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Neutralization of proteases from *Bothrops* snake venoms by the aqueous extract from *Casearia sylvestris* (Flacourtiaceae)

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Abstract

Aqueous extract from *Casearia sylvestris* leaves, a typical plant from Brazilian open pastures, was able to neutralize the hemorrhagic activity caused by *Bothrops asper*, *Bothrops jararacussu*, *Bothrops moojeni*, *Bothrops neuwiedi* and *Bothrops pirajai* venoms. It also neutralized two hemorrhagic metalloproteinases from *Bothrops asper* venom. Proteolytic activity on casein induced by bothropic venoms and by isolated proteases, including Bn2 metalloproteinase from *B. neuwiedi* venom, was also inhibited by the *C. sylvestris* extract in different levels. The α-fibrinogen chain was partially protected against degradation caused by *B. jararacussu* venom, when this venom was incubated with *C. sylvestris* extract. We also observed that this extract partially increased the time of plasma coagulation caused by *B. jararacussu*, *B. moojeni* and *B. neuwiedi* venoms. *C. sylvestris* extract did not induce proteolysis in any substrate assayed.

Keywords: Snake venoms; Proteases; Antiophidian activity; *Casearia sylvestris* extracts

Animal venoms, including snake venoms, are complex mixtures of proteins. Among these are hemorrhagins, proteases, phospholipases A_2 (PLA_2) and myotoxins that act by different mechanisms. Venom composition may vary according to nutritional, geographic and seasonal factors (Assakura et al., 1992; Rodrigues et al., 1998).

Envenomation caused by snake venoms of the genus *Bothrops* induces many local effects such as myonecrose, edema and hemorrhage (Rosenfeld, 1971; Gutiérrez et al., 1995). These venoms contain many proteolytic enzymes that degrade a variety of natural substrates such as casein, hemoglobin, collagen, elastin, fibrinogen, fibronectin and others (Iwanaga and Suzuki, 1979). Hemorrhagic toxins are among these enzymes and are responsible for degradation of proteins from the extracellular matrix or alterations in blood coagulation (Matrisian, 1992; Markland, 1998). Hemorrhage induced by snakebite occurs due to hemorragins, zinc-dependent metalloproteinases, capable of disrupt the basement membrane of capillaries and causing alterations on capillary vessels (Bjarnason and Fox, 1994).

Medicinal plants play a key role in world health, as they are source of many pharmacologically active compounds such as flavonoids and tannins. Many of these substances structurally resemble biological compounds and this similarity is the basis of their physiological action (Havsteen, 1983). The use of plant extracts as antidote for animal venoms is an old option found in many communities that do not have a prompt access to serum therapy. In addition, depending on the time between the accident and treatment, the ability of the antiserum to neutralize local effects of envenomation is only partial. Vegetal extracts become then an attractive research material as an alternative substitute for antiserum (Rizzini et al., 1988; Ruppelt et al., 1990; Pereira et al., 1992; Martz, 1992).

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Abbreviations: Cs, *Casearia sylvestris* aqueous extract; BaP1 and BH4, metalloproteinases from *Bothrops asper* venom; Bn2, metalloproteinase from *Bothrops neuwiedi* venom; PBS, Phosphate-buffered saline solution

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In Brazil, the popular name of *Casearia sylvestris* (Flacourtiaceae) is Guaçatonga and it has a widespread use in folk medicine as antiseptic, healer, topical anaesthetic, antitumor agent, antiulcer and antiophidian (Itokawa et al., 1988; Basile et al., 1990). The neutralization activity of *C. sylvestris* against crude venoms and purified toxins from several genera (*Bothrops*, *Crotalus*, *Micrurus* and *Heloderma*) of snakes and *Apis mellifera* bee venom was investigated (Borges et al., 2000). This neutralization may be due to the presence of enzymatic inhibitors, chemical inactivators or immunomodulators (Hart et al., 1989). The mechanism of action is still unknown.

This study shows the ability of the aqueous extract from *C. sylvestris* to neutralize the hemorrhagic, coagulant and proteolytic activity on casein or fibrinogen, induced by five bothropic venoms (*Bothrops asper*, *Bothrops jararacussu*, *Bothrops moojeni*, *Bothrops neuwiedi* and *Bothrops pirajai*) and by three metalloproteinases (BaP1 and BH4 from *B. asper* and Bn2 from *B. neuwiedi*).

Snake venoms were collected in the serpentarium of Ribeirão Preto School of Medicine, SP, Brazil, and in Clodomiro Picado Institute, UCR, Costa Rica. The proteases BaP1 and BH4 from *B. asper* and Bn2 from *B. neuwiedi* were previously isolated by Gutiérrez et al., 1995; Franceschi et al., 2000; Rodrigues et al., 2000, respectively. The leaves of *C. sylvestris*, collected in Uberlândia-MG, Brazil, were washed, stirred with deionized water in a waring blender for 15 min at room temperature and then sieved. The filtrate was centrifuged at $30.000 \times g$ for 20 min and the supernatant was lyophilized and stored at −20°C. Leaf extract was weighed and dissolved in deionized water before use (Borges et al., 2000). The extract concentration was expressed in terms of dry weight. Adults Swiss mice were obtained from the vivarium of Ribeirão Preto School of Medicine, SP, Brazil.

Hemorrhagic activity was assayed according to the method of Nikai et al. (1984). Mice (20–25 g) received three minimum hemorrhagic doses (MHD) of crude venoms or isolated metalloproteinases (5 and 15 μg) in 50 μl of PBS, intradermally. Three hours later, the animals were sacrificed and the inner surface of the skin was examined. The MHD (defined as the amount of venom that results in a hemorrhagic spot of about 1 cm in diameter) for the *B. jararacussu* venom is 50 μg, *B. neuwiedi* is 8 μg, *B. moojeni* and *B. asper* is 5 μg.

Fibrinogenolytic (Edgar and Prentice, 1973) or caseinolytic activities (Franceschi et al., 2000) were evaluated trough incubation of the whole venom (*B. asper*, *B. moojeni*, *B. pirajai*, *B. neuwiedi* and *B. jararacussu*) or purified proteases (BaP1, BH4 and Bn2) with fibrinogen or casein at 37°C for 5 min. For caseinolytic activity, 40 μg each venom or 20 μg of each protease were utilized, while 1 μg of *B. jararacussu* venom was used to verify fibrinogen proteolysis.

Coagulant activity on bovine plasma of *B. jararacussu* (50 μg), *B. moojeni* (50 μg) and *B. neuwiedi* (5 μg) venoms was assayed according to Assakura et al. (1992); 50 μl of venom solutions were added to 200 μl of bovine plasma at 37°C. The time to clot the plasma solution, in s, was recorded.

Venoms and toxins were dissolved in phosphate-buffered saline, pH 7.2 (PBS). Proteins were estimated by the methods of Itzhaki and Gill (1964) and Bradford (1976). For neutralization assays of the hemorrhagic, proteolytic and coagulant activities, toxins and venoms were previously incubated with the extract at different ratios (1:1; 1:3; 1:5 and 1:10 w/w, venom:extract) for 30 min at room temperature.

The hemorrhagic activity caused by intradermal injection of 3MDH of *B. asper*, *B. jararacussu*, *B. neuwiedi* and *B. pirajai* venoms and by the proteases BaP1 and BH4 isolated from *B. asper* were significantly neutralized by the *C. sylvestris* extract at a ratio of 1:3 (w/w venom:extract). Fig. 1(A) shows the inner surface of the skin and Fig. 1(B) represents the diameter of the hemorrhagic spot.

Antihemorrhagic compounds have purified from different sources. The compounds Ar turmerone has been isolated from *Curcuma longa* roots, Zingiberaceae (Ferreira et al., 1992) and Wedelolactone was isolated from *Eclipta prostata* leaves, Asteraceae (Melo et al., 1994). Furthermore, many antihemorrhagic proteins have already been isolated from the serum of resistant animals (Soares et al., 1997; Pérez and Sánchez, 1999).

Our results clearly indicate that *C. sylvestris* extract contains compounds capable of neutralizing the hemorrhagic activity induced by crude venoms or by isolated toxins. PLA_2 activity also was inhibited by this extract. PLA_2 enzymes are related with a wide variety of pharmacological activities and among these is miotoxicity, which was neutralized by *C. sylvestris* too (Borges et al., 2000).

Hemorrhagins and phospholipases are enzymes that need a divalent metal ion for their activity. So, we can suggest that the extract has compounds that bind these ions, causing inhibition. Similar results were obtained when Wedelolactone, isolated from *E. prostrata* plant, neutralized PLA_2 and proteolytic enzymes in venoms responsible for myotoxic and hemorrhagic activities (Melo and Ownby, 1999).

Fig. 2 shows the fibrigenolytic activity exerted by *B. jararacussu* venom. Proteolytic enzymes present in this venom degraded preferentially the Aα chain of bovine fibrinogen in few minutes. Partial inhibition of the Aα chain degradation was observed when venom and extract were incubated together before being mixed to fibrinogen at a ratio of 1:10 (w/w, venom:extract).

Caseinolytic activity induced by crude venoms [Fig. 3(A)] or isolated proteases [Fig. 3(B)] showed a significant inhibition when these venoms or toxins were incubated with *C. sylvestris* leaf extract at different ratios. Neither fibrinogenolytic activity (results not shown) nor caseinolytic activity were induced by *C. sylvestris* extract alone (Fig. 3).

Inhibition of caseinolytic and fibrinogenolytic activities by other compounds has already been shown. ABC

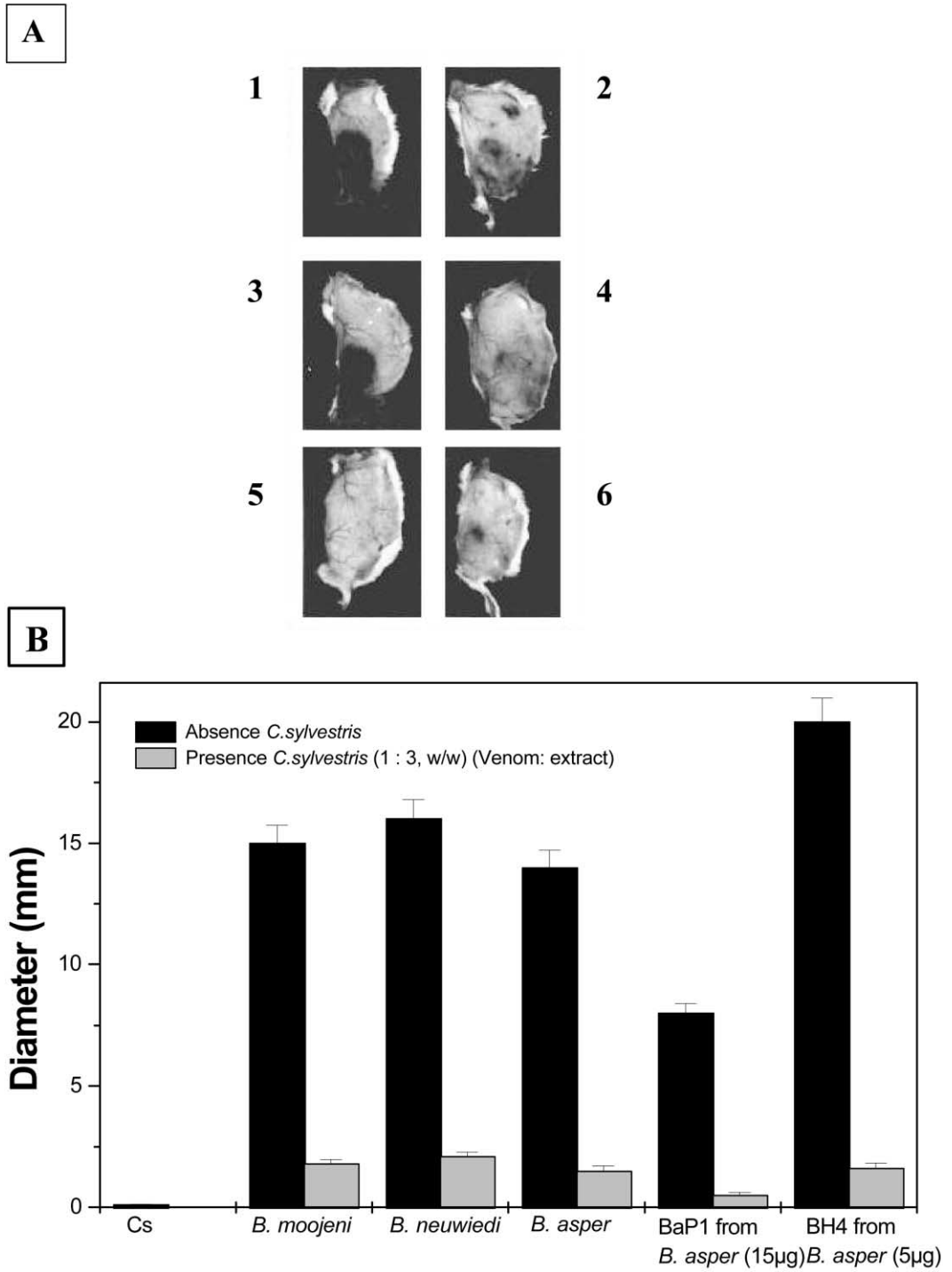

Fig. 1. Effect of *C. sylvestris* extract on the hemorrhagic activity of crude venoms or isolated metalloproteinases. 3MDH crude venom or 5 and 15 μg isolated proteases were incubated for 30 min at room temperature, with either PBS or *C. sylvestris*. Fifty μl this mixture was injected intradermally in mice. The skins were removed after 3 h and the diameters of the hemorrhagic spot were measured. Ratio venom:extract 1:3(w/w, venom:extract). (A) Skins show the spot in the inner side: 1, *B. neuwiedi* (24 μg); 2, *B. neuwiedi* + *C. sylvestris*; 3, *B. jararacussu* (150 μg); 4, *B. jararacussu* + *C. sylvestris*; 5, PBS; 6, *C. sylvestris*. (B) Diameter of the skin lesion. Each bar represents the mean ± SD ($n = 6$). The difference between Absence *C. sylvestris* and Presence *C. sylvestris* is significant ($P < 0.05$ One-way ANOVA).

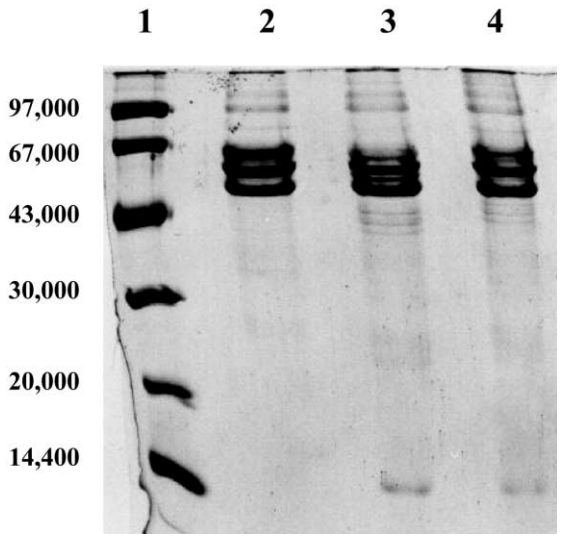

Fig. 2. Inhibition of proteolysis of bovine fibrinogen induced by *B. jararacussu*. *B. jararacussu* venom (1 μg) was pre-incubated with *C. sylvestris* extract for 30 min at room temperature and was mixed with 50 μl of fibrinogen solution (1 mg/ml) at 37°C for 5 min. A SDS-polyacrilamide gel electrophoresis (16%) shows fibrinogen degradation. Lane 1: molecular mass markers: phosporilase b (97,000); bovine serum albumin (67,000); ovoalbumin (43,000); carbonic anhydrase (30,000); soybean trypsin inhibitor (20,000) and α-lactoalbumin (14,400). Lane 2: fibrinogen control (without venom or extract). Lane 3: fibrinogen incubated with *B. jararacussu*. Lane 4: fibrinogen incubated with *B. jararacussu* in presence of *C. sylvestris* extract at a ratio 1:10 (w/w, venom:extract).

complex, an antihemorrhagic factor purified from *Didelphis marsupialis* serum, inhibited the caseinolytic and fibrinogenolytic activities of *B. jararaca* venom. Electrophoretic evidences have shown a non-covalent complex formation between the ABC complex and the component(s) of *B. jararaca* venom (Neves-Ferreira et al., 1997).

B. jararacussu, *B. neuwiedi* and *B. moojeni* venoms induced bovine plasma coagulation in 10–50 s. When these venoms were preincubated with *C. sylvestris* at a ratio of 1:10(w/w, venom:extract), an increase in coagulation time was observed, but total inhibition was not verified (Table 1).

Hemostatically active components are largely distributed in snake venoms. These compounds interact with proteins of the coagulation cascade and fibrinolytic pathway. Markland (1998) purified many fibrin(ogen)olytic enzymes from different snake venoms, the majority of which were metalloproteinases.

This work shows that *C. sylvestris* extract is more effective in neutralizing hemorrhagic metalloproteinases from bothropic venoms than the serine-proteinases that cause alterations in the coagulation system. This extract also neutralizes Bn2 protease from *B. neuwiedi* venom. Bn2 is considered a weak hemorrhagic protease and has moderate proteolytic activity (Rodrigues et al., 2000). The antihemorrhagic factor from *Didelphis marsupialis* serum inhibits snake venom metalloproteinases by non-covalent complex formation, but no action on venom serine-proteinases was recorded (Neves-Ferreira et al., 1997). Both *C. sylvestris* extract and the antihemorrhagic factor appear to have specificity for metalloproteinases.

To better understand these inhibition mechanisms, it is necessary to study isolated compounds. Purification of these compounds from *C. sylvestris* extract is in progress, but the isolation of an active substance can be a problem. Purification may exclude components that act in combination, resulting in synergism.

Regarding a possible action mechanism, our previous studies have shown that no alteration occurs in the electroforetic pattern of *B. moojeni* venom and isolated myotoxin, after incubation with *C. sylvestris* extract, excluding proteolytic degradation as a potential mechanism (Borges et al., 2000). Because of the preference for metalloproteinases, we could suggest that *C. sylvestris* extracts may be a natural chelanting agent, interacting with metals. On the other hand, components of the extract may occupy sites in the venoms/toxins, preventing binding of the substrate to the enzyme, and this interaction may be covalent or non-covalent.

Tannins (Santos et al., 2000) and flavonoids (Havsteen, 1983) are able to bind metal ions. Lindahl and Tagesson (1997) reported that flavonoids appear to inhibit PLA_2-class II but not PLA_2-class I, but how this occurs is not

Table 1
Coagulant activity on bovine plasma ($n = 3$) of crude venoms incubated or not with *C. sylvestris* extract

Sample	Controls	Time (s)	
		Venom: *C. sylvestris* (1:3 w/w)	Venom: *C. sylvestris* (1:10 w/w)[a]
B. jararacussu (53 μg)	50.81 ± 2.30	85.3 ± 5.03	134.22 ± 4.08
B. moojeni (50 μg)	10.89 ± 5.40	58.75 ± 3.20	127.65 ± 3.90
B. neuwiedi (5 μg)	35.60 ± 5.10	27.66 ± 1.50	100.21 ± 5.60
PBS[b]	245.00 ± 0.04	–	–
C. sylvestris[b] (540 μg)	245.05 ± 0.10	–	–

[a] Inhibition of coagulant activity by *C. sylvestris* is significant ($P < 0.05$ One Way ANOVA).
[b] Incubation was performed for 245 s. No coagulation was observed.

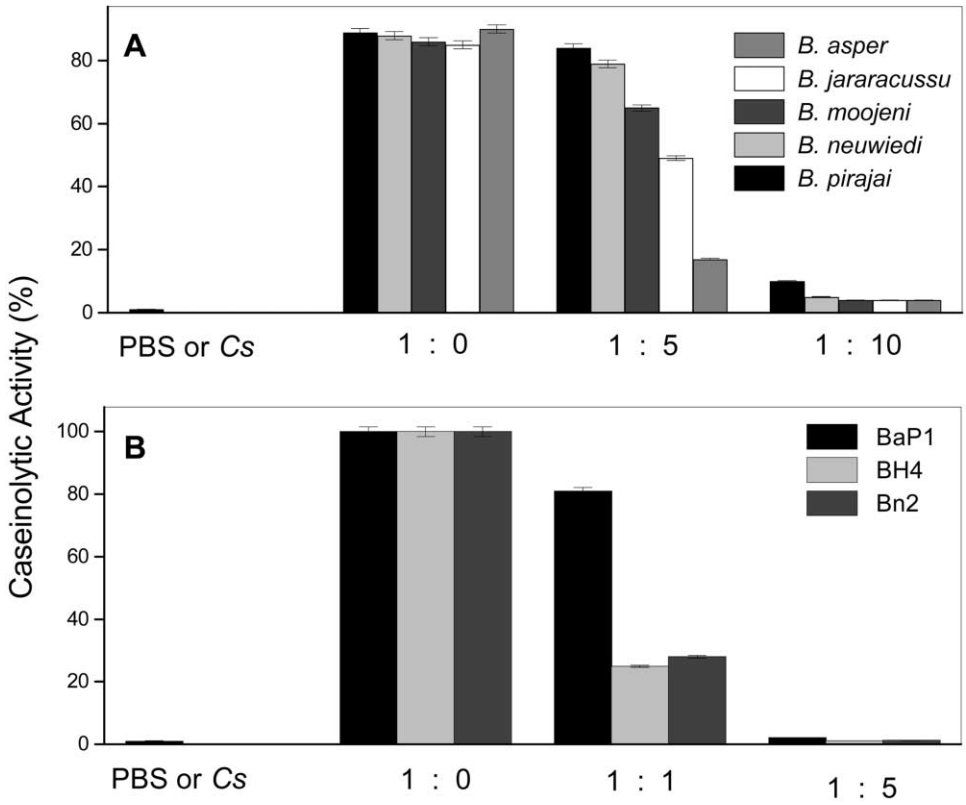

Fig. 3. Neutralization of proteolytic activity on casein of the crude venoms (A) or isolated proteases (B) by *C. sylvestris* extract. The venoms (40 μg) or toxins (20 μg) were previously incubated with *C. sylvestris* at different ratios for 30 min at room temperature and were mixed with substrate at 37°C for 5 min. Bars represent the mean ± SD ($n = 3$). The difference between incubated or not incubated is statistically significant ($P < 0.05$ One Way ANOVA).

clear. Furthermore, flavonoids have a high chemical reactivity, can bind to biological polymers, and are able to catalyze electron transport, and numerous studies show inhibition of a variety of enzymes by flavonoids (Havsteen, 1983).

Snake venom inhibitors have been purified from many plants. Ar turmerone was capable of abolishing the hemorrhagic activity caused by *Bothrops* venom and inhibited about 70% of the lethal effect of *Crotalus* venom (Ferreira et al., 1992). Wedelolactone neutralized hemorrhagic and myotoxic activity induced by *Crotalus* and *Bothrops* venoms and isolated toxins (Mors et al., 1989; Melo et al., 1994), and also inhibited myotoxic PLA_2 from *Crotalus d. durissus*, *Crotalus v. viridis* and *Agkistrodon contortrix laticinetus* venoms (Melo and Ownby, 1999).

The mechanisms of action of these compounds are still unknown. Interactions between venom and extract and the involvement in immunological mechanisms cannot be excluded. *C. longa* and Ar turmerone extracts inhibited lymphocyte proliferation. 2-hidroxy-4-methoxy benzoic acid isolated from a root extract of the Indian plant *Hemidesmus indicus*, directly neutralized viper venom-induced lethal, hemorrhagic and coagulant activities. This compound also indirectly neutralized both lethal and hemorrhagic activities of these venoms by increasing the antibody production in hyper immunized rabbits. The mechanism of action is not clear, but this study indicates that 2-hidroxy-4-methoxy benzoic acid may act as an adjuvant thus triggering the high title of antibodies, which effectively neutralizes the venoms (Alam and Gomes, 1998).

In conclusion, our results show that the *C. sylvestris* aqueous extract contains compounds that neutralize proteases present in snake venoms. We tested many others plant extracts in the same conditions and we observed that these extracts were not capable of inhibiting the same proteases. It is not clear yet if this inhibition occurs due to specific interactions between specific groups from vegetal extract and metalloproteinases hemorrhagic domains, but this study show that metalloproteinases are neutralized by smaller amounts of extract. Fibrinogenolytic and coagulant activities were only partially inhibited even when higher ratios of the extract were used.

Identification of metalloproteinase inhibitors has medical importance, because proteases structurally similar to snake venom metalloproteinases are found in mammalian cells. Proteases and their inhibitors are in delicate equilibrium

with each other to maintain a steady state in the cell. When this balance is broken disturbs such as neoplasia and others diseases can occur, so effective drugs capable of inhibiting metalloproteinases could be developed using these inhibitors. Furthermore, snake venom inhibitors can be useful tools for the elucidation of the mechanisms of action of purified toxins.

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