. These plants have been used in traditional indigenous medicine for over 2,000 years [4] due to their anti-inflammatory properties, helping in the symptomatic treatment of acute joint and muscle pain. They also act as immunostimulants, antiviral and antitumor agents[5-7]. Due to its preeminent biological activity, *U. tomentosa* has been incorporated into the Brazilian Unified Health System (SUS) as a phytotherapic [8].

Uncaria tomentosa and *U. guianensis* have several bioactive compounds responsible for their pharmacological properties, especially pentacyclic alkaloids, tetracyclic alkaloids, polyphenolics, flavonoids, triterpenes, and saponins [9]. Several *in vitro* and *in vivo* tests with extracts of *U. tomentosa* and *U. guianensis* confirmed their antioxidant, anticancer, anti-inflammatory, antidiabetic, antimicrobial, immunostimulant, and anti-Parkinson effects [10]. Although some of the applications of these species are related to blood physiology [11], there is little scientific evidence that addresses their modulatory effect on hemostasis-related processes.

In this sense, the present study aimed to evaluate the effects of aqueous extracts of *U. tomentosa* and *U. guianensis* on hemostasis and enzymatic activity exerted by phospholipases A_2 and proteases, using snake venom of the species *Bothrops moojeni* to prospect for effects of extracts on the human body.

2. MATERIALS AND METHODS

2.1 Snake venom and chemical compounds

Uncaria tomentosa (Florien brand) and *Uncaria guianensis* (Purifarma brand) were obtained from compounding pharmacies in Lavras, Brazil. The herbal medicine of *U. tomentosa* is from the brand Florien, internal batch 19G15-FL00-000011, manufacturer batch NPT.0219/147. The herbal medicine of *U. guianensis* is from the brand Purifarma, internal batch PURI013952, manufacturer batch 23/5. For both medicines, the part used was the bark from the vine. Aqueous extracts were prepared according to Simão et al. (2015) [12]. The samples were solubilized in ultrapure water, at a ratio of 1:25 (w/v), then incubated in a thermostatic bath at 36 °C under 60 rpm for 24 hours. The aqueous extract was centrifuged (FanemBaby®I Model 206 BL) at 2000 rpm for 30 minutes, and the supernatant was collected and stored at -20ºC. The extract consisted of a final concentration of 40 µg/µL.

The crystallized venom of *Bothrops moojeni* was commercially obtained from the Bioagentes serpentarium located in Batatais, São Paulo. The venom was weighed (10 mg) and dissolved in 1 mL of phosphate-buffered saline (PBS, pH 7.4). All venom concentrations were previously established for each activity tested based on pilot assays. The use of snake venom as a laboratory research tool was registered in SisGen under the number ADF95EA.

Our assays used synthetic anti-inflammatory drugs prednisolone and diclofenac resinate as positive controls. Both chemicals were purchased in Lavras, Minas Gerais. Diclofenac resinate, a non-steroidal anti-inflammatory drug (NSAID), was produced by Biosintética in the concentration of 3 mg/mL. Prednisolone, a steroidal anti-inflammatory drug (SAID), was produced by CIMED in a concentration of 15 mg/mL.

2.2 Identification and quantification of phenolic compounds

The chromatographic analyses were carried out at the Central Laboratory of Chemical Analysis and Prospecting of the Federal University of Lavras. A Shimadzu HPLC equipment was used, equipped with two LC-20AT high-pressure pumps, a UV-visible detector model SPD-M20A, a CTO-20AC oven, a CBM-20A interface, and an automatic injector with a SIL-20A sampler. Separations were performed using a Shim-pack VP-ODS-C18 column (250 mm x 4.6 mm) connected to a Shim-pack Column Holder pre-column (10 mm x 4.6 mm). The aqueous extracts of *U. tomentosa* and *U. guianensis*, and the phenolic standards were filtered through a 0.45 µm membrane (Millipore®) and injected into the chromatograph. Compounds were identified by comparing retention times to standards. The standards used were: gallic acid, catechin, chlorogenic acid, caffeic acid, vanillic acid, *p*-coumaric acid, ferulic acid, *m*-coumaric acid, *o*-coumaric acid, resveratrol, and trans-cinnamic acid. Quantification was performed through the construction of analytical curves obtained by linear regression, considering the coefficient of determination (R^2) of 0.99 [13].

2.3 Obtaining human blood

The blood used for the tests was obtained from volunteers, of both sexes aged between 20 and 40 years old, who declared that they had not used medication for a period of 30 days before collection. Blood was collected by venipuncture into tubes containing citrate for coagulant activity, heparin for anti-inflammatory and hemolytic activity, and tubes without anticoagulants were used for the thrombolytic activity. All experiments were previously approved by the Ethics Committee for Research with Human Beings (COEP) of the Federal University of Lavras, under registration number (Nº CAAE/41967420.0.0000.5148).

2.4 Phospholipase and hemolytic activities in a solid medium

Phospholipase and hemolytic activities were evaluated in a solid medium, as described by [14]. The gel to evaluate the phospholipase activity was prepared with 0.01 mol/L $CaCl_2$, egg yolk phospholipids 1:3 (v/v), PBS (pH 7.4), 1% bacteriological agar and 0.005% sodium azide. The solution was poured into Petri dishes at 50 ºC. After the gel solidified, the treatments were applied in holes of 0.4 cm in diameter made in the gel, to hold a final volume of 30 µL. The plates were kept in a cell culture chamber for 24 hours at 37°C. Phospholipase assay was performed using *B. moojeni* venom, in which the minimum dose (10 µg) of venom was previously incubated with aqueous extracts of the herbal medicines for 30 minutes at 37°C using doses of 5; 10; 25; 50; 100; 200; 500 µg.

For hemolytic activity, the gel was prepared by replacing the phospholipids with a concentrate of erythrocytes. To obtain the cells, freshly collected blood was centrifuged at 2000 rpm for 5 minutes. Plasma was removed and red blood cells were suspended in PBS (pH 7.4) and centrifuged under the same conditions, this washing step is repeated twice. Inhibition of hemolytic activity was evaluated using *B. moojeni* venom (30 µg), under the same conditions previously mentioned.

Both activities were evaluated by measuring, in millimeters, the diameter of the translucent halo formed around the holes in the gels, and the results were converted into a percentage of activity. Controls were also carried out with prednisolone (SAID) and diclofenac resinate (NSAID) at doses of 5 and 10 µg. Controls containing only venom were considered to have 100% activity.

2.5 Hemolytic activity in liquid medium

The thermal hemolysis assay was performed according to the protocol described by Nkeh-Chungag et al. (2014) [15]. Blood was collected and centrifuged (FanemBaby®I Model 206 BL) at 3600 rpm for 5 minutes. Plasma was discarded and red blood cells were suspended in

PBS (pH 7.4) and centrifuged under the same conditions, this washing step is repeated twice. A 2% (v/v) suspension of erythrocytes was prepared in PBS (pH 7.4). The treatments (200 µl) at different doses (8000; 4000; 2000 µg) were added to 1200 µL of the erythrocyte suspension and incubated in a thermostatic bath for 30 minutes at 37 ºC. The incubates were centrifuged (Corning® LSE™ Model 6765-HS) at 1200 g for 10 minutes, the supernatant was collected and evaluated in a spectrophotometer (Biochrom Libra S12) at 540 nm. Then, the treatments were incubated for another 20 minutes at 54 ºC, centrifuged and the product was quantified in a spectrophotometer at 540 nm. Positive and negative controls consisted of distilled water and PBS, respectively. Prednisolone and diclofenac resinate were also evaluated at a concentration of 50 µL/mL. The positive control (distilled water) was considered 100% thermal hemolysis. The negative control (PBS) corresponded to mechanically induced hemolysis during the tests.

2.6 Proteolytic activity on casein

To evaluate this activity, the methodology described by Gutiérrez et al. (1988) [14] was used, with the replacement of phospholipids by casein solution at the same concentration described by Wang et al. (2004) [16]. The casein solution was at a concentration of 5 mg/mL, in 50 mmol/L Tris-HCl buffer (pH 8.0).

Bothrops moojeni venom (10 µg) and extracts of *U. tomentosa* and *U. guianensis* were incubated at doses of 5; 10; 25; 50; 100; 200; 500 µg for 30 minutes at 37°C. Then, the samples were applied to the holes made in the gel, in a final volume of 30 µL. The plates were kept in a cell culture chamber for 18 hours at 37°C. After this period, the gel was subjected to staining with 1% black starch solution for 10 minutes, followed by bleaching with 10% acetic acid for 30 minutes, allowing the measurement of the diameter of the translucent halos formed. The results were expressed as a percentage, where controls containing only venom were considered to have 100% proteolytic activity.

2.7 Thrombolytic activity

The thrombolytic activity was evaluated in human blood clots formed *in vitro* according to the methodology described by Cintra et al. (2012) [17]. Blood collected without the presence of anticoagulant was immediately distributed (100 µL) into wells of a 96-well microplate and allowed to clot for 20 minutes. On the clots, treatments were added (final volume of 30 µL per sample/well) corresponding to PBS (negative control), *B. moojeni* venom (30 µg), controls containing only the aqueous extracts of *U. tomentosa* and *U. guianensis*, and venom previously incubated with the extracts at doses of 15; 30; 75; 150; 300; 600; 1500 µg for 30 minutes at 37°C. The microplates were kept in a cell culture chamber for 24 hours at 37 °C.

The thrombolytic activity was estimated by measuring the volume of fluid released by each thrombus. The average of the volume obtained in the negative control (PBS) was subtracted from the other treatments. The total reaction volume (130 µL) was considered 100% lysis.

2.8 Coagulant activity

The assessment of the clotting time of citrated human plasma was performed as described by Rodrigues et al. (2000) [18]. The aqueous extracts were previously incubated for 10 minutes at 37 ºC with the venom of *B. moojeni* (10 µg), at the final reaction doses of 50, 100, 200, 500 µg. Tubes containing citrated plasma (200 µL) were kept in a thermostatic bath at 37 °C. The incubated samples were added to the plasma and the time until a hard clot formed was recorded. Controls containing only venom were also performed. The minimum coagulant dose was previously established, corresponding to the smallest amount of venom capable of inducing coagulation in a period between 50 and 120 seconds [19].

2.9 Statistical analysis

Results were presented as mean ± standard deviation of three replicates. Then, the normality of the data was evaluated by the Shapiro-Wilk test. Data were compared by Analysis of Variance (ANOVA), followed by Dunnett's *post-hoc* test ($p< 0.05$) using the GraphPad v9 software (La Jolla, CA, USA).

3. RESULTS AND DISCUSSION

3.1 Phenolic compounds

The phenolic compounds content of the aqueous extracts of *U. tomentosa* and *U. guianensis* are shown in Table 1. The extract of *U. guianensis* showed a higher content of phenolic compounds than that obtained for *U. tomentosa*. The extracts also showed different constituents between the two species. *U. tomentosa* extract had the highest caffeic acid content, followed by *p*-coumaric acid and catechin. However, *U. guianensis* extract showed the highest content of chlorogenic acid, followed by *p*-coumaric acid and *m*-coumaric acid. Our results are consistent with a study that investigated the chemical differences between the stem bark extract of both species [20].

Table 1: Content of phenolic compounds in the aqueous extracts of Uncaria tomentosa and Uncaria guianensis.

Phenolic compounds (mg/100g)	***U. tomentosa***	***U. guianensis***
gallic acid	4.88 ± 0.11	-
catechin	5.33 ± 0.02	91.10 ± 0.51
chlorogenic acid	5.24 ± 0.05	237.04 ± 2.11
caffeic acid	39.02 ± 0.80	11.02 ± 0.17
vanillic acid	2.37 ± 0.05	9.02 ± 0.21
p-coumaric acid	10.33 ± 0.03	144.77 ± 0.46
ferulic acid	nq	130.93 ± 0.34
m-coumaric acid	-	134.33 ± 0.22
o-coumaric acid	-	86.51 ± 0.37
trans-cinnamic acid	0.73 ± 0.00	-
∑ phenolic compounds	**67.9**	**844.72**

Phenolic compounds expressed as equivalent milligrams of each compound per 100g of herbal medicine (mg/100g). The data correspond to means of triplicates and the standard deviation. nq= not quantified (the compound was identified but was below the quantification limit).

The phenolic compounds present in *Uncaria* sp. are possibly responsible for their pharmacological potential and synergistic interactions between the different compounds of these species may occur [10]. These chemicals act by modifying the expression of several pro-inflammatory genes, contributing to the regulation of inflammatory signaling. Their action is mainly related to the ability to inhibit enzymes such as phospholipase A_2, cyclooxygenase, and lipoxygenase [21, 22]. Moreover, a study suggested that the anti-inflammatory activity of the aqueous extract of *U. tomentosa* is possibly correlated to the antioxidant mechanism exerted by the phenolic compounds present in the extract [23].

3.2 Phospholipase and hemolytic activities

The phospholipase activity (%) induced by *B. moojeni* venom previously incubated with aqueous extracts of both plants is shown in Figure 1. *U. tomentosa* ($F_{(11, 24)} = 16.64$, $p < 0.0001$)

and *U. guianensis* ($F_{(11, 24)} = 55.74$, $p < 0.0001$) significantly inhibited the phospholipase activity of *B. moojeni* venom at all doses evaluated, showing inhibitions on phospholipase activity between 10 and 18% and 10 and 46%, respectively. The highest percentages of inhibition were observed for the highest doses of both extracts.

Although only steroidal anti-inflammatory drugs are described as phospholipase A_2 inhibitors, both synthetic chemicals exerted inhibitions between 8 and 15%. Low inhibitions were expected since high inhibitions of these enzymes are associated with several adverse reactions. However, the physical barrier imposed by the solid medium in which the substrate is inserted can also configure a factor of activity reduction, since molecules with different gels will have different diffusion rates [24].

The inhibitions of the phospholipase activity observed in this study are possibly related to the presence of phenolic compounds in the extracts of *U. tomentosa* and *U. guianensis*. Phospholipases A_2 are stable, relatively small (~14 kDa), Ca^{2+}dependent, and rich in disulfide interactions. These enzymes are fundamental in the regulation of the arachidonic acid pathway through which pro-inflammatory mediators such as prostaglandins, leukotrienes, and thromboxanes are released, and in the regulation of lipid metabolism. These mediators are responsible for changes in hemostasis and their disordered production can trigger various inflammatory diseases [25].

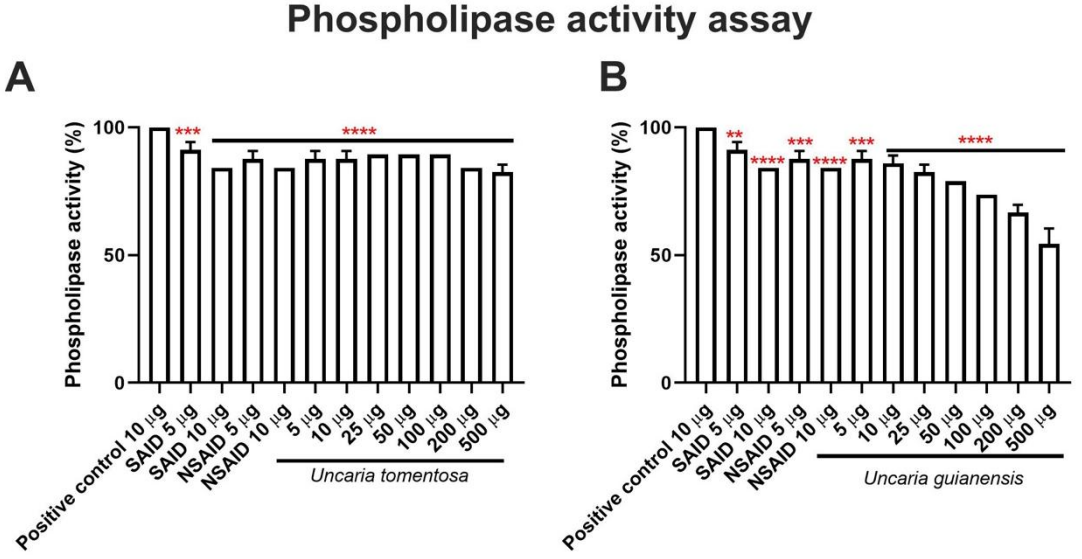

*Figure 1: Phospholipase activity (%) induced by Bothrops moojeni venom, previously incubated with anti-inflammatory drugs (SAID: steroidal anti-inflammatory drug - prednisolone; NSAID: non-steroidal anti-inflammatory drug - diclofenac resinate) and with the aqueous extracts of Uncaria tomentosa and Uncaria guianensis. The control (+) containing only venom (10 µg) was considered as 100% activity. The results correspond to means of triplicates and the standard deviation obtained at each dose (µg). Asterisk denotes a significant difference at **p ≤ 0.01, ***p ≤ 0.001, **** p ≤ 0.0001 (one-way ANOVA followed by Dunnett's post-hoc test).*

Several plant extracts have already been described with inhibitory activity on phospholipase A_2 [26-28]. Isolated phenolic compounds such as *p*-coumaric acid, catechin, and epicatechin also showed significant inhibitions on phospholipase activity [21, 22].

Some interaction mechanisms between phenolic compounds and phospholipases A_2 have already been scientifically described [29] highlighted that tannins can form complexes with Ca^{2+}, the enzyme cofactor of phospholipases A_2, and modulate the activity of these enzymes. Another mechanism that has been described is the ability of flavonoids to bind to amide groups of proteins through hydrogen bonds, forming complexes that precipitate [30]. Phenolic compounds can also inhibit the activity of phospholipases A_2 through hydrogen bonds with the active site of this enzyme, phenolic hydroxyls interact with amino acid residues, preventing or hindering the

binding of the enzyme to the substrate [31]. In this context, the study of the modulation of phospholipase A_2 activity by natural compounds is an important tool to elucidate the mechanisms of action and to prospect potential anti-inflammatory agents and modulators of hemostasis.

Hemolytic activity in a solid medium (Figure 2) was performed to evaluate the direct effects of the extracts on human erythrocytes, as well as their potential to inhibit *B. moojeni* venom-induced cytotoxicity. Pure extracts up to 500 µg (without incubation with the venom) did not induce hemolysis under the conditions evaluated (data not shown), confirming the absence of cytotoxicity in this assay and corroborating previous studies [32].

The extracts of *U. tomentosa* ($F_{(11, 24)} = 58.78$, $p < 0.0001$) and *U. guianensis* ($F_{(11, 24)} = 173.6$, $p < 0.0001$) significantly inhibited the hemolytic activity induced by B. *moojeni* venom at all doses evaluated. The *U. tomentosa* extract inhibited hemolytic activity between 14 and 41%, while the *U. guianensis* extract exerted inhibitions between 14 and 60%. Erythrocyte lysis was reduced with increasing doses of both extracts. It was also observed that the extract of *U. guianensis* was more efficient in inhibiting the hemolytic activity induced by the venom, with inhibitions of 60% for the doses of 100, 200, and 500 µg.

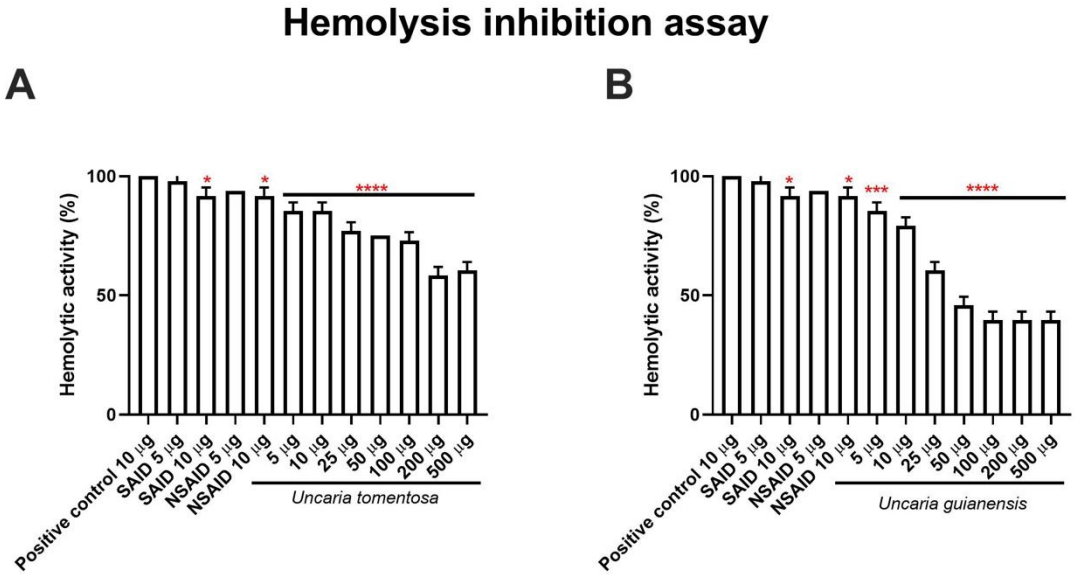

*Figure 2: Hemolytic activity (%) induced by Bothrops moojeni venom, previously incubated with anti-inflammatory drugs (SAID: steroidal anti-inflammatory drug - prednisolone; NSAID: non-steroidal anti-inflammatory drug - diclofenac resinate) and with the aqueous extracts of Uncaria tomentosa and Uncaria guianensis. The control (+) containing only venom (10 µg) was considered as 100% activity. The results correspond to means of triplicates and the standard deviation obtained at each dose (µg). Asterisk denotes a significant difference at *p ≤ 0.05, ***p ≤ 0.001, **** p ≤ 0.0001 (one-way ANOVA followed by Dunnett's post-hoc test).*

3.3 Thermal hemolysis

Erythrocyte membranes are used as useful tools to study potential anti-inflammatory agents, due to the similarity between the lysosome and erythrocyte membranes. During inflammatory reactions, lysosomes are mobilized, and their content is released, resulting in a series of physiological changes [33]. Non-steroidal anti-inflammatory drugs (NSAIDs) act by inhibiting the release of lysosomal enzymes or stabilizing membranes [34]. Exposure of erythrocytes to heat or harmful substances results in membrane lysis, accompanied by hemolysis and hemoglobin oxidation [35]. In this sense, the inhibition of heat-induced hemolysis was related to the anti-inflammatory potential of the evaluated extracts.

Table 2 shows that both *U. tomentosa* and *U. guianensis* extracts at all doses (2000-8000 µg) significantly promoted stabilization of the human erythrocyte membrane at 37 ºC ($F_{(9, 20)} = 2289$,

$p < 0.0001$) and 54 ºC ($F_{(9, 20)} = 437.9$, $p < 0.0001$). A similar pattern was observed for the SAID and NSAID drugs.

Membrane stabilization leads to the prevention of leakage of proteins and serum fluids into tissues during a period of increased permeability caused by inflammatory mediators [36], or in the case of this study, by high temperature simulating the fever induced by the human organism as part of the inflammatory response. Thus, membrane protection possibly promoted by phenolic compounds present in the extracts would reduce erythrocyte lysis. From this perspective, we can infer that both extracts were able to stabilize the erythrocyte membrane against heat, preventing the release of cellular contents, due to the presence of phenolic compounds. These compounds act on the erythrocyte surface and may be responsible for their protective properties, in addition to acting as free radical scavengers and inhibitors of lipid peroxidation, increasing cellular resistance to oxidative damage [37].

Table 2: Evaluation of thermal hemolysis in the presence of anti-inflammatories and aqueous extracts of Uncaria tomentosa and Uncaria guianensis.

	Concentration/Dose	**% Hemolysis at 37°C**	**% Hemolysis at 54°C**
PBS	-	1.08 ± 0.12	19.00 ± 0,03
SAID	50 µg.mL	5.23 ± 0.10**	21.79 ± 0,02^{ns}
NSAID	50 µg.mL	3.76 ± 0.12*	21.62 ± 0,04^{ns}
Uncaria tomentosa	2000 µg	8.12 ± 0.09****	28.14 ± 0,16***
	4000 µg	4.78 ± 0.17**	18.59 ± 0,21^{ns}
	8000 µg	4.32 ± 0.01*	17.72 ± 0,08^{ns}
Uncaria guianensis	2000 µg	12.90 ± 0.02****	51.03 ± 0,05****
	4000 µg	8.51 ± 0.15****	35.33 ± 0,10****
	8000 µg	5.51 ± 0.03***	19.42 ± 0,14^{ns}

The values correspond to means of triplicates and the standard deviation. SAID: steroidal anti-inflammatory drug – prednisolone. NSAID: non-steroidal anti-inflammatory drug - diclofenac resinate. The assays were evaluated in a 2% (v/v) hematocrit solution. The control containing only water was considered 100% hemolysis. Asterisk denotes a significant difference at *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, **** p ≤ 0.0001 (one-way ANOVA followed by Dunnett's post-hoc test).

3.4 Proteolytic activity on casein

The extracts of *U. tomentosa* ($F_{(7, 16)} = 55.62$, $p < 0.0001$) and *U. guianensis* ($F_{(7, 16)} = 28.88$, $p < 0.0001$) significantly inhibited the proteolytic activity on casein induced by the venom of *B. moojeni* at all doses evaluated, with the increase in inhibition being directly proportional to the increase in doses (Figure 3).

The proteases present in snake venoms have high homology with human enzymes, thus being useful as research tools in the search for diagnosis and treatment of various thrombotic conditions and hemostatic disorders [38, 39]. Several medicinal plants and phytocompounds have been described as inhibitors of snake toxins [40]. These bioactive compounds present in plant extracts can interact with proteases through hydrophobic interactions with aromatic residues present in the enzyme structure and complexation with metal ions, resulting in a reduction or even an increase in enzymatic activity [41].

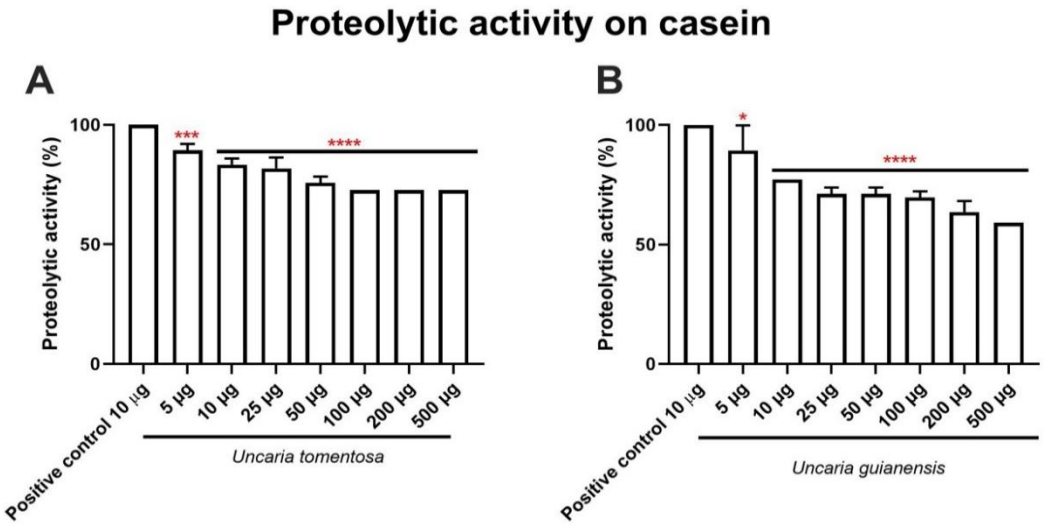

*Figure 3: Proteolytic activity (%) induced by Bothrops moojeni venom, previously incubated with the aqueous extracts of Uncaria tomentosa and Uncaria guianensis. The control (+) containing only venom (10 µg) was considered as 100% activity. The results correspond to means of triplicates and the standard deviation obtained at each dose (µg). Asterisk denotes a significant difference at *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, **** p ≤ 0.0001 (one-way ANOVA followed by Dunnett's post-hoc test).*

3.5 Thrombolytic activity

The results of the inhibition of the thrombolytic activity of *B. moojeni* venom by the extracts are presented in Figure 4. The extracts of *U. tomentosa* ($F_{(8, 18)}$ = 234.0, p < 0.0001) and *U. guianensis* ($F_{(8, 18)}$ = 664.4, p < 0.0001) significantly inhibited the thrombolytic activity of *B. moojeni* venom. Controls containing only extracts (without incubation with venom) showed higher inhibitory potential compared to the incubated extracts. The aqueous extract of *U. tomentosa*, at the different concentrations evaluated, inhibited between 20% and 46% of the activity induced by *B. moojeni* venom. The aqueous extract of *U. guianensis* inhibited between 31% and 53% of the activity induced by *B. moojeni* venom. The highest inhibition was observed at the highest concentration of 1500 µg, with a 53% reduction in thrombus lysis. This result may reflect the presence of phenolic:proteolytic-hemorrhagic enzyme ratios more ideal for interactions to occur.

Thrombosis, or the formation of a blood clot, is associated with several inflammatory diseases and is one of the main causes of human death. Thrombus formation results in abnormal hemostatic conditions due to uncontrolled action of thrombin on fibrinogen or excess thrombin production [42, 43].

Thus, it is important to evaluate natural compounds capable of inhibiting enzymes that act on the blood clotting cascade, such as phospholipases A2 and proteases. The inhibition of thrombolytic activity induced by venoms through the extracts of *U. tomentosa* and *U. guianensis* may be related to the ability to inhibit thrombin enzymes, mainly serine proteases, which have structures and functions similar to thrombin, suggesting possible pharmaceutical applications. Several plant extracts and isolated active principles have demonstrated both thrombolytic and antithrombotic activities, researchers suggest that phenolic compounds may be related to thrombus dissolution [21, 22, 44, 45].

3.6 Coagulant activity

The previous incubation of the venom with *U. tomentosa* and *U. guianensis* extracts at doses of 50, 100, 200, and 500 µg, significantly increased the clotting time between 27 and 57 seconds and between 74 and 384 seconds, respectively, compared to the control ($F_{(8, 18)}$ = 731.6, p < 0.0001) (Table 3). For both extracts, a dose-dependent response was observed, with the highest clotting time observed at 500 µg. Indeed, the clotting time at 500 µg was two times bigger

for *U. tomentosa* and eight times bigger for *U. guianensis* compared with the control containing only *B. moojeni* venom. The effect of *U. tomentosa* and *U. guianensis* extracts in prolonging the clotting time induced by proteases suggests the presence of inhibitors in the extract's composition. Data on clotting enzyme inhibition corroborate the data on thrombolysis inhibition (Figure 4) since venom proteases have several target substrates and are responsible for clotting and hemorrhage [46]. In addition, the extracts themselves have components with thrombolytic action, favoring blood fluidity. Thus, the extracts evaluated may have significant potential for use in the treatment and prevention of thrombotic diseases.

Table 3: Effect of aqueous extracts of Uncaria tomentosa and Uncaria guianensis on the coagulant activity induced by Bothrops moojeni venom in citrated human plasma.

	Dose (µg)	Clotting time (s)
Control (*B. moojeni* venom)	10	56.12 ± 2.80
***Uncaria tomentosa* + venom**	50	84.08 ± 1.95*
	100	87.84 ± 2.75**
	200	93.66 ± 4.74***
	500	113.35 ± 1.18****
***Uncaria guianensis* + venom**	50	130.93 ± 13.18****
	100	245.03 ± 5.18****
	200	410.50 ± 19.42****
	500	440.34 ± 13.17****

The data correspond to means of triplicates and standard deviation. Samples: aqueous extracts of U. tomentosa and U. guianensis previously incubated with the venom for 10 minutes at 37 ºC. The control was carried out with 10 µg of venom. Asterisk denotes a significant difference at *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, **** p ≤ 0.0001 (one-way ANOVA followed by Dunnett's post-hoc test).

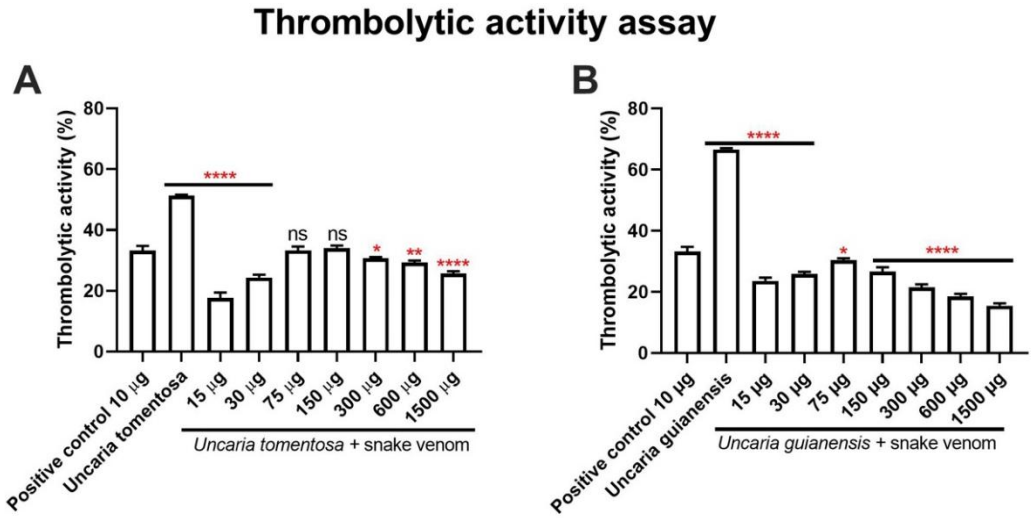

*Figure 4: Thrombolytic activity (%) induced by Bothrops moojeni venom, previously incubated with the aqueous extracts of Uncaria tomentosa and Uncaria guianensis. The total reaction volume (130 µl) was considered as 100% lysis. The control (-) containing PBS was discounted from the other treatments. C = corresponds to controls treated with extracts only, without venom. The results correspond to means of triplicates and the standard deviation obtained at each dose (µg). Asterisk denotes a significant difference at ***p ≤ 0.001, **** p ≤ 0.0001 (one-way ANOVA followed by Dunnett's post-hoc test).*

A pioneering study evaluated the effects of *U. tomentosa* on the hemostatic system [11]. The extracts demonstrated anticoagulant potential, antiplatelet effects, thrombolytic and fibrinolytic

activity. In the *in silico* study, the researchers demonstrated the interactions of thrombin with the main components of the extracts. These compounds interacted with thrombin inside and outside the active site, depending on the compound. The highest binding affinity was found for procyanidins B2 and C1 (flavonoids).

In this context, considering the presence of phenolic compounds in the composition of the extracts of *U. tomentosa* and *U. guianensis*, we can infer that the enzymatic inhibitions observed result from molecular interaction mechanisms between phenolic compounds and phospholipase A_2 and proteases. Thus, these herbal medicines act in the modulation of mechanisms related to hemostasis and inflammatory processes.

4. CONCLUSIONS

The extracts of *U. tomentosa* and *U. guianensis* have several bioactive substances that confer potential for treating and preventing inflammatory diseases and hemostasis disorders. In the present study, the aqueous extract of *U. guianensis* showed a higher content of phenolic compounds compared to *U. tomentosa*. These compounds are probably related to the modulatory effects on the activity of enzymes, such as phospholipases A_2 and proteases. These enzymes act in physiological processes such as blood clotting, thrombus formation, fibrinogenolysis, inflammatory response, and platelet aggregation. However, complementary studies are needed to expand the knowledge about the mechanisms of interactions between bioactive compounds and enzymes, enabling different uses for the phytotherapics of *U. tomentosa* and *U. guianensis*.

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