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# INHIBITION OF HARMFUL EFFECTS FROM *Bothrops alternatus* VENOM BY *Casearia arborea* EXTRACT

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**Abstract:** This study aimed to assess the action of methanol extract from *Casearia arborea* (Salicaceae) branches against the harmful effects of *Bothrops alternatus* venom. The venom was tested combined or not with *Casearia arborea* methanol extract (CAME) at different ratios (1:10, 1:50 (w/w)). Coagulant activity was tested using bovine plasma at 37°C and crude venom (10μg). The hemorrhagic activity of the venom (15μg) was assessed 150 min after inoculating venom samples in the dorsal region of mice. Myotoxic activity was tested by injecting venom into the gastrocnemius muscle of mice, followed by determination of creatine kinase (CK) levels. Phospholipase $A_2$ activity was evaluated using egg yolk as substrate. Histologic sections of gastrocnemius muscles were analyzed under an optical microscope. The results indicated that CAME inhibited coagulant activity by 59% and reduced hemorrhagic activity by 91%. Myotoxicity analysis demonstrated that serum CK levels declined and phospholipase $A_2$ activity assessment showed a 50% reduction. The histological sections analyzed indicated decreased local tissue damage in the presence of CAME. These results suggest that CAME may be a potential adjuvant in conjunction with the serum therapy currently applied to neutralize botropic venoms.
**Keywords:** Ophidism, snake venom**,** tissue damage, vegetal extract.

## INTRODUCTION

Snakebite envenoming, listed among the World Health Organization's (WHO) Neglected Tropical Diseases (NTD), is a particularly important public health problem in rural areas, where access to healthcare services is limited (Chippaux, 2017).

The most clinically relevant snakes belong to the families Elapidae and Viperidae (Cardoso & Wen, 2009; Félix-Silva et al., 2014). In Brazil, for example, accidents caused by vipers from the genera *Bothrops* and *Crotalus* are common, while those related to *Lachesis* and *Micrurus* snakes are rare. For example, snakebite accidents reported in southwestern Goiás state in March and April between 2007 and 2013 occurred in rural areas and are predominantly attributed to the Bothrops genus. Victims are mostly male rural workers aged between 41 and 50 years old, with the lower limbs (feet and legs) being bitten most frequently (NUNES et al., 2020).

*Bothrops* snake venom induces local tissue damage such as hemorrhaging, swelling, inflammation and necrosis. One of the effects of *Bothrops* snakebites is muscle necrosis, which leads to permanent tissue or functional loss and, in more severe cases, amputation of the affected limb (França & Málaque, 2009; Luna et al., 2011; Roriz et al., 2018, Silva Jr. et al., 2016).

The only specific treatment currently available for snakebite envenoming is antivenom, a set of neutralizing immunoglobulins or fragments of purified immunoglobulin from the plasma of animals hyperimmunized to snake venoms or specific toxins. Its effectiveness lies in its potential to provide patients with antibodies against snake venom in order to eliminate the toxins responsible for envenomation and mitigate its toxic effects (Ahmed et al., 2008; Wen, 2009; Félix-Silva et al., 2017).

However, antivenom has some limitations, including its poor ability to treat local effects, high cost, the risk of immunological reactions and difficult access in some regions. If administered soon after envenomation, it generally successfully neutralizes the systemic effects, but local tissue damage is more difficult to counteract (Wen, 2009; Gutiérrez et al., 2014; Félix-Silva et al., 2014).

In this context, the search for complementary therapies to treat snakebites is relevant and medicinal plants stand out as

a rich source of potential natural inhibitors and pharmacologically active compounds (Amui et al., 2011; Abhijit and Jitendra, 2012; Goswani et al., 2014; Hasan et al., 2015).

Herbal drugs used to treat snakebites in traditional medicine have gained widespread attention from toxinologists, as a tool to detect potential inhibitors of the effects caused by snake venom. The potential benefits of antiophidic plants are their low cost, accessibility, stability under ambient temperature and ability to neutralize a broad spectrum of toxins, as well as local tissue damage (Félix-Silva et al., 2014; Atanasov et al., 2015).

In this respect, several plants have been studied for their potential antiophidic activity, including *Schizolobium parahyba* (Mendes et al., 2008, 2013), *Bombacopsis glabra* (Mendes et al., 2012), *Bellucia dichotoma, Connarus favosus* (Moura et al., 2015), *Tamarindus indica*, *Paullinia pinnata* (Molander et al., 2015), *Bredemeyera floribunda* (Alves et al., 2019), *Zanthoxylum monogynum* (Oliveira et al., 2019, Santos et al., 2021) and *Paquira aquática* (Vieira et al., 2021).

The wide variety of secondary metabolites produced by plants exhibit a range of biological activities that not only protect these organisms, but also have medicinal properties beneficial to humans, such as anti-inflammatory, antitumor and analgesic action, among others (Maciel et al., 2002).

Plants from the Salicaceae family are recognized for producing important classes of secondary metabolites, with multiple biological properties. In the genus *Casearia* alone, 287 metabolites have been described, the most prominent being terpenoids, coumarins, glycosides, alkaloids and flavonoids (Fernandes et al., 2009; Martelli et al., 2018).

Scientific studies demonstrate the antiophidic potential of *Casearia* aqueous extract against *Bothrops jararacussu* venom in inhibiting phospholipase ($A_2$) activity, as well as its antiiflammatory and wound healing properties in mice (Borges et al., 2000; Campos et al., 2015). *Casearia mariquitenses* aqueous extract also exhibits antiophidic potential by neutralizing hematological changes induced by crude *Bothrops neuwiedi pauloensis* venom (Izidoro et al., 2003; Sanchez et al., 2008).

As such, the present study aimed to neutralize urutu (*Bothrops alternatus*) venom using *Casearia arborea* methanolic extract.

## MATERIALS AND METHODS

### VENOM AND ANIMALS

*Bothrops alternatus* venom used here was donated by the Center for Biological Studies and Research (CEPB) of the Pontifical Catholic University of Goiás (PUC-Goiás). The lyophilized venom (crude venom - BV) was weighed, dissolved in NaCl (0.9%) and centrifuged at 2000rpm for 10 minutes at 4ºC. The supernatant was collected, dosed using the biuret method (Itzhaki & Gill, 1964) and used in the experiments. Male Swiss mice were supplied by the Bioterium of the Federal University of Goiás (UFG) (Samambaia Campus). The experiments carried out at the Animal Experimentation Vivarium (BEA) of the Federal University of Jataí. The animals were kept under standard light conditions (12h light/dark cycle), with water and food *ad libitum*. For use in the experiments, they were anesthetized using 10% ketamine (0.05mL/Kg) and 2% xylazine (0.025mL/Kg), in accordance with the protocol approved by the institutional Animal Ethics Committee, under protocol number 022/18.

### PLANT ACTIVE INGREDIENT AND CONTROL SUBSTANCES

The *Caesaria arborea* methanolic extract (CAME) was provided State University of Southwestern Bahia, and made from ground *Caesaria arborea* branches. The *Casearia*

*arborea* plants were collected on June 28, 2012, in a forest fragment at Fazenda Brejo Novo (13º56'41"S and 40º06'33.9 "W; between 617 and 755 m a.s.l.), 9 km from the city of Jequié, in Bahia state, Brazil. The plant material was dried in a Tecnal oven (model TE 394 \ 2) at 40 ° C for 48 h and submitted to cold maceration with methanol. For experiments, the control groups were C1- using only BV; C2- the extract alone; C3- only NaCl (0.9%). The test groups (T) were prepared by incubating the BV and CAME at ratios of 1:10 and 1:50 (w/w, venom:vegetal extract).

## COAGULANT ACTIVITY

The method used was based on that described by Assakura et al. (1992) with some modifications. Citrated bovine plasma (200µL) was used, heated in a water bath at 37°C for 10 minutes, and then added with venom samples combined or not with vegetal extract. The reaction was timed and the time at which fibrin network formation occurred recorded, the tests were carried out in triplicates.

## HEMORRHAGIC ACTIVITY

Hemorrhagic activity was analyzed using the methodology described by Nikai et al. (1984), with some modifications. The tests were conducted with 3 MDH (minimum hemorrhagic doses) of *Bothrops alternatus* venom and *Casearia arborea* (CAME). Saline solution (NaCl 0.9%; m / v) and *Casearia arborea* methanolic extract (CAME) were each used as control substances and individually applied to the mice. The following groups were used: (A) BV; (B) BV + CAME (1:10, w / w); (C) BV + CAME (1:50, w / w); (D) CAME control, (E) Saline solution. The mice were intradermally inoculated in the dorsal region with crude venom combined or not with plant extract. After 2.5 hours, the animals were anesthetized in accordance with the protocol approved by the animal ethics committee and then euthanized. The skin from the dorsal region where the mice were inoculated was removed and the hemorrhagic halos measured with a pachymeter.

## HISTOLOGICAL ANALYSIS

Myonecrosis was assessed based on morphological changes induced by inoculation crude venom (25µg) into the gastrocnemius muscle of mice (n=3, 18 - 22g). After 3, 6, 24 and 48 hours, mice were anesthetized, euthanized and the gastrocnemius muscle dissected and fixed in a 10% formaldehyde solution with PBS (v/v). Dehydration was performed with increasing ethanol concentrations and the samples were processed for embedding in paraffin blocks. The slides were prepared using 2.5µm sections stained with hematoxylin and eosin (HandE) and Gomori trichrome and examined under optical microscope.

## MYOTOXIC ACTIVITY

Groups of mice (n=3, 18-25g) were injected in the right gastrocnemius muscle with 25 µg of crude venom. Three hours later, they were anesthetized, euthanized and exsanguinated by cardiac puncture. The serum was used after the blood collected had coagulated. Serum creatine kinase activity was determined using a commercial kit. Activity was expressed in units per liter, with each unit corresponding to the production of 1µmol of NADH per minute at 30ºC.

## PHOSPHOLIPASE A2 ACTIVITY ON EGG YOLK AGAR PLATES

Phospholipase $A_2$ activity was evaluated using egg yolk agar plates (Haberman & Hardt, 1972).

Agar solution (0.3 g in 20 mL of PBS at 50°C) was added to 500 µL of egg yolk solution diluted in PBS (25%, v/v) and 5 µL of 1M $CaCl_2$. The solution was poured into Petri

dishes and, after solidification, 5 holes (0.31 cm in diameter) were made in the agar for sample application.

Three plates were prepared to evaluate the groups: BV control, BV + CAME (1:10, w/w), BV + CAME (1:50, w/w), CAME control, NaCl 0.9% control. After applying the samples, the Petri dishes were incubated in a humid chamber at 37°C for 20 h. All experiments were carried out in triplicate. Phospholipase $A_2$ activity was assessed by measuring the transparent halos produced around the wells.

### HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS

High performance liquid chromatography was performed using a Waters Alliance system with an e2695 separation module and 2998 photodiode array detector, and data were obtained with Empower software. Chromatographic separations were carried out in a Zorbax Eclipse XDB-C18 reversed-phase column (250 x 4.6 mm, 5 µm). Column temperature was maintained at 35 °C with an injection volume of 10 µL. The mobile phases contained 0.05% formic acid in acetonitrile (pH = 3.45) (solvent A) and 0.05% formic acid in water (pH = 3.15) (solvent B) at a flow rate of 1 mL min-1. The following gradient was applied: 0% ± 5% A (0 ± 5 min); 5% ~ 10% A (5 ~ 15 min); 10% ~ 15% A (15 ~ 25 min), 15% ~ 20% A (25 ~ 35 min), and a 15-minute isocratic segment of 20% A (35 ~ 50 min). The mobile phases were previously filtered through a 0.45 µm PVDF membrane and degassed in an ultrasonic cleaner.

For analysis, 30 mg of extract was diluted in 5 mL of methanol in a volumetric flask. To identify the peaks separated by HPLC, the following stock solutions (0.25 mg mL-1 in methanol) supplied by Sigma Aldrich were used: caffeic acid, caffeine, catechin, chlorogenic acid, ellagic acid, epicatechin, gallic acid, naringin, p-Coumaric acid and rutin. The chromatograms were recorded at wavelengths of 254, 327 and 366 nm. Identification was achieved by comparing the retention times and UV spectra at 190 a 400 nm of the HPLC chromatograms of the extract with those of the pure standards. Before injection, the solutions were filtered through a 0.45 µm PVDF membrane.

### STATISTICAL ANALYSIS

The results were expressed as mean ± standard deviation. Statistical significance was assessed using the Kruskal- Wallis and Tukey's tests and GraphPad Prism 5 software.

## RESULTS

### COAGULANT ACTIVITY

After incubation of *B. alternatus* venom and CAME with bovine plasma at 37°C, the results were expressed in Table 1 as coagulation time per dose (µg).

The average blood coagulation times recorded for the BV (positive control (15µg), BV+CAME (1:10, w/w) and BV+CAME (1:50, w/w) groups were 60, 108 and 133 seconds, respectively. Comparison between the BV and test groups showed a significant difference (p>0.05). Coagulation time for the negative control was 303 seconds, with no significant difference (p>0.05) in relation to the saline solution.

| | Coagulation Time (sec) | | | |
|---|---|---|---|---|
| | Test 1 | Test 2 | Test 3 | Mean x SD |
| BV | 57 | 56 | 57 | 56.6 ± 0.57 |
| BV+CAME(1/10) | 108 | 110 | 107 | *108.3 ± 1.52 |
| BV+CAME(1/50) | 135 | 132 | 134 | *133.6 ± 1.52 |
| CAME | 303 | 303 | 303 | >303 ± 0.0 |
| Saline | 303 | 303 | 303 | > 303 ± 0.0 |

* - p-values below 0.05 (p<0.05) were considered significant.

Table 1. Coagulation time of *Bothrops alternatus* venom mixed or not with *Caesaria arborea* methanolic extract (CAME).

In relation to BV, coagulation time in BV+CAME (1:10, w/w) increased by 89.47% and 136.84% in BV+CAME (1:50, w/w), with the latter group showing a 25% rise when compared to the former.

### HEMORRHAGIC ACTIVITY

The hemorrhagic activity of *B. alternatus* venom was evaluated in mice 150 min after BV application combined or not with CAME. Negative controls received only saline (NaCl0,9%) or CAME. Results are expressed as mean ± standard deviation (n = 4). Hemorrhagic activity was neutralized by the vegetal extract at 1:50 (w/w) (Figure 1 [D]), which proved more efficient than a ratio of 1:10 (w/w) (Figure 1 [C]), with an 80% decline in the hemorrhagic halo diameter.

### MYOTOXIC ACTIVITY

For myotoxic activity assessment, the right gastrocnemius muscle of groups of mice was injected with 25 μg doses of crude *B. alternatus* venom, followed by determination of the creatine kinase (CK) level. The CK release in the plasma of BV + CAME (1:50, w/w) mice was smaller in relation to BV control, exhibiting 70% venom-induced myotoxicity inhibition (Figure 2).

### PHOSPHOLIPASE A2 ACTIVITY

Phospholipase $A_2$ activity was evaluated using egg yolk as a substrate. Figure 3 shows the average values obtained for B. alternatus venom in the absence and presence of plant extracts and controls. The crude venom generated a halo greater than 6 $cm^2$; however, when combined with the CAME at a ratio of 1:50 (w / w), the halo decreased considerably to 1.5 $cm^2$ ($p <0.05$).

### HISTOLOGICAL ANALYSIS

Images of the histologic sections analyzed showed normal muscle tissue in mice treated with saline and CAME (control substances) 3, 6, 24 and 48 hours after venom administration (Fig. 4). However, after the same periods, treatment with *B. alternatus* venom resulted in hemorrhaging and muscle tissue necrosis, which declined for the BV + CAME treatment, particularly at ratio of 1:50 (w/w), with leukocyte clumps evident in the spaces between muscle fibers after 24 and 48 hours.

### HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS

The data obtained by HPLC revealed that the major compound in *C. arborea* extract was chlorogenic acid (CGA), with a retention time of 17.267 min in the extract and 17.301 min in the standard, exhibiting the same UV spectrum (Fig. 5).

## DISCUSSION

In the present study, the coagulation time of *B. alternatus* venom increased after incubation with CAME. Several studies have been conducted to investigate the effects of plants against the venom of Bothrops snakes. An important finding is that the aqueous extract of *Jatropha gossypiifolia* leaves (bellyache bush; Euphorbiaceae), rich in sugars, alkaloids, flavonoids, tannins, terpenes and proteins, inhibited the enzymatic and biological activities induced by *Bothrops jararaca* in vitro and in vivo, exhibiting an anticoagulant effect due to prolonged coagulation time in fibrinogen solution (6 mg / mL) and plasma (Félix-Silva et al., 2014).

Mourão et al., (2014) found that the medicinal plant *Bellucia dichotoma* (Melastomataceae) inhibited *Bothrops atrox* venom, showing a greater effect in preincubation tests and declining significantly or disappearing when the extract was injected without the venom to simulate traditional use. According to the authors, the plant's

high tannin content, metabolites capable of precipitating proteins, may be the main contributing factor to venom inhibition in preincubation tests.

In the present study, CAME combined with *B. alternatus* venom at a ratio of 1:50 (w/w) resulted in longer coagulation times, suggesting that higher concentrations of the plant compounds present may be more effective in venom inhibition.

The results obtained for the chelating activity of the vegetal extract (CAME), assessed by preincubation of the extract with calcium chloride, were the same as those observed for coagulant activity (results not shown).

Based on the hemorrhagic results of previous studies, Moura et al. (2015) investigated plants used by a community in Pará state, Brazil to treat *B. jararaca* bites and identified a total of 24 plants from 19 families. The authors also reported that, in preincubation tests, venom-induced hemorrhaging was completely inhibited by aqueous extracts of *Bellucia dichotoma, Plathymenia reticulata* and *Philodendron megalophyllum* (climbing vine), which exhibit high concentrations of phenolic compounds as well as condensed and hydrolyzable tannins. However, hemorrhagic activity was not correlated with the effect of the vegetal extracts on the venom proteins.

Because it is rich in snake venom metalloproteinases (SVMPs), a hemorrhagic toxin whose catalytic domain contains zinc, the venom of *Bothrops* snakes needs this ion as a co-factor in order to produce an effect. According to Ramirez (2008), the ethanolic extract of *Casearia arborea* branches collected in November 2006 in Porto Grande, Amapá state (AP), Brazil, contains two steroids, two diterpenes and a mixture of the diterpene trans-13-hydroxy-trans-cleroda-3,14-dieno (Fl-4a=Fl-3) and the fatty acid ester ethyl palmitate (Fl-4b).

The presence of these metabolites in CAME may infer that the decline in *B. alternatus* venom-induced hemorrhaging may be due to interaction between terpenes and SVMPs, blocking the catalytic sites of these enzymes or chelating the zinc ions that are vital to ensuring the effect of metalloproteases (Flausino et al., 2009; Moura et al., 2015).

Strauch et al. (2013) found that some plant metabolites have the potential to bind to and neutralize snake venom toxins, inhibiting their catalytic effect, independent of concentration. In general, thrombin-like enzymes in snake venom accelerate fibrin production, causing a procoagulant effect and depletion of fibrinogen, resulting in hemorrhage (Sajevic et al., 2011). Thus, the fact that CAME inhibited the hemorrhagic effect of *B. alternatus* venom may be directly related to the potential of the extract's components to inhibit the proteolytic action of the venom. Flausino et al. (2009) reported that the diterpenes present in plant species from the family Salicaceae, to which *Caesaria arborea* belongs, may inhibit protease activity.

The myotoxic effect of *Bothrops* snake venom has been attributed to the proteolytic influence of its components and phospholipase $A_2$, as well as polypeptides that directly modify the sarcolemma (Fernandes et al., 2014). The myotoxic action of $PLA_2$ causes local and systemic tissue damage, largely because it binds to receptors on the plasma membrane, lipids or proteins, which differ in their affinity with $PLA_2$s. This allows myotoxic $PLA_2$s to compromise the integrity of the plasma membrane through dependent or independent catalytic events, generating a $Ca_2$ + inflow that triggers a complex series of degenerative mechanisms associated with muscle hypercontraction (Harris and Scott-Davey, 2013; Strauch et al., 2013; Mukherjee et al., 2014).

Previous studies have reported that vegetal extracts inhibit the myotoxic effect of snake venom from this genus. The use of *Jatropha gossypiifolia* extract (Euphorbiaceae, such as the rubber tree and castor bean) resulted in almost 100% inhibition of the myotoxic effect of *B. jararaca* venom. Enzymatic tests suggest that this extract may have antiophidic potential due to the inhibitory effect of SVMPs or SVSPs (snake venom serine proteases) (Silva-Félix, 2014). A study conducted with the aqueous extract of *Casearia sylvestris* leaves (wild sage; Flacourtiaceae) found that it displayed inhibitory action against *B. moojeni* venom and myotoxin II (Borges et al., 2000).

Studies with *Casearia mariquitenses* aqueous extract also demonstrate the antiophidic activity of the species in neutralizing hematological changes induced by neuwiedase, a P-I class metalloproteinase present in *Bothrops neuwiedi pauloensis* venom (Izidoro et al., 2003; Sanchez et al., 2008).

A study with *Casearia gossypiosperma* Briquet hydroalcoholic extract observed its antiophidic activity in blocking the neuromuscular action induced by *B. jararacussu* venom in the phrenic nerve of mice (Camargo et al., 2010).

Strauch et al. (2013) conducted an experiment on the isolated long extensor muscles of mice and observed an increase in the release of CK from the muscles 90 minutes after exposure to (usar italic no nome das espécies, revisar todo o texto) *B. jararacussu*, *B. atrox* and *B. jararaca* venom (25 μg / mL). However, the addition of increasing concentrations (100-300 μg/mL) of *Humirianthera ampla* ethanolic extract significantly reduced the CK release rate, suggesting that the decline in *Bothrops* venom myotoxicity may be linked to $PLA_2$ inhibition.

Fernandes et al. (2014) assessed the in vivo myotoxicity of *B. jararacussu* venom by analyzing the increase in plasma creatine kinase activity in mice treated with muscular injection of isolated venom or venom associated with *Combretum leprosum* extract (Mofumbo; Combretaceae) and found that the extract decreased the high venom-induced CK rate, suggesting that interaction between the two resulted in antagonism.

The results obtained in the present study for phospholipase $A_2$ activity of *B. alternatus* venom were similar to those reported by Strauch et al. (2013) using egg yolk and agar as substrate, whereby *B. atrox*, *B. jararacussu* and *B. jararaca* venom significantly reduced the turbidity of egg yolk solutions when compared to the negative control. After application of *Humirianthera ampla* ethanolic extract (1-500 μg/mL), the authors observed a decline in the enzymatic activity of all three venoms, with inhibition of up to 92%.

A previous study demonstrated that the aqueous extract of *Casearia sylvestris* leaves (wild sage; Flacourtiaceae) induces partial inhibition of $PLA_2$ activity in venoms containing class I, II and III $PLA_2$. When the extract was tested against the isolated toxins, it showed greater efficacy against class II $PLA_2$ activity in viper venom (Borges et al., 2000).

The inhibition of $PLA_2$ is pharmacologically and therapeutically relevant because these enzymes are related to inflammatory pathologies (Carvalho et al., 2013).

Research has shown that vegetal extracts may have anti-inflammatory properties due to possible direct binding of certain metabolites to the activated site of $PLA_2$.

Some extracts may contain a rich mixture of polyphenols, terpenoids and fatty acids (Dhananjaya and Shivalingaiah, 2016).

Carvalho et al. (2013) argued that the main class of plant secondary metabolites capable of inhibiting $PLA_2$ are phenolic compounds,

including flavonoids, alkaloids, steroids and terpenoids (mono-, di- and triterpenes) as well as polyphenols (tannins).

Another study revealed that the presence of flavonoids, tannins, steroids, saponins and terpenoids in the aqueous extract of *Achyranthes aspera* stems (chaff flower; Amaranthaceae) favored phospholipase $A_2$ inhibition in *Bitis arietans* venom (Nwune et al., 2017). These findings suggest that the possible presence of multiple compounds in the CAME used here may have contributed to the $PLA_2$ inhibition observed.

Reports indicate that *B. alternatus* venom damages the endothelium and basement membrane due to the proteolytic action generated by SVMPs (Gay et al., 2009; Palacio et al., 2012; Matias et al., 2017).

The reduced hemorrhaging observed here corroborates previous findings that the aqueous extract of *Casearia* species decreases hemorrhagic activity (Borges et al., 2000).

According to Silva et al. (2006), the antiophidic activity of aqueous *Casearia* extract is due to its inhibition of SVMPs and $PLA_2$. In a previous study, Oshima-Franco et al. (2005) found that hydroalcoholic *Casearia sylvestris* leaf extract blocked the action of bothropstoxin-I (BthTX-I), isolated from *Bothrops jararacussu*, which affects neuromuscular activity in mice. This protective mechanism is associated with the presence of flavonoids and phenolic compounds in the extract. Chlorogenic acids (CGA) detected in *C. arborea* methanol extract are phenolic compounds formed by the esterification of cinnamic acids such as caffeic, ferulic and p-coumaric acids, with (-) quinic acid (Farah et al., 2008). In a study of *C. sylvestris* leaves, qualitative analysis by thin-layer chromatography (TLC) (Oshima-Franco et al., 2005) and HPLC – PDA – ESI + / MS (Ameni et al., 2015) indicated the presence of CGA.

There is growing scientific evidence to support the health benefits of CGA for humans, especially because it acts as a protective agent by inhibiting oxidative stress (Liang and Kitts, 2016) and inflammation (Girsang et al., 2019). This may be associated with the biological activities of *C. arborea* extract detected here, such as the decline in coagulability and hemorrhaging induced by *B. alternatus* venom. A study with *Argusia argentea* ethanol extract demonstrated its anti-hemorrhaging effect against crude *Trimeresurus flavoviridis* venom, attributed to the association of CGA with caffeic and rosmarinic acids (Aung et al., 2010).

It is important to note that the association between groups such as caffeic acid with CGA may contribute to the anti-hemorrhagic effect. For example, a study found that caffeic acid can bind allosterically to snake venom through the carboxyl group, causing conformational changes and producing anti-inflammatory and anti-myotoxic activity (Ticli et al., 2005).

In a more recent study, Ferreira et al. (2019) tested isolated CGA in association with *Bothrops brazili* venom and found that CGA completely inhibited venom-induced proteolytic activity at ratios of 1:5 and 1:10 (w/w). However, the compound was unable to significantly inhibit the phospholipase $A_2$ activity and fibrinogen chain degradation caused by the venom.

In the present study, given that *C. arborea* extract inhibited phospholipase $A_2$ activity at a ratio of 1:50 (w/w) and that CGA was not tested in isolation, it can be inferred that its association with other compounds may be responsible for this inhibition.

These findings contribute to a better understanding of the chemical composition of *C. arborea*. Further research could highlight its potential as an alternative source of CGA, which can also be used as a chemical marker

of the species for quality control. However, in terms of its antivenom effect, additional studies are needed to investigate the mechanism of action of this compound.

## CONCLUSIONS

*Bothrops* venom is recognized for its proteolytic, myotoxic and hemorrhagic activity. In the present study, after analysis of the harmful effects of *B. alternatus* venom, it was concluded that the venom exhibits rapid coagulant action and phospholipase activity, in addition to promoting muscle damage in mice via creatine kinase. High performance liquid chromatography identified CGA as the major compound in was the *Casearia arborea* methanol extract (CAME) used here. In turn, the extract significantly inhibited the harmful effects of *B. Alternatus* venom, suggesting that CGA may be related to the inhibitory action observed. Additionally, the data obtained here indicate that CAME may be employed as a potential adjuvant in conjunction with the serum therapy currently used to neutralize the effects of *B. alternatus* venom. However, additional studies should be conducted to determine the mechanisms of action of antiophidic activity of this extract, emphasizing the effect of CGA in the extract.

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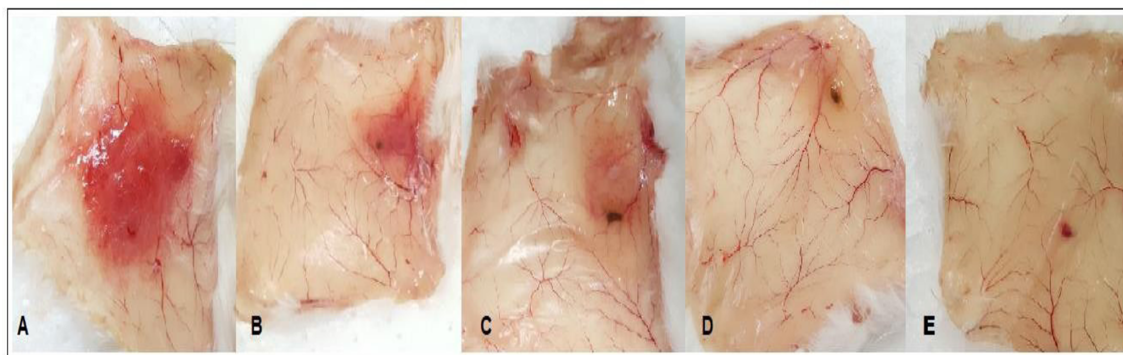



Figure 1 – Inhibition of hemorrhagic activity by 3 MDH (minimum hemorrhagic doses) of *Bothrops alternatus* venom and *Casearia arborea* methanolic extract *(*CAME). Saline solution (NaCl 0.9%; m/v) and *Casearia arborea (*CAME) were applied to the mice individually as control substances. The following groups were used: (A) BV; (B) BV + CAME (1:10, w/w); (C) BV + CAME (1:50, w/w); (D) CAME control, (E) Saline solution. Results are expressed as mean ± SD (n=4). * Significant inhibition difference ($p<0.05$)

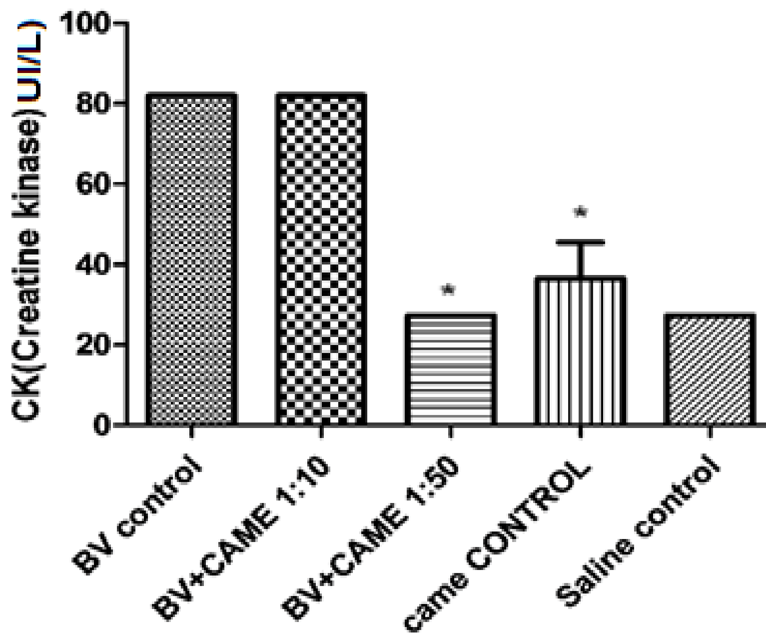



Fig. 2. Neutralization of myotoxic activity induced by crude *B. alternatus* venom (BV - 20µg PB) three hours after inoculation. Activity was expressed in U/L and the results as mean ± SD. * Significant difference (p< 0.05) in percentage inhibition in relation to controls (BV - 20µg PB).

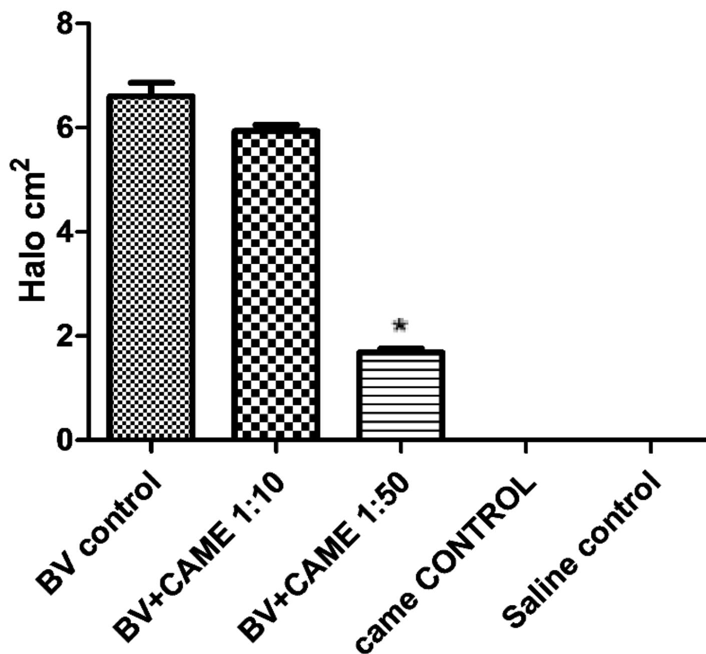
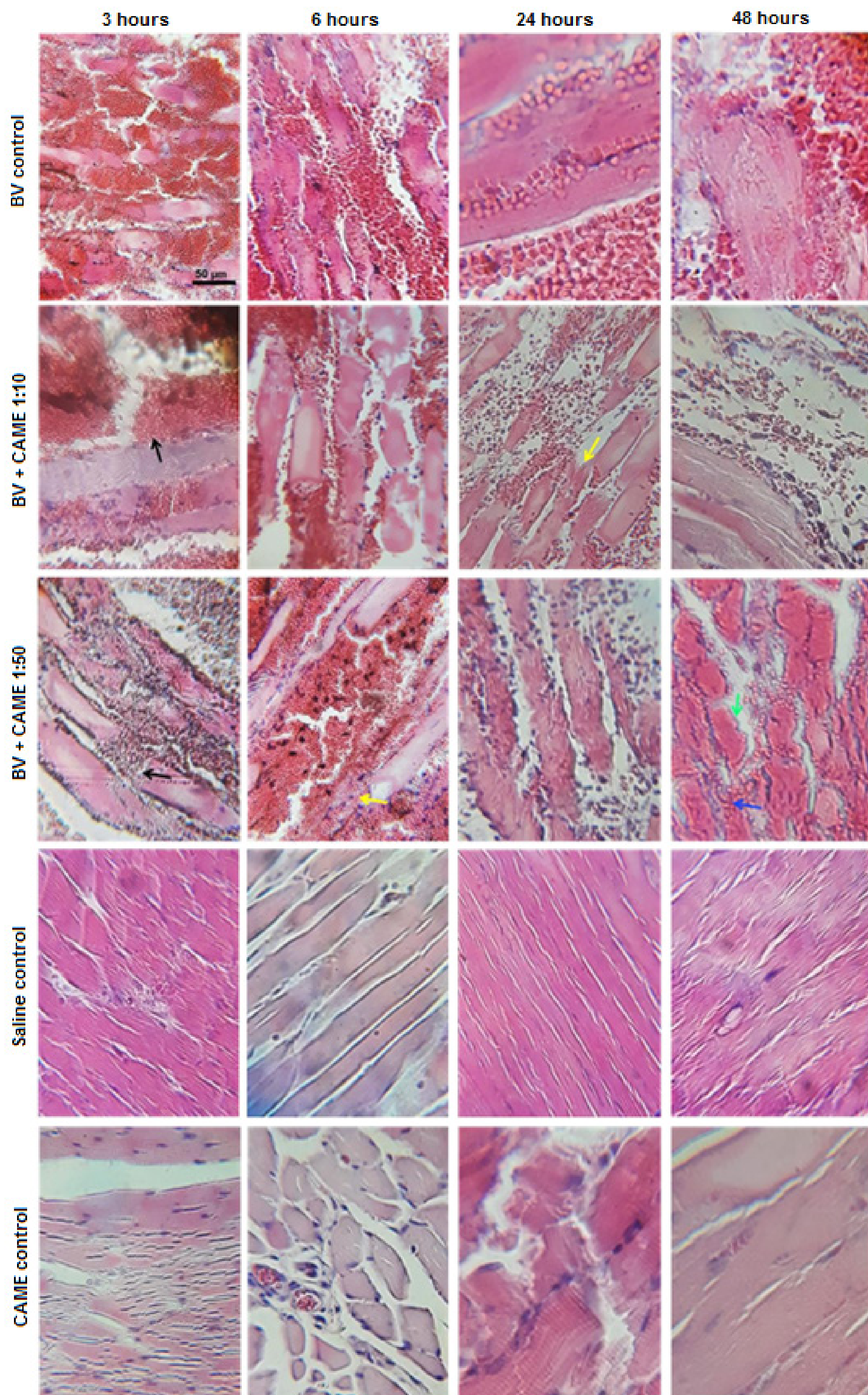



Fig. 3. Phospholipase $A_2$ activity of *B. alternatus* venom in the absence and presence of vegetal extracts (BV + CAME (1: 10, w/w); BV + CAME (1: 50, w/w)). * Significant difference (p< 0.05) in halo in relation to controls (BV - 20µg PB).




Arrows: black = hemorrhage; yellow = necrosis; blue = necrotic bands; green = regenerative cells.

Fig. 4. Analysis of histologic sections of the gastrocnemius muscles of mice 3, 6, 24 and 48 hours after treatment with 20µg of crude *B. alternatus* venom (BV), (BV +CAME(1:10, w/w)), (BV + CAME(1:50,w/w)), Saline (0.9% NaCl; m/v) and CAME alone.



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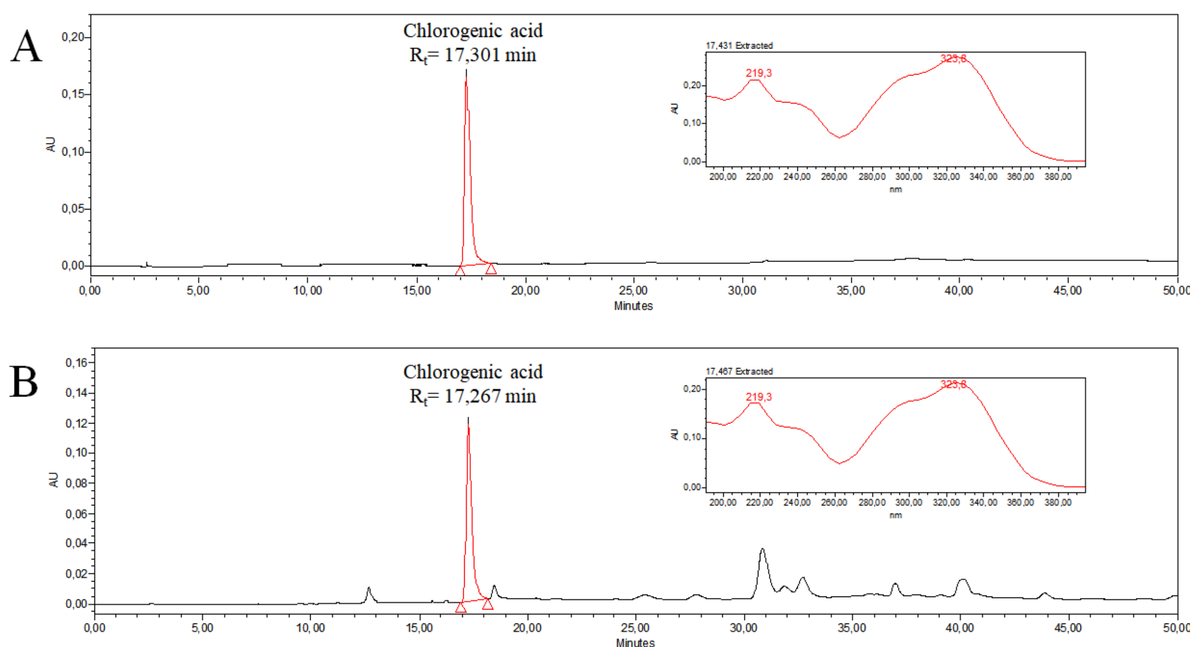


Fig. 5. HPLC-PDA chromatograms (λ = 327 nm) of: chlorogenic acid standard (A); C. arborea extract (B) with UV spectrum (190-400 nm).

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