Journal of Biotechnology and Biodiversity

journal homepage: *https://sistemas.uft.edu.br/periodicos/index.php/JBB/index*

Modulation of hemostasis by inhibiting enzymes with the extract of *Averrhoa carambola* leaves

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INFO

Keyworks
enzymes inhibitors
natural products
medicinal plants
phenolic compounds
venoms as tools

ABSTRACT

Herbal medicines represent an advantageous alternative for the prevention and treatment of several diseases when compared to allopathic medicines. *Averrhoa carambola* (Oxalidaceae) is a plant rich in phenolic compounds and popularly known for its medicinal properties such as anti-inflammatory, antioxidant, and hypoglycemic. Different enzymes of the human organism participate in physiological processes which involve hemostasis, inflammation, and formation of new tissue. These enzymes are highlighted as pharmaceutical targets for the treatment of numerous pathologies. The present work evaluated the aqueous and ethanolic extracts from *A. carambola* leaves on phospholipase, hemolytic, caseinolytic, thrombolytic, coagulant, and fibrinogenolytic activities induced by phospholipases A_2 and proteases. Phenolic compounds and total flavonoids were quantified in the aqueous and ethanolic extracts of the leaves of *Averrhoa carambola*. These extracts were evaluated, in vitro, on phospholipase, proteolytic, hemolytic, thrombolytic and fibrinogenolytic activities induced by snake venoms. The results confirm the pharmacological potential of *A. carambola* since the extracts were able to modulate all evaluated activities related to hemostasis through inhibitions or potentiation of the enzymatic activities (phospholipases A_2 and proteases). The constituents of *A. carambola* may act interfering in processes such as coagulation, thrombus dissolution, and fibrinogenolysis.

RESUMO

Palavras-chaves
inibidores enzimáticos
produtos naturais plantas
medicinais compostos
fenólicos peçonhas como
ferramentas

Modulação da hemostasia por inibição enzimática exercida por folhas de Averrhoa carambola

Os medicamentos fitoterápicos representam uma alternativa vantajosa para a prevenção e tratamento de diversas doenças quando comparados aos medicamentos alopáticos. *Averrhoa carambola* (Oxalidaceae) é uma planta rica em compostos fenólicos e popularmente conhecida por suas propriedades medicinais como anti-inflamatória, antioxidante e hipoglicêmica. Diferentes enzimas do organismo humano participam de processos fisiológicos que envolvem hemostasia, inflamação e formação de novos tecidos. Essas enzimas são destacadas como alvos farmacêuticos para o tratamento de inúmeras patologias. O presente trabalho avaliou os extratos aquoso e etanólico das folhas de *A. carambola* sobre as atividades fosfolipásica, hemolítica, caseinolítica, trombolítica, coagulante e fibrinogenolítica induzidas por fosfolipases A_2 e proteases. Compostos fenólicos e flavonoides totais foram quantificados nos extratos aquoso e etanólico das folhas de *A. carambola*. Esses extratos foram avaliados, *in vitro*, sobre as atividades fosfolipásica, proteolítica, hemolítica, trombolítica e fibrinogenolítica induzidas por peçonhas de serpentes. Os resultados confirmam o potencial farmacológico de *A. carambola* uma vez que os extratos foram capazes de modular todas as atividades avaliadas relacionadas à hemostasia por meio de inibições ou potencialização das atividades enzimáticas (fosfolipases A_2 e proteases). Os constituintes de *A. carambola* podem atuar interferindo em processos como coagulação, dissolução de trombos e fibrinogenólise.

Received 07 May 2021; Received in revised from 01 June 2021; Accepted 28 October 2021

© 2021 Journal of Biotechnology and Biodiversity
ISSN: 2179-4804
DOI: https://doi.org/10.20873/jbb.uft.cemaf.v9n4.oliveira

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INTRODUCTION

The most popular and ancient method in the treatment of diseases is using herbal medicine. Plants constitute a rich source of bioactive compounds, and many drugs that are available in the market were obtained directly or indirectly from them (Dehelean et al., 2021). Approximately 50% of all medicines have their origin from plants, which demonstrate their essential role in the development of the pharmaceutical industry (Glasl and Khan, 2018). Data from the World Health Organization (WHO) show that more than 80% of the world population depends on herbal medicines for disease prevention and treatment. Many herbal medicines have advantages compared to the allopathic ones, such as low cost to obtain, greater safety in their use, and fewer adverse effects (Ahmad and Sharma, 2020).

One of the aggravating factors of ophidian accidents is the local effect, which is conditioned to tissue damages such as necrosis, edema, myotoxicity, and hemorrhage. Envenoming is caused by the collective action of different classes of toxins, such as phospholipases A_2, metalloproteases, serine proteases, L-amino acid oxidases, and hyaluronidases (Gutiérrez et al., 2007).

Some plants are rich in bioactive compounds that are responsible for inhibiting the toxic and pharmacological effects induced by snake venoms, acting as enzymatic inhibitors (Urs et al., 2013). Due to the large number of biological activities induced by these toxins and the high degree of homology that many of them present with enzymes of the human organism, numerous researches have been developed using snake toxins and venoms as tools to induce pharmacological effects and simulate the modulation of human enzymes (Romero et al., 2010).

Several investigations involve mechanisms of action of natural compounds that act as enzymatic inhibitors, and the main classes of toxins used as targets are those involved in inflammatory response and hemostasis (phospholipases A_2, metalloproteases, and serine proteases) (Bijak et al., 2014; Braga et al., 2019; Marques et al., 2019). However, mechanisms of interaction between enzymes and natural compounds are poorly described and require extensive studies of structural and functional characterization (Moura-da-Silva et al., 2016), thus enabling future applications of these compounds in the therapy of human diseases.

Studies point out to different parts of *Averrhoa carambola* L. to have pharmacological properties such as antioxidant, anti-inflammatory, hepatoprotective, and anti-ulcer activity in leaves and stems (Cabrini et al., 2011). Phytochemical analyses of the leaves revealed the presence of bioactive compounds such as saponins, alkaloids, flavonoids, and tannins (Thomas et al., 2008).

The objective of this study was to perform the toxicological and pharmacological characterization of the aqueous and ethanolic extracts of *A. carambola* leaves, evaluating their action on hemostasis and enzymatic activities (exerted by phospholipases A_2 and proteases). Venoms of different snakes were used as tools once they are rich sources of these enzymes and are already broadly characterized.

MATERIAL AND METHODS

Collection and identification of plant material

Leaves of *Averrhoa carambola* (Oxalidaceae) were collected in an orchard in the city of Lavras, MG, Brazil (altitude 845 m, latitude 21.15 ° S, and longitude 45.22 ° W), in December of 2015. The leaves were dried on a forced air oven at 35 °C and then milled in a Willey type mill. The powder obtained was kept at room temperature. The exsiccates of the species were deposited at the Herbarium of the Federal University of Lavras, Brazil, under registration no. 24201.

Preparation of aqueous and ethanolic extracts

The powder obtained from the leaves of *A. carambola* was used to prepare extracts in two different solvents: water (infusion for 30 minutes of 40 g in 400 mL of freshly boiled water) and ethanol (percolation at 92.8 °C and room temperature). The residual solvent in the ethanolic extract was removed in a rotary evaporator at 40 °C. After this process, the aqueous and ethanolic extracts were frozen and then lyophilized. Both extracts were weighed and dissolved in PBS for analysis.

Total phenolic compounds

The determination of the total phenolic compounds of the aqueous and ethanolic extracts from *A. carambola* leaves was performed according to the method of Folin-Denis (Anvisa, 2010). The results were expressed as mg compounds/100 g of sample. The analytical curve was performed with five concentrations of tannic acid (4, 8, 12, 16, and 20 µg mL^{-1}).

Total flavonoids

The methodology described by Woisky and Salatino (1998) was used for the determination of flavonoid contents. Lyophilized (0.1 g) samples of the aqueous and ethanolic extracts from *A.*

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ISSN: 2179-4804
DOI: https://doi.org/10.20873/jbb.uft.cemaf.v9n4.oliveira

carambola leaves were weighed and dissolved in 25 mL of methanol. Aliquots of 50, 200, and 400 µL of each extract were added to 500 µL of a methanolic solution of aluminum chloride 2% (w:v^{-1}) and the volume filled with methanolic acetic acid solution 5% (v:v^{-1}). After 30 minutes, absorbances were read at 425 nm. The analytical curve was performed with aliquots of 50, 100, 200, 300, 400, and 500 µL of quercetin (QE) at 100 µg mL^{-1}.

Snake venoms

Venoms of *Bothrops, Crotalus durissus terrificus* (*C.d.t.*), and *Lachesis muta* were used as the source of enzymes to induce the different activities. They were purchased from the serpentarium Bioagents (Batatais city, state of São Paulo, Brazil) and previously evaluated in different doses to define the minimum effective dose adequate for each activity. The venoms were weighed and dissolved in PBS, pH 7.4, to perform the assays.

Obtaining human blood

The blood was obtained from healthy volunteers, and collected in tubes containing heparin (hemolytic activity), citrate (coagulant activity), and without anticoagulant (thrombolytic activity).

All tests using human blood or its components were carried out with the previous authorization of the Ethics Committee on Human Research (COEP) of the Federal University of Lavras, under the registration number: CAAE: 56619816.6.0000.5148.

Phospholipase and hemolytic activity

The phospholipase and hemolytic activities were evaluated in a solid medium, as described by Gutiérrez et al. (1988). The gel for the evaluation of the phospholipase activity was prepared with 0.01 mol L^{-1} $CaCl_2$, egg yolk lecithins 1:3 v:v, PBS (pH 7.4), 1% bacteriological agar, and 0.005% sodium azide. The medium was poured into Petri dishes at 45-50 °C. After gel solidification, the treatments were applied in 0.5 cm diameter holes, and the dishes were maintained in a cell culture chamber for 12 hours at 37 °C.

The gel was made, for the hemolytic activity, replacing the egg yolk lecithins with a human erythrocyte concentrate calculated to get a hematocrit of 1%. The newly collected blood was centrifuged at 900 *g* for 5 minutes to obtain the erythrocytes. The erythrocytes was then suspended in 5 mmol L^{-1} of PBS (pH 7.4) and centrifuged under the same conditions, repeating this washing step twice.

The inhibition of phospholipase (*Bothrops jararacussu*, *B. moojeni*, and *B. alternatus*) and hemolytic (*B. moojeni*, *B. atrox*, and *Crotalus durissus terrificus (C.d.t.)*) activities induced by snake venoms was evaluated by their previous incubation with extracts from *A. carambola* leaves for 30 minutes at 37 ºC in different proportions (venom: extract, w:w). Controls containing only venoms or extracts were also performed.

Caseinolytic activity

A gel was prepared following the methodology of the phospholipase activity described by Gutiérrez et al. (1988), replacing egg yolk lecithins with a casein solution. The casein concentration was adjusted to 1% per test. The venoms of *B. jararacussu*, and *B. moojeni* (30 µg) and *A. carambola* leaf extracts were preincubated in the proportions of 1:0.5, 1:1, 1:2, 1:4, and 1:8 (venom: extract, w:w) for 30 minutes at 37 °C. Samples were then applied to orifices made in the gel, followed by incubation for a period of 12 hours at 37 °C in a cell culture chamber.

The activities described in phospholipase, hemolytic, and caseinolyticwere evaluated by measuring the diameters of the translucent halos formed in the gel around the holes. The results were expressed in percentage, in which the controls containing snake venom corresponded to 100% of activity.

Thrombolytic activity

The thrombolytic activity was assessed on human blood clots formed *in vitro*, according to the methodology described by Cintra et al. (2012). The clots were incubated for 24 hours at 37 ºC with the samples containing *B. atrox*, *B. moojeni*, and *Lachesis muta* (40 µg) venoms, PBS, or the venoms previously incubated (30 minutes at 37 ºC) with *A. carambola* leaf extracts in the proportions of 1:0.5, 1:1, 1:2, and 1:4 (venom: extract, w:w). The activities were calculated by measuring the volume of fluid released by each thrombus. The data were converted in percentage, and the controls containing only venoms were considered as 100% activity.

Coagulant / anticoagulant activity

The evaluation of clotting time was performed according to Rodrigues et al. (2000). The leaf extracts from *A. carambola* were previously incubated with the *C.d.t.*, *L. muta*, and *B. atrox* venoms for 10 minutes at 37 °C in the proportions of 1:0.1, 1:0.25, 1:0.5, 1:1, 1:2, and 1:4 (venom: extract, w:w). Tubes containing citrated plasma were kept in a 37 °C bath. The incubated samples were added to the plasma (200 µL), and then the

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ISSN: 2179-4804
DOI: https://doi.org/10.20873/jbb.uft.cemaf.v9n4.oliveira

time until the formation of a rigid clot was measured. The minimum coagulant dose was previously defined, which was the lowest amount of venom capable of inducing plasma coagulation in a range between 50 and 180 seconds.

Fibrinogenolylis activity

The protease inhibition assays were performed through the preincubation of *B. moojeni* venom (60 µg) with leaf extracts of *A. carambola* in the proportions of 1:2.5, 1:5, and 1:10 (venom: extract, w:w), for 30 minutes at 37 °C. Then, the fibrinogen (60 µg) was added to the samples, and they remained at the same temperature for another 90 minutes. The samples were analyzed in 12% polyacrylamide gel (w:v), allowing observation of the α, β, and γ fibrinogen chains (Laemmli, 1970). Controls containing venom and fibrinogen were also carried out.

Statistical analysis

The results were presented as the mean of triplicates ± standard deviation. The data were evaluated by analysis of variance, and the means were compared using the Scott Knott test ($p < 0.05$) (R Core Team, 2012).

RESULTS AND DISCUSSION

Total phenolic compounds and total flavonoids

The leaves of *Averrhoa carambola* are rich in flavonoids (Cazarolli et al., 2012; Islam et al., 2020), and their medicinal properties are largely attributed to these compounds. *A. carambola* is scientifically proven to contain properties such as antioxidant, anti-inflammatory, and antihyperglycaemic (Moresco et al., 2012; Siddika et al., 2020; Yang et al., 2020) The ethanolic extract from leaves of *A. carambola* presented the highest total phenolic content (72.49 mg tannic acid/g) and total flavonoids (67.95 mg quercetin/g) (Table 1). In the literature, it was found that the ethanolic extract of *A. carambola* leaves produced 79.07 mg GAE/g for phenolic compounds and 35.25 mg QE/g for flavonoids (Moresco et al., 2012). However, in the present work, the flavonoid content was higher than in the literature. The different percentage of flavonoid concentration observed is possibly due to climatic disturbances and other regional variables to which the plants are subjected.

Table 1 - Contents of yields, total phenolic compounds, and total flavonoids in extracts of leaves of *Averrhoa carambola*

Extracts	Yield %	mg phenolic/ 100g sample	mg flavonoids /100g sample
Aqueous	18.48	4950	1628
EtOH	7.10	7249	6795

Flavonoids are the most relevant products derived from plant material. Many of the biological activities reported for flavonoids (antitumor, antioxidant, and anti-inflammatory) (Ansari and Akhtar, 2019; Jucá et al., 2020), make these compounds to be pharmacologically active. They may interfere in the cell growth regulating pathways, energy metabolism, apoptosis, cell division, transcription, repair genes, inflammation, stress response, and may act on tumor formation (Havsteen, 2002). Thus, flavonoids are promising molecules in the treatment of tumors (Kapoor et al., 2021).

Flavonoids act on cells that are involved in inflammation by inhibiting the proliferation of T lymphocytes, inhibit the production of proinflammatory cytokines (TNF-α and IL-1). They also act by modulating the activity of enzymes on the arachidonic acid pathways such as phospholipases A_2, cyclooxygenase, and lipoxygenase (López-Posadas et al., 2008), which are essential mediators of the inflammatory response (Kim et al., 2004). Quercetin was the first flavonoid to inhibit the phospholipase A_2 activity of human neutrophils. Other flavonoids, such as hesperetin, naringenin, kaempferol, and myricetin, also inhibited the activity of phospholipase A_2 (Lättig et al., 2007). The flavonoid luteolin inhibited the activity of the enzyme cyclooxygenase (COX) (Baumann et al., 1980). Flavonols such as baicalin and catechin are inhibitors of the enzyme lipoxygenase (Burnett et al., 2007).

Phospholipase activity

The aqueous extract inhibited 30% of the phospholipase activity induced by *B. moojeni* venom in 1:0.5 and 1:2 (w:w) ratios and 40% and 25% in the proportions of 1:1 and 1:8 (w:w), respectively. The ethanolic extract inhibited the activity induced by the same venom by 25% when evaluated in the ratios of 1:1 and 1:2 (w:w) and 30% in 1:4 (w:w) ratio (Figure 1).

When using the *B. alternatus* venom, the aqueous extract was responsible for 25% of inhibition after incubation at 1:1 and 1:2 (w:w)

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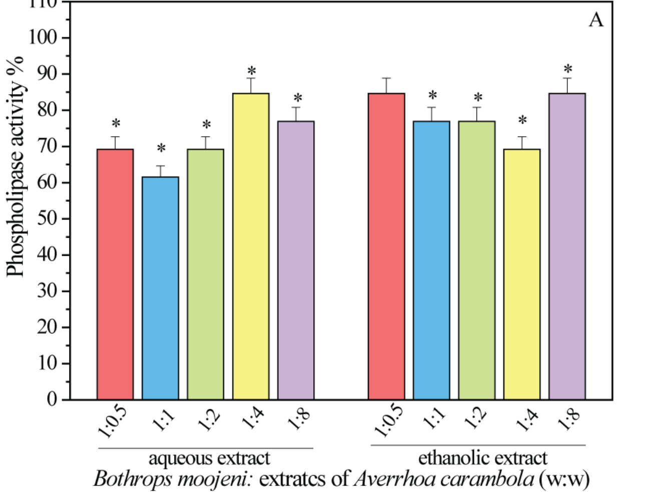
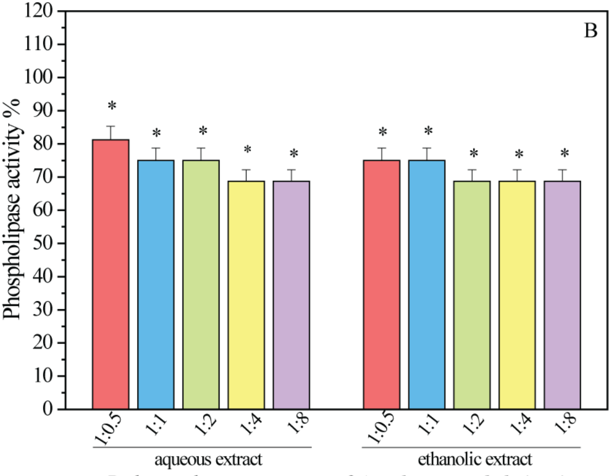
ISSN: 2179-4804
DOI: https://doi.org/10.20873/jbb.uft.cemaf.v9n4.oliveira

ratios, and 32% at 1:4 and 1:8 (w:w). Meanwhile, the ethanolic extract reduced 25% of phospholipase activity at 1:0.5 and 1:1 (w:w) ratios, and 32% in 1:2, 1:4, and 1:8 (w:w) ratios (Figure 1).





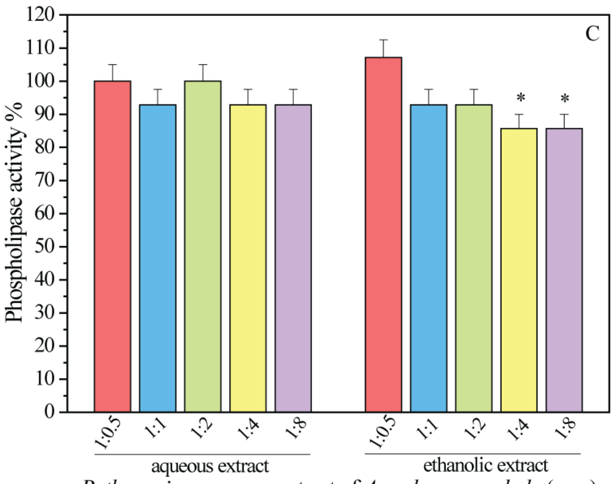


Figure 1 - Phospholipase activity (%) induced by *Bothrops moojeni*, *B. alternatus*, and *B. jararacussu* venoms, previously incubated with the extracts of *Averrhoa carambola*. Control (+) containing only venom (10 µg) was considered as 100% activity. The results correspond to the means of triplicates obtained in each proportion (venom: extract, w:w) and their calculated standard deviations
*Statistically different from the positive control.

The inhibition of phospholipases A_2 by natural compounds is substantial in the scientific context, mainly when aiming at the prospecting mechanisms of action of plant molecules present in therapeutically-used formulations for the treatment of numerous diseases. In addition, this inhibition is related to physiological processes involving inflammatory responses and blood coagulation (Dey and Nath, 2012; Hage-Melim et al., 2013).

One of the inhibition mechanisms suggested in the literature demonstrate the action of plant compounds in the formation of complexes with calcium ions, interfering in their binding to the phospholipases A_2 and, consequently, in the performance of their function as cofactors (Dey and Nath, 2012).

Polyhydroxylated flavonoids are inhibitors of PLA_2s (Kim et al., 2004). They can bind to amide groups of different proteins by strong hydrogen bonds (Mors et al., 2000). This interaction might be one of the possible mechanisms of inhibition that explains the reductions of the phospholipase activity observed in the present work.

Hemolytic activity

The extracts of *A. carambola* did not induce hemolysis under the conditions, concentrations,

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ISSN: 2179-4804
DOI: https://doi.org/10.20873/jbb.uft.cemaf.v9n4.oliveira

and incubation time evaluated.

The ethanolic extract showed inhibitory activity on the *B. atrox* venom, reducing 22% of its hemolytic activity at the 1:0.25 (w:w) ratio. On the other hand, the aqueous extract reduced 30% of the activity induced by the same venom at the 1:0.1 and 1:0.25 ratios and 38% in the ratio of 1:0.5 (w:w) (Figure 2A). 37% of the hemolytic activity induced by the venom of *C.d.t.* was reduced after incubation with the aqueous extract at the ratio of 1:0.5 (w:w), and 50% at 1:0.1 and 1:0.25 (w:w) ratios (Figure 2B). The *B. moojeni* venom had its activity inhibited in 22% when previously incubated with the aqueous extract in the ratio of 1:0.1 (w:w), and 27% when incubated with the ethanolic extract at the 1:0.25 and 1:0.5 (w:w) ratios (Figure 2C).

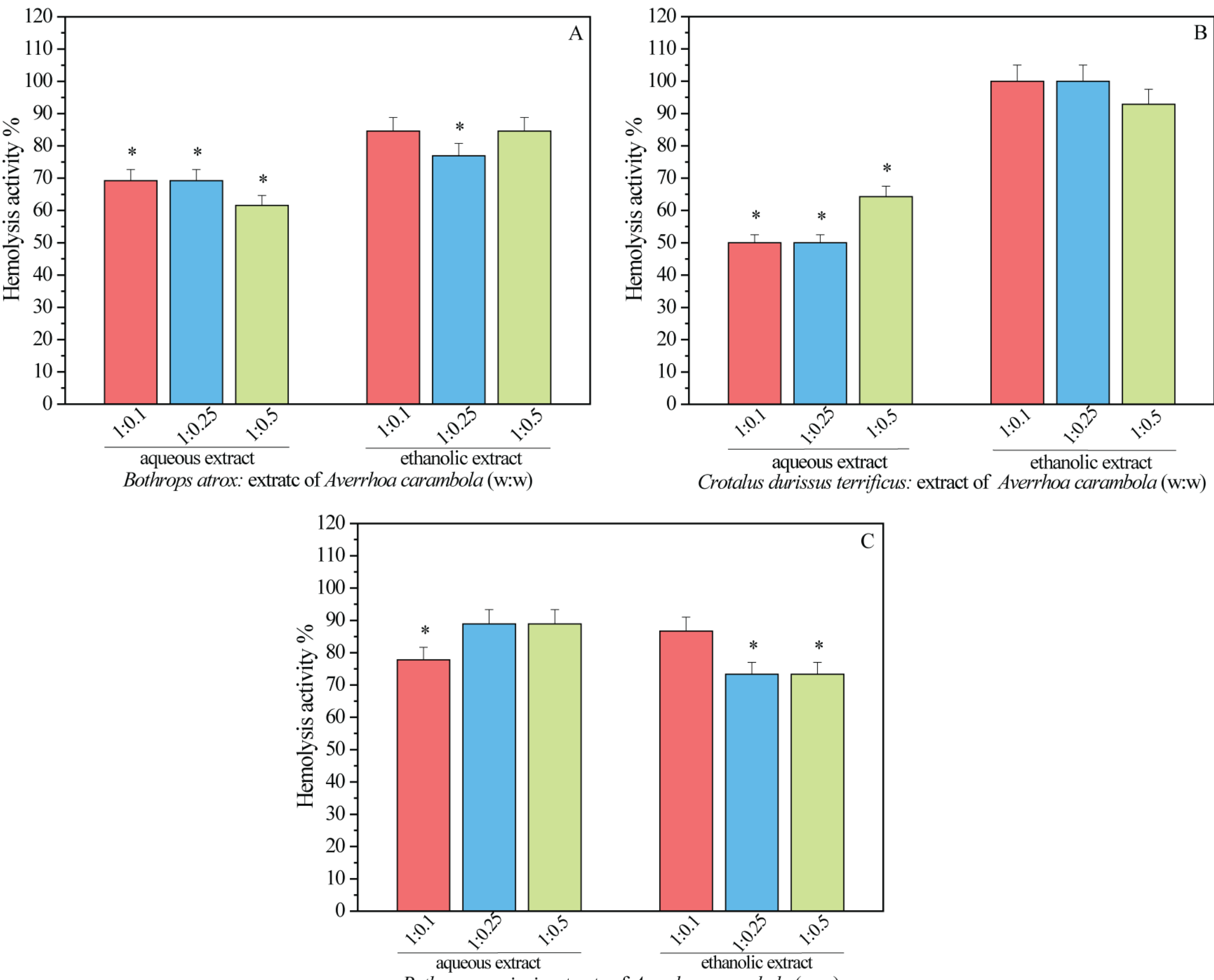

Figure 2 - Hemolytic activity (%) induced by *Bothrops atrox* (A), *Crotalus durissus terrificus* (*C.d.t.*) (B), and *B. moojeni* (C) venoms, previously incubated with the extracts of *Averrhoa carambola*. Controls (+) containing only venom were considered as 100% activity (*B. atrox*, 30 µg, *C.d.t.,* 30 µg, and *B. moojeni*, 60 µg). The results correspond to the means of triplicates obtained in each proportion (venom: extract, w:w) and their calculated standard deviations
*Statistically different from the positive control.

Félix-Silva et al. (2014), when evaluating the aqueous extract of leaves of *Jatropha gossypiifolia* L., observed the absence of hemolytic activity. In this type of extraction, a considerable amount of molecules are primary metabolites (e.g., proteins, amino acids, and carbohydrates), and would justify the absence of toxicity even when the extracts are evaluated in high concentrations.

The hemostatic system components are the main physiological targets of venom toxins (Queiroz et al., 2017). It is suggested that the inhibitory effects presented by these extracts are due to the action of metabolites as enzymatic inhibitors, chemical inactivators, or

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ISSN: 2179-4804
DOI: https://doi.org/10.20873/jbb.uft.cemaf.v9n4.oliveira

immunomodulators that interacted with target macromolecules (Mors et al., 2000).

Caseinolytic activity

The most significant inhibitions of the caseinolytic activity were observed for the *B. moojeni* venom, with an inhibition of 20% in the ratio of 1:8 (w:w) by the aqueous extract and 20 to 27% in the ratios of 1:0.5; 1:4, and 1:8 (w:w) by the ethanolic extract (Figure 3A). Only the ethanolic extract, in the ratios 1:1 and 1:2 (w:w), exerted inhibition on the proteolytic activity induced by the *B. alternatus* venom (Figure 3B).

The aqueous extracts potentiated the proteolytic activity induced by *B. jararacussu* venom in the 1:1, 1:2, 1:4, and 1:8 (w:w) ratios and inhibited the proteolysis in 1:0.5 (w:w). The ethanolic extract, when previously incubated with the same venom, potentiated the activity by inducing 10% more proteolysis at the ratios of 1:4 and 1:8 (w:w) (Figure 3C).

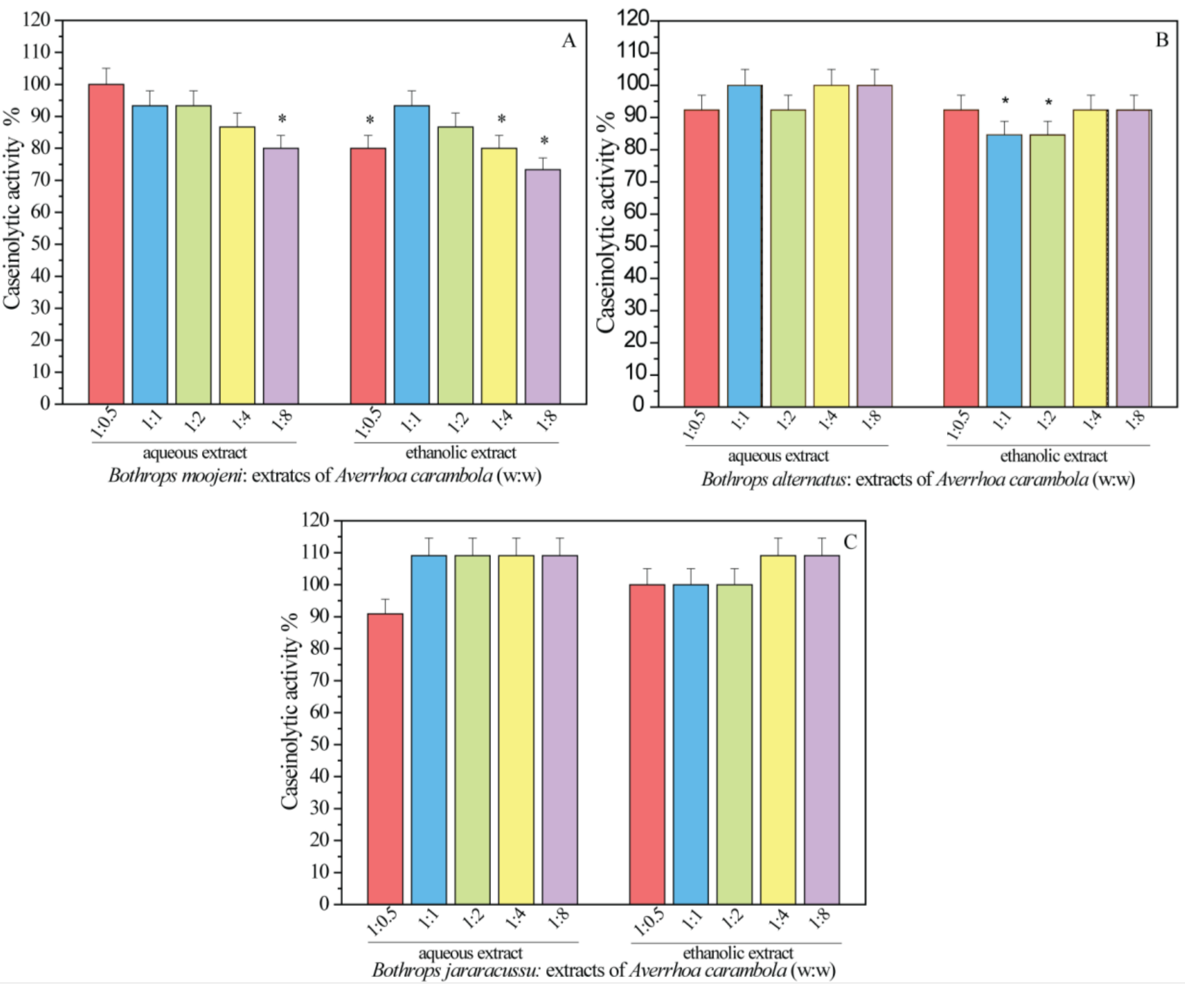

Figure 3 - Caseinolytic activity (%) induced by *Bothrops moojeni* (A), *B. alternatus* (B), and *B. jararacussu* (B) venoms, previously incubated with the extracts of *Averrhoa carambola*. Controls (+) containing only venom (10 µg) were considered as 100% activity. The results correspond to the means of triplicates obtained in each proportion (venom: extract, w: w) and their calculated standard deviations
*Statistically different from the positive control.

The aqueous extracts from *Lannea acida* and *Bauhinia thonningii* and the ethanolic extracts from *Grewia mollis*, *Waltheria indica*, and *Pentanisia prunelloides* inhibited 90% of the proteolysis activity induced by *Bitis arietans* venom. In these extracts were found significant amounts of polyphenols, such as tannins (Molander et al., 2014). They have vast medical applications and can interact with enzymes present in venoms, inactivating or reducing their activities (Pithayanukul et al., 2010).

The ethanolic extract from the leaves of *Renealmia alpinia* inhibited a metalloprotease isolated from the venom of *B. atrox* by 25.9%, 35.2%, 48.4%, 65.1%, and 75.8% in the proportions of 1:1, 1:2.5, 1:5, 1:10, and 1:20

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ISSN: 2179-4804
DOI: https://doi.org/10.20873/jbb.uft.cemaf.v9n4.oliveira

(w:w), respectively. Flavonoids, terpenoids, and coumarins were found in this extract (Patiño et al., 2013). The flavonoids gallocatechin and myricetin-3-o-glycoside were inhibitors of the metalloprotease activity (Vale et al., 2011).

The direct action on enzymes present in venoms, through weak interactions, as well as interactions of plant compounds with cofactors essential for the catalytic activity of these enzymes, have already been described as mechanisms of action (Núñez et al., 2005). Plant extracts containing significant amounts of flavonoids can act as metal chelators and sequestration of zinc ions, which are essential for the catalytic activity of several proteases present in venoms (Mira et al., 2002).

Thrombolytic activity

The aqueous extract from leaves of *A. carambola* resulted in a significant increase in the thrombolytic activity induced by *B. atrox* venom [75%, 85%, and 50% in the proportions of 1:0.5, 1:1, and 1:2 (w:w), respectively]. The ethanolic extract also increased the thrombolytic activity by 80%, 120%, 140%, and 40% in the ratios of 1:0.5, 1:1, 1:2, and 1:4 (w:w), respectively (Figure 4A).

Differently, the thrombolytic activity induced by *L. muta* venom was reduced in 30%, 43%, and 45% after incubation with the aqueous extract in the ratios of 1:0.5, 1:2, and 1:4 (w:w), respectively. Significant inhibitions were also observed to the ethanolic extract, with reduced activity of 20% and 50% for the proportions 1:0.5 and 1:4 (w:w), respectively (Figure 4B).

The aqueous extract significantly increased the thrombolysis induced by *B. moojeni* venom in 50% and 70% in the proportions of 1:1 and 1:2 (w:w), respectively. However, when evaluated at the ratio of 1:0.5 (w:w), the aqueous extract inhibited 50% of the thrombolytic activity. The ethanolic extract, in the proportions of 1:1, 1:2 and 1:4 (w:w), induced a significant increase (between 30 and 48%) under the same conditions (Figure 4C).

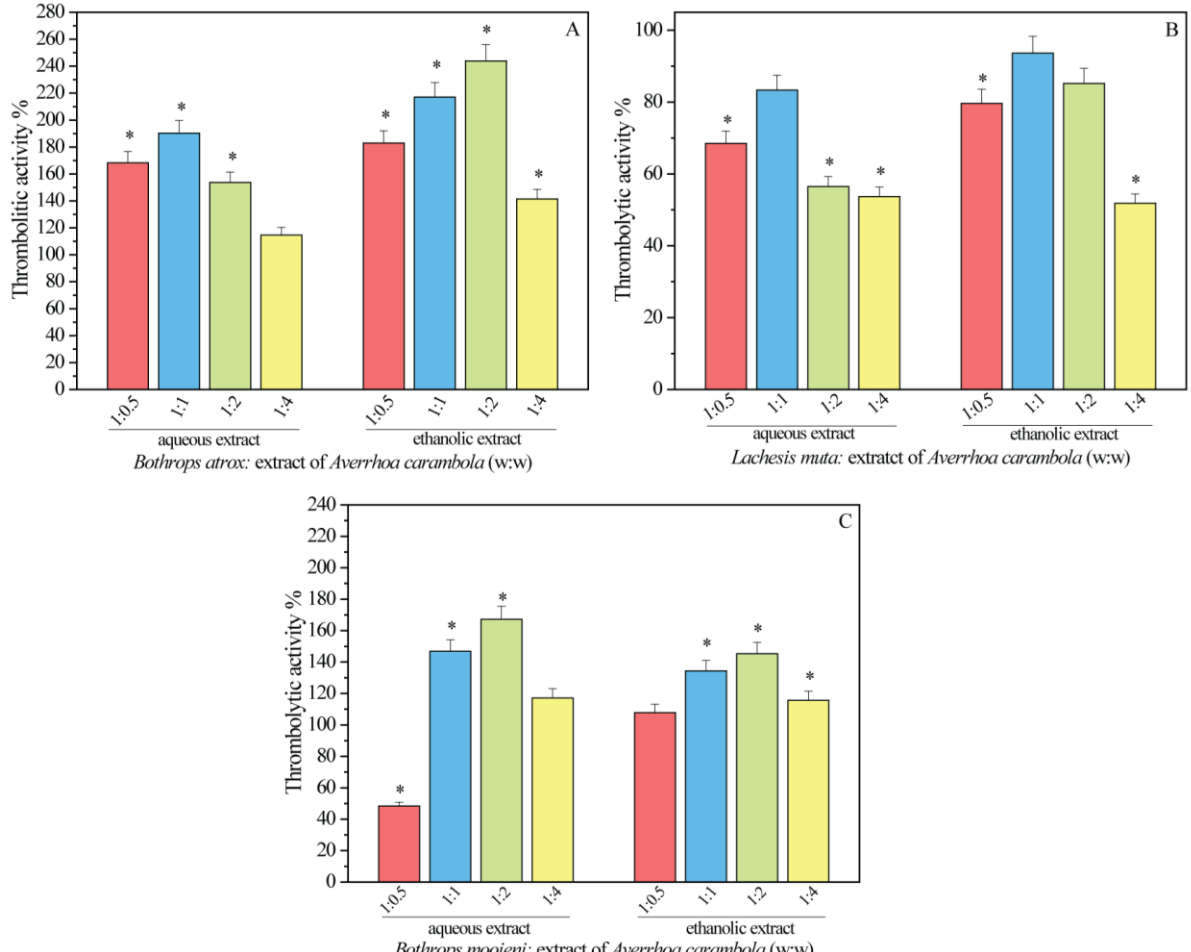

Figure 4 - Thrombolytic activity (%) induced by the venoms from *Bothrops atrox* (A), *Lachesis muta* (B), and *B. moojeni* (C), previously incubated with the extracts of *Averrhoa carambola*. Controls (+) containing only venom (40 µg) were considered as 100% activity.The results correspond to the means of data triplicates obtained in each proportion (venom: extract, w:w) and their calculated standard deviations
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The inhibition by natural compounds of some proteases present in snake venoms simulates the anticoagulant effects of these compounds on animal organisms since these proteases act on the factors of the coagulation cascade and exert similar thrombin function, exhibiting high homology with human enzymes (Marsh, 1994).

The metalloproteinases in the *Bothrops* snakes venoms induce mainly hemorrhage and debridement of tissues, and these enzymes require bivalent ions as cofactors (e.g., zinc, calcium, magnesium) (Fox and Serrano, 2008). Tannins and flavonoids present in plant extracts inhibit metalloproteases by zinc chelation (Castro et al., 1999), consequently preventing the degradation of the components responsible for the hemostatic balance.

Some authors suggest the use of plant extracts in local administration to allow their inhibitory action in metalloproteases (Assafim et al., 2011).

In the present study, the aqueous and ethanolic extracts of *A. carambola* showed anticoagulant activity (Table 2). In addition to the proposed mechanisms related to enzymatic inhibition, the inhibiyion may also be the result of the activity exerted by the flavonoids present in *A. carambola* leaves on the production of nitric oxide in blood platelets, inhibiting platelet aggregation and delaying the formation of blood clots (Bhowmick et al., 2014).

Different proportions of the *A. carambola* extracts incubated with *B. atrox* and *B. moojeni* venoms presented thrombolytic activity (Figure 4A and 4C), suggesting that flavonoids contained in the extracts may be acting as inhibitors of thrombin. Therefore, they have great potential for application in the treatment of thrombotic diseases (Bijak et al., 2014).

On the other hand, when observing the results obtained for the activity of the extracts on the *C.d.t.* venom in the coagulation test (Table 2) and on the *L. muta* venom in the thrombolytic test (Figure 4B), it is suggested their potential application in the treatment of hemorrhagic diseases, since they accelerated the formation of a clot (procoagulant action) and significantly decreased the thrombolytic activity.

Coagulant/anticoagulant activity

The aqueous extract from *A. carambola* leaves exerted procoagulant activity, reducing the clotting time by 8 to 50 seconds, when previously incubated with the *C.d.t.* at the ratios 1:0.1, 1:0.25, 1:1, 1:2, and 1:4 (w:w). The ethanolic extract showed procoagulant activity in the proportions of 1:0.1, 1:0.25, and 1:1 (w:w), with reductions between 6 and 24 seconds. It also demonstrated anticoagulant activity at the ratios 1:2 and 1:4 (w:w), which increased the clotting time by 15 and 38 seconds, respectively (Table 2).

Table 2 - Effect of the extracts from *Averrhoa carambola* leaves on the coagulant activity induced by different snake venoms

		Clotting time (s)		
		Crotalus durissus terrificus	*Lachesis muta*	*Bothrops atrox*
*Control		175.00 ± 8.75	76.00 ± 3.80	51.00 ± 2.55
Sample	Ratio (w:w)			
Aqueous extract	1:0.1	136.00 ± 6.80 a	85.00 ± 4.25 b	90.00 ± 4.50 b
	1:0.25	167.00 ± 8.35 a	51.00 ± 2.55 a	49.00 ± 2.45
	1:1	125.00 ± 6.25 a	77.00 ± 3.85	50.00 ± 2.50
	1:2	153.00 ± 7.65 a	96.00 ± 4.80 b	88.00 ± 4.40 b
	1:4	158.00 ± 7.90 a	90.00 ± 4.50 b	69.00 ± 3.45 b
Ethanolic extract	1:0.1	151.00 ± 7.55 a	85.00 ± 4.25 b	57.00 ± 2.85 b
	1:0.25	155.00 ± 7.75 a	94.00 ± 4.70 b	39.00 ± 1.95 a
	1:1	169.00 ± 8.45 a	81.00 ± 4.05 b	65.00 ± 3.25 b
	1:2	190.00 ± 9.50 b	72.00 ± 3.60	83.00 ± 4.15 b
	1:4	213.00 ± 10.65 b	74.00 ± 3.70	81.00 ± 4.05 b

a – Differ from the positive control ($p < 0.05$) - reduces time.
b – Differ from the positive control ($p < 0.05$) - increases time.
*The controls were carried out with 10 µg of each evaluated venom.
The results are presented as the average of triplicates ± standard deviation $p < 0.05$.

Incubations of *L. muta* venom with the aqueous extract increased the clotting time by 9 up to 20 seconds, characterizing an anticoagulant action at the ratios of 1:0.1, 1:2, and 1:4 (w:w). It was observed procoagulant action only in the ratio of 1:0.25 (w:w), with a reduction of 25 seconds in

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ISSN: 2179-4804
DOI: https://doi.org/10.20873/jbb.uft.cemaf.v9n4.oliveira

clotting time (Table 2). The ethanolic extract was responsible for increasing the clotting time induced by the venom of *L. muta* when evaluated at the proportions of 1:0.1, 1:0.25, and 1:1 (w:w), with values between 5 and 18 seconds higher than the positive control (Table 2).

Previous incubations of the aqueous extract with the *B. atrox* venom in the ratios 1:0.1, 1:2, and 1:4 (w:w) resulted in anticoagulant activity, with the clotting time increasing by 18 to 39 seconds (Table 2). Similarly, the ethanolic extract increased the clotting time up to 32 seconds in the proportions of 1:0.1, 1:1, 1:2, and 1:4 (w:w). However, it induced procoagulant activity when evaluated at the ratio of 1:0.25 (w:w) (Table 2).

Procoagulant and anticoagulant activities observed for the same extract (in different proportions) acting on the same venom suggests the presence of active substances that inhibits some classes of proteases or molecules that potentiate some classes of enzymes. Some hemorrhagic metalloproteinases act as anticoagulants, and the fibrinogenolytics ones act as procoagulants, generating fibrin networks. Serine proteases are responsible for forming friable clots that can also act as anticoagulants but with less efficiency. In addition, phospholipases A_2 can act as pro-coagulants (Lu et al., 2005).

Whether the extracts will be used to form a clot to stagnate hemorrhages or in the dissolution of clots, acting as anticoagulant agents of wide application in the treatment of cardiovascular diseases, knowing the different enzymes that act in blood coagulation is necessary to evaluate different concentrations of the extracts. Once that is properly done, defining the appropriate amounts of active substances that will provide a given plant's expected action is much easier.

Fibrinogenolysis activity

The two extracts evaluated were efficient in inhibiting fibrinogen molecules' degradation, induced by the *B. moojeni* venom, in all ratios evaluated (Figure 5).

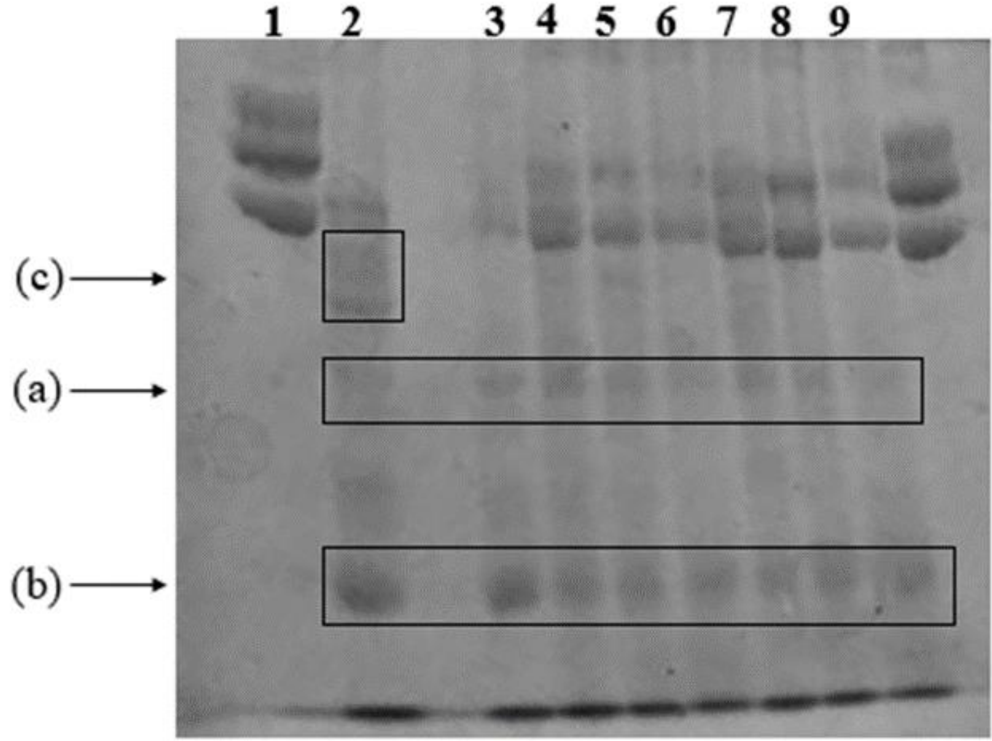

Figure 5 - Electrophoretic profile (SDS-PAGE) of the proteolytic action of *Bothrops moojeni* venom on fibrinogen. Effect of extracts from *Averrhoa carambola* leaves on the activity of the venom. 1- Fibrinogen (60 µg); 2- *B. moojeni* (60 µg) + fibrinogen; 3- *B. moojeni*; 4- *B. moojeni* + fibrinogen + aqueous extract of *A. carambola* (1:2.5 w:w); 5- *B. moojeni* + fibrinogen + aqueous extract of *A. carambola* (1:5 w:w); 6- *B. moojeni* + fibrinogen + aqueous extract of *A. carambola* (1:10 w:w); 7- *B. moojeni* + fibrinogen + ethanolic extract of *A. carambola* (1:2.5 w:w); 8- *B. moojeni* + fibrinogen + ethanolic extract of *A. carambola* (1:5 w:w); 9- *B. moojeni* + fibrinogen + ethanolic extract of *A. carambola* (1:10 w:w). (a) Proteases present in the venom. (b) Phospholipases A_2 present in the venom. (c) Fibrinopeptides released from the breakdown of the α and β chains of the fibrinogen molecules.

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ISSN: 2179-4804
DOI: https://doi.org/10.20873/jbb.uft.cemaf.v9n4.oliveira

In our study, the extracts prevented the breakdown of fibrinogen (Figure 5) by proteolytic enzymes present in the venom, which results in the non-formation of the fibrin networks and consequently reducing the formation of clots. The coagulant activity results corroborate the inhibition presented in the fibrinogenolysis activity since clotting time prolongations were observed for the extracts in different ratios (Table 2).

One of the proposed mechanisms of action for plant molecules involves the formation of complexes with zinc ions in the binding sites present in the structures of metalloproteases, resulting in the inhibition of these enzymes (Moura-da-Silva et al., 2016). Thus, the inhibitions observed in the caseinolytic, thrombolytic, fibrinogenolytic, and coagulant activities of the present study, whose proteases play a fundamental role, can be partially attributed to the formation of ionic complexes with plant compounds.

The ability of flavonoids to bind to amide groups of proteins by hydrogen bonds (Pithayanukul et al., 2010) can result in structural alterations that lead to a partial or total loss of activities performed by enzymes. This is one more type of mechanism of action that would explain the inhibitions of proteases observed in the present work.

CONCLUSIONS

The extracts of *Averrhoa carambola* leaves present bioactive coumpounds that have the therapeutic potential to treat several diseases related to hemostasis disorders. The present work highlights the high content of phenolic compounds and total flavonoids present in the extracts, which are probably related to the enzymes inhibition observed. The leaves of *A. carambola* significantly inhibit phospholipases A_2 and proteases (mainly fibrinogenolytic and thrombin-like). These classes of enzymes presented in the venoms have high structural and functional homology with human enzymes and are involved in processes such as blood coagulation cascade, platelet aggregation, tissue regeneration, and inflammatory and immune responses. Thus, the *A. carambola* leaves extract have a grea grerat potential for future use as therapeutic drugs. However, it is still needed further studies for the refinement of doses and the development of a suitable formulation for human consumption.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial support by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Nível Superior (CAPES). The authors express their gratitude to the Federal University of Lavras for the structural and logistical support.

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ISSN: 2179-4804
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DOI: https://doi.org/10.20873/jbb.uft.cemaf.v9n4.oliveira