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## Anti-hemorrhagic effect of hydro-alcoholic extract of the leaves of *Mikania glomerata* in lesions induced by *Bothrops jararaca* venom in rats<sup>1</sup>

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### ABSTRACT

**PURPOSE:** To investigate the effects of hydroalcoholic leaf extract of *Mikania glomerata* Spreng (Asteraceae) on the activity of *Bothrops jararaca* snake venom in Wistar rats.

**METHODS:** Fifty four rats Wistar were divided into six groups of nine animals in each: control treated with saline; control treated with *B. jararaca* venom; control treated with *M. glomerata* extract; *B. jararaca* venom incubated with *M. glomerata* extract at proportions of 1:1, 1:2, and 1:4.

**RESULTS:** Histopathological and morphometric analysis showed that intradermal administration of snake venom incubated with the hydroalcoholic extract at proportions of 1:1, 1:2 and 1:4 promoted a significant reduction in the number of inflammatory cells and a marked decrease in edema after the third hour. There was also a significant reduction in the intensity of the hemorrhagic halo in animals receiving the snake venom incubated with the extract, with the observation of a progressive and parallel inhibition with increasing proportion of *M. glomerata*.

**CONCLUSION:** The *Mikania glomerata* hydroalcoholic extract exerted effective anti-inflammatory and antihemorrhagic activity against the effects induced by *Bothrops jararaca* snake venom.

**Key words:** Snake venoms. Plants, Medicinal. Mikania. Bothrops. Rats.

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## Introduction

Snake venoms comprise several substances, including proteins, enzymes and peptides that can vary depending on the place, alimentation, age and gender of the animals. The Viperidae family represents an important group of snakes in public health. They are responsible for most serious ophidian accidents reported in Brazil and other occidental countries. The *Bothrops* genus (family Viperidae) encloses some of the most remarkable species from the medical point of view, since it contributes 73.1% of the ophidian annual accidents which occur in Brazil<sup>1-3</sup>.

Morbidity related to snake envenomation not only depends on the quantity of snake venom inoculated, but also on the chemical composition of the venom which is species specific. The venom of *Bothrops jararaca* primarily causes shock, tissue necrosis, intravascular coagulation, local and systemic hemorrhage, and edema<sup>3</sup>. Snake venom is a modified form of saliva that consists of a complex mixture of proteins and toxic enzymes such as phospholipases A<sub>2</sub>, myotoxins, hemorrhagic/non hemorrhagic metalloproteinases, serine proteases, neurotoxins, and cytokines, among others<sup>4</sup>. These proteins account for 90 to 95% of the dry weight of snake venoms<sup>5</sup>.

Plant extracts have been used for the treatment of snakebites because they contain a wide variety of chemical compounds with pharmacological activities that exert inhibitory activity against the toxins present in snake venom<sup>6-9</sup>. This plant has antifungal, antimicrobial, bronchodilatory, antiallergic, antihemorrhagic, and anti-inflammatory properties<sup>10</sup>. Phytochemical studies have shown that *Mikania* contains terpenes, essential oils<sup>11</sup>, di- and sesquiterpene lactones<sup>12</sup>, flavonoids, and stigmasterol<sup>13</sup>, alcohols, acids, esters, aldehydes, organic esters and steroids. Regarding the safety of the extracts, phytomedicines and isolated compounds, guaco species did not present significant toxic and genotoxic effects in humans<sup>14</sup>.

In a study of plants with antiophidic properties used in folk medicine, Ruppelt<sup>15</sup> also observed anti-inflammatory activity of *M. glomerata*. The main chemical compound with medicinal activity, which has been reported to exert antiophidic activity<sup>16</sup>, is coumarin<sup>17,18</sup>. Hadjipavlou-Litina<sup>19</sup> observed that coumarin may reduce edema and inflammation by inhibiting the synthesis of prostaglandins. Aqueous extracts prepared from several plant parts efficiently inhibit the different toxic, pharmacological, and enzymatic effects induced by the venom of *Bothrops* and *Crotalus* snakes<sup>14</sup>. Maiorano<sup>20</sup> observed that guaco root extracts reduced the hemorrhage zone stimulated by the intradermal injection of *Bothrops* venom by 80% in rats suggesting that there is an interaction between the components of guaco and metalloproteinases involving the catalytic sites of these enzymes or

essential metal ions, thereby inhibiting their hemorrhagic activities.

Serum therapy is used as an approach to treat the local and systemic effects of snakebites. However, studies indicate that this therapy is partially effective in inhibiting the effects of the venom at the site of inoculation, which usually result in severe and irreversible injuries. Therefore, there is increasing interest in alternative coadjuvant antiophidic therapies<sup>21</sup>.

The objective of the present study was to evaluate the effects of a hydroalcoholic leaf extract of *M. glomerata* Spreng. (Asteraceae) activity on inflammatory and haemorrhagic of *B. jararaca* snake venom in Wistar rats.

## Methods

The study was approved by the Ethics Committee for Animal Use of Herminio Ometto University Center - UNIARARAS (Protocol N° 063/2010), and was conducted in accordance with international regulations on animal experimentation and biodiversity.

Fifty-four male Wistar rats (*Rattus norvegicus*), 120 days old and weighing on average 300g, were obtained from the Center of Animal Experimentation "Prof. Dr. Luiz Edmundo de Magalhães" of Hermínio Ometto University Center - UNIARARAS. The animals were maintained in individual cages at a constant temperature of 23 ± 2°C under a 12-h light/dark cycle, and received commercial chow and water *ad libitum*. No differences in behavior were observed between animals during the experiment.

### *Plant material*

For the preparation of the hydroalcoholic extract, leaves of *Mikania glomerata* Spreng were collected in the Medicinal Plant Garden of Herminio Ometto University Center - UNIARARAS, through the "Project Health & Harmony". The samples were identified by the botanist Gerson de Oliveira Romão of Higher School of Agriculture "Luiz de Queiróz" - ESALQ/USP, and deposited in the ESA Herbarium (N° 114299).

### *Preparation of the hydroalcoholic extract*

After collection, the *M. glomerata* leaves were selected, washed, dried at room temperature, and weighed. For extraction, the leaves were repeatedly macerated with 70% ethanol for 14 days. The resulting hydroalcoholic extract was evaporated at 40°C (Fisatom 802 Rotary Evaporator) until the complete removal of ethanol. The extract was lyophilized (VirTis® 8L Freeze Dryer) at Research Center for Chemical, Biological and Agricultural (CPQBA) - UNICAMP.

For the inhibition studies of the hemorrhagic halo the extract was solubilized in PBS and then used the soluble fraction.

#### *Snake venom*

The *Bothrops jararaca* snake venom was purchased from Bioactive proteins (Serpentário Bioagents Ltda., Batatais-SP, Brazil).

#### *Experimental protocol*

The *M. glomerata* hydroalcoholic extract (soluble fraction) was incubated with the snake venom at different proportions (1:1, 1:2, and 1:4) for 30 min at 37°C<sup>20</sup>.

The animals were divided into six groups of nine animals each. All animals were anesthetized intraperitoneally with Xylazine hydrochloride (20mg/kg) and Ketamine hydrochloride (50mg/kg) and a trichotomy was performed in the dorsal region. After 48h, the animals were submitted to the following procedures after anesthesia: group G1, intradermal administration of 100µL saline; group G2, intradermal administration of 100µL *B. jararaca* venom at a concentration of 3.2µg/µL; group G3, intradermal administration of 100µL *M. glomerata* hydroalcoholic extract at a concentration of 15µg/µL; group G4, intradermal administration of 100µL *B. jararaca* venom + *M. glomerata* hydroalcoholic extract at a concentration of 3.2µg/µL (1:1); group G5, intradermal administration of 100µL *B. jararaca* venom + *M. glomerata* hydroalcoholic extract at a concentration of 6.4µg/µL (1:2); group G6, intradermal administration of 100µL *B. jararaca* venom + *M. glomerata* hydroalcoholic extract at a concentration of 12.8µg/µL (1:4). The volume inoculated in each group was always the same: 100µL.

One, two and three hours after intradermal administration of the venom and extract, three animals from each group were euthanized with an overdose of the anesthetic and the wound areas were collected from the different groups for photodocumentation. For this purpose, the animals were anesthetized as described above. Next, the skin was removed, photographed, and prepared for histopathological analysis. The macroscopic images were used to measure the size of the hemorrhagic halo with a pachymeter. The data were analyzed using the Posturograma 3.0 software (Fisiometer) and were compared by ANOVA (p<0.001) using the Origin 6.0 program. The results are reported as the mean and standard deviation of each group.

#### *Histopathological analysis*

The wound area was removed and fixed in 10% formalin

in Millonig buffer, pH 7.4, for 24h at room temperature. Next, the specimens were washed in the same buffer and processed for embedding in Paraplast™ (Merck). Longitudinal sections (5µm) were stained with hematoxylin-eosin for routine histology and by the Dominici method for the quantification of inflammatory cells. The specimens were examined and photographed with a Leica DM 2000 photomicroscope. The following histopathological parameters were evaluated: acute inflammatory reaction in the dermis, edema, eosinophilic collagen fibers (denaturation and disarrangement), subcutaneous muscle necrosis, and number of inflammatory cells.

The morphological results are reported as the mean and standard deviation of each group and were compared by ANOVA and the Tukey's post-hoc test (p<0.05).

#### *Determination of minimum hemorrhagic dose*

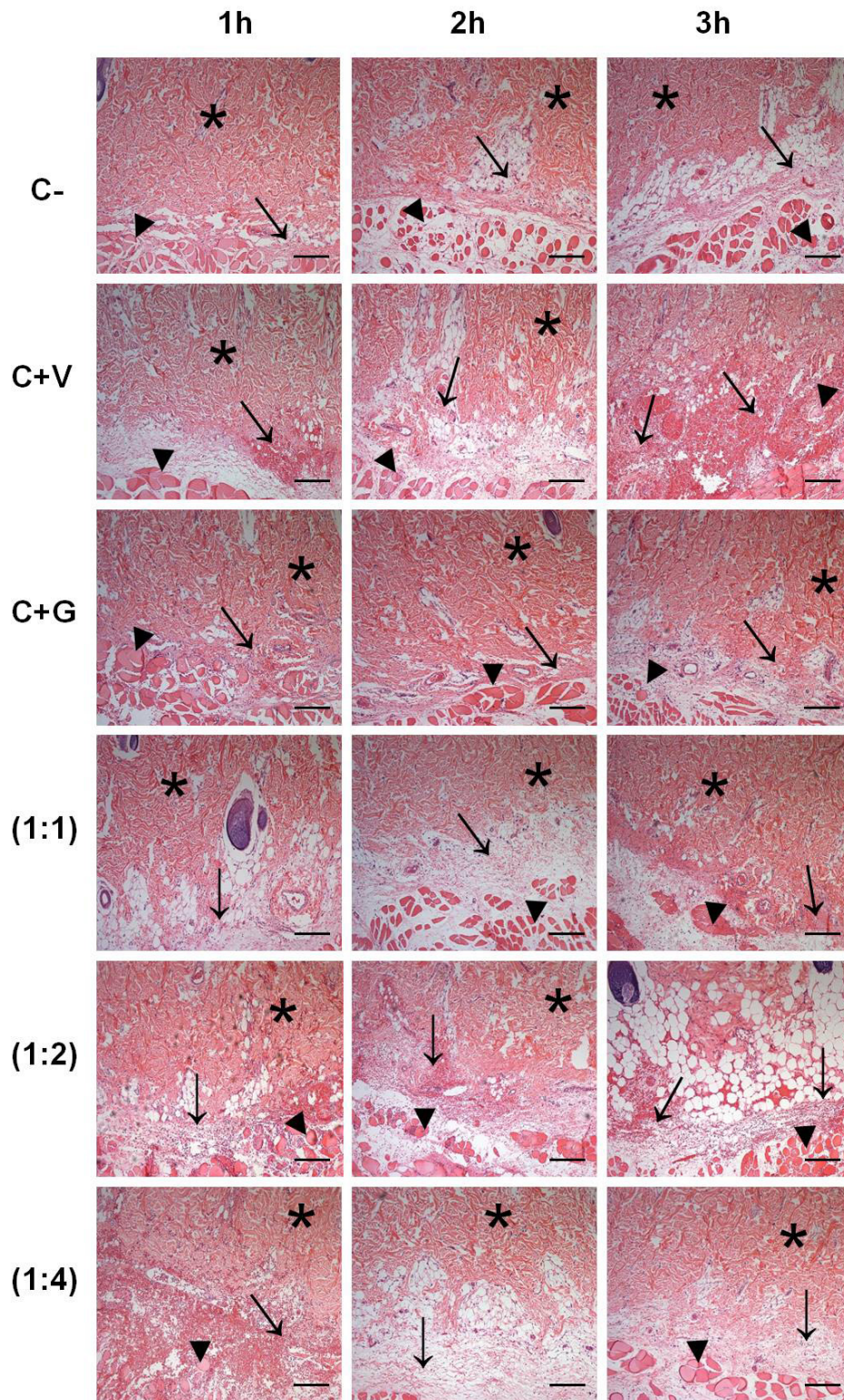
The hemorrhagic activity of the venom was quantified according to the method of Ownby<sup>22</sup>, with some modifications. The animals were trichotomized in the dorsal region and anesthetized with Xylazine hydrochloride (20mg/kg) and Ketamine hydrochloride (50mg/kg). Next, different concentrations of crude *B. jararaca* venom (5, 10, 20, 40, 80, 160, 320, and 640 µg) were injected intradermally. The animals were euthanized with an overdose of the anesthetic and after 1, 2 and 3 h and their skin was removed and photographed for analysis of the formation of a hemorrhagic halo using the Posturograma program. The measurements were done in triplicate and the minimum hemorrhagic dose was defined as the minimum concentration of snake venom necessary to produce a hemorrhagic halo measuring cm in diameter.

The minimum hemorrhagic dose determined in the present study was 160µg. However, a dose of 320µg was established for the inhibition assays to account for the dilution of the snake venom by the plant extract.

#### **Results**

Figure 1 shows the results of analysis of the histopathological effects of *B. jararaca* snake venom combined with the *M. glomerata* hydroalcoholic extract. Tissue samples of group G1 presented some inflammatory foci, mild edema, and areas of muscle necrosis at all time points studied. In addition, structural alterations in collagen fibers were observed after the third hour, which were due to the presence of eosinophilia seen during this period.





**FIGURE 1** – Cross-sections obtained from the site of intradermal injury induced by *Bothrops jararaca* snake venom incubated with hydroalcoholic extract of *Mikania glomerata* Spreng. **G1**: intradermal administration of 100  $\mu$ L saline; **G2**: intradermal administration of 100  $\mu$ L *B. jararaca* venom at a concentration of 3.2  $\mu$ g/ $\mu$ L; **G3**: intradermal administration of 100  $\mu$ L *M. glomerata* hydroalcoholic extract at a concentration of 15  $\mu$ g/ $\mu$ L; **G4**: intradermal administration of 100  $\mu$ L *B. jararaca* venom + *M. glomerata* hydroalcoholic extract at a concentration of 3.2  $\mu$ g/ $\mu$ L (1:1); **G5**: intradermal administration of 100  $\mu$ L *B. jararaca* venom + *M. glomerata* hydroalcoholic extract at a concentration of 6.4  $\mu$ g/ $\mu$ L (1:2); **G6**: intradermal administration of 100  $\mu$ L *B. jararaca* venom + *M. glomerata* hydroalcoholic extract at a concentration of 12.8  $\mu$ g/ $\mu$ L (1:4). Tissue samples were collected 1, 2 and 3 h after administration. The sections were stained with hematoxylin-eosin and examined under a bright-field microscope. (\*) Collagen fibers of the deep dermis; (→) areas of inflammatory infiltration and hemorrhage; (▶) areas of tissue necrosis. Bar = 100  $\mu$ m.

The dermis of group G2 animals was characterized by the presence of significant inflammatory foci seen as early as in the first hour, which increased over time. Interstitial edema was observed at all time points studied. Alterations of collagen fibers were seen as early as in the first hour and intense eosinophilia was observed in deeper layers of the dermis. Extensive areas of deep muscle necrosis were noted at all time points and the number of hemorrhagic foci increased over time.

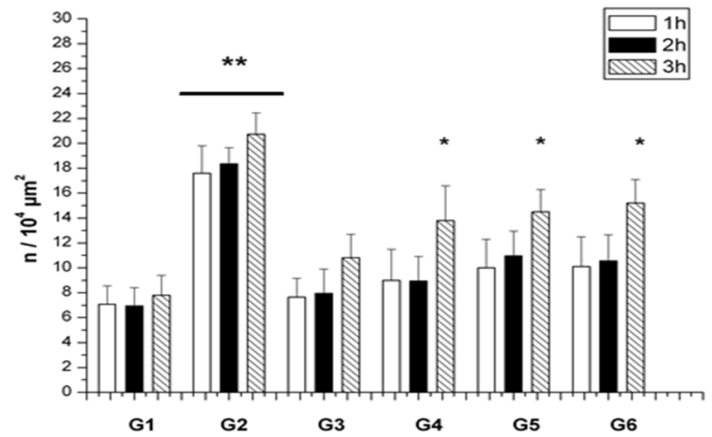
The findings of group G3 were similar to those seen in group G1. However, a greater inflammatory response was observed at all time points, but important hemorrhagic foci were absent.

In group G4, interstitial edema was seen as early as in the first hour and was accompanied by hemorrhagic foci that became more intense after the third hour of observation. Deep muscle necrosis was observed throughout the study period, but was more evident in the third hour. The intensity of eosinophilic collagen fibers in the dermis was similar to that seen in group G2, especially in the third hour of observation.

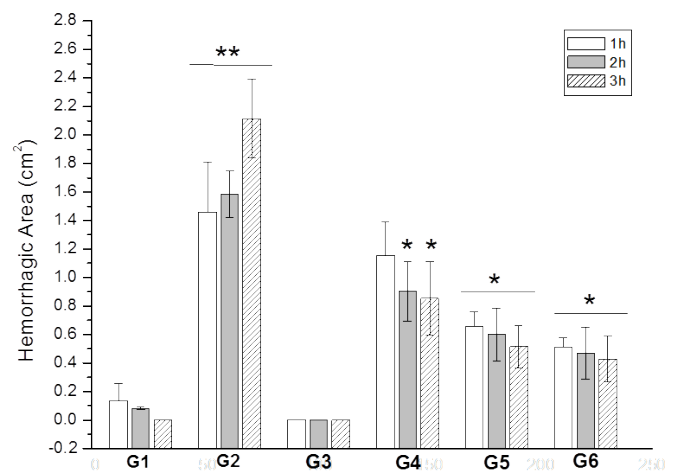
The results obtained for groups G5 and G6 were similar. In these groups, the deep muscle necrosis and eosinophilic collagen fibers in the dermis were less intense than those observed in group G4. Tissue edema appeared more intense in these groups and the inflammatory infiltrate was more diffuse. In addition, hemorrhagic foci were more frequently found in the interstitium, but were less intense than those seen in group G2.

The largest number of inflammatory cells was observed in group G2, whereas the smallest number was seen in group G1 when compared to the other groups (Figure 2). No significant difference in the number of inflammatory cells was observed between groups G1, G3, G4, G5 and G6 in the first hour, but these numbers were significantly lower than those seen in group G2. In the third hour, the number of inflammatory cells in groups G4, G5 and G6 was significantly higher when compared to groups G1 and G3 but lower when compared to group G2. The results obtained for groups G4, G5 and G6 were similar (Figure 2).

A significant reduction in the intensity of hemorrhagic activity was observed at the various time points in the groups receiving an intradermal injection of *B. jararaca* venom incubated at different proportions with the *M. glomerata* hydroalcoholic extract when compared to group G2 (Figure 3). This reduction was most marked at the proportion of 1:4. These results indicate a progressive and parallel inhibition with increasing proportion of *M. glomerata*.



**FIGURE 2** – Total number of inflammatory cells ( $n/10^4 \mu\text{m}^2$ ) at the site of intradermal injection of *Bothrops jararaca* snake venom incubated with hydroalcoholic extract of *Mikania glomerata* Spreng. **G1**: intradermal administration of 100  $\mu\text{L}$  saline; **G2**: intradermal administration of 100  $\mu\text{L}$  *B. jararaca* venom at a concentration of 3.2  $\mu\text{g}/\mu\text{L}$ ; **G3**: intradermal administration of 100  $\mu\text{L}$  *M. glomerata* hydroalcoholic extract at a concentration of 15  $\mu\text{g}/\mu\text{L}$ ; **G4**: intradermal administration of 100  $\mu\text{L}$  *B. jararaca* venom + *M. glomerata* hydroalcoholic extract at a concentration of 3.2  $\mu\text{g}/\mu\text{L}$  (1:1); **G5**: intradermal administration of 100  $\mu\text{L}$  *B. jararaca* venom + *M. glomerata* hydroalcoholic extract at a concentration of 6.4  $\mu\text{g}/\mu\text{L}$  (1:2); **G6**: intradermal administration of 100  $\mu\text{L}$  *B. jararaca* venom + *M. glomerata* hydroalcoholic extract at a concentration of 12.8  $\mu\text{g}/\mu\text{L}$  (1:4). Tissue samples were collected 1, 2 and 3 h after administration. Results are reported as the mean and standard deviation of each group and were compared by ANOVA and the Tukey's post-hoc test ( $p < 0.05$ ). \* and \*\* indicate significant differences between time points.



**FIGURE 3** – Inhibition of hemorrhagic activity of *Bothrops jararaca* snake venom by the hydroalcoholic extract of *Mikania glomerata* Spreng. **G1**: intradermal administration of 100  $\mu\text{L}$  saline; **G2**: intradermal administration of 100  $\mu\text{L}$  *B. jararaca* venom at a concentration of 3.2  $\mu\text{g}/\mu\text{L}$ ; **G3**: intradermal administration of 100  $\mu\text{L}$  *M. glomerata* hydroalcoholic extract at a concentration of 15  $\mu\text{g}/\mu\text{L}$ ; **G4**: intradermal administration of 100  $\mu\text{L}$  *B. jararaca* venom + *M. glomerata* hydroalcoholic extract at a concentration of 3.2  $\mu\text{g}/\mu\text{L}$  (1:1); **G5**: intradermal administration of 100  $\mu\text{L}$  *B. jararaca* venom + *M. glomerata* hydroalcoholic extract at a concentration of 6.4  $\mu\text{g}/\mu\text{L}$  (1:2); **G6**: intradermal administration of 100  $\mu\text{L}$  *B. jararaca* venom + *M. glomerata* hydroalcoholic extract at a concentration of 12.8  $\mu\text{g}/\mu\text{L}$  (1:4). The assays were performed 1, 2 and 3 h after administration. The results are expressed as the size of the hemorrhagic area (in  $\text{cm}^2$ ) and are reported as the mean  $\pm$  standard deviation ( $n = 3$ ). \* and \*\* indicate significant differences between time points.



## Discussion

Plants with antiophidic properties have been studied by different investigators, who observed their inhibitory effects in animals inoculated with venom of snakes of the genus *Bothrops*<sup>20,23</sup>.

The presence of some inflammatory foci, mild edema, and areas of muscle necrosis observed in the group G1 is related to early stages of healing. The initial inflammatory response involves the recruitment of cells that fight potential bacterial contamination of the wound and activate cytokine secretion to activate dermal and epidermal processes<sup>24</sup>. However, if inflammation increases beyond a certain level, it will lead to healing impairment, destruction of the early migratory effect and an arrest of the healing progression<sup>25</sup>.

The intradermal administration of *B. jararaca* snake venom incubated with *M. glomerata* hydroalcoholic extract promoted a significant reduction in the number of inflammatory cells and a marked decrease of edema in the third hour in groups G4, G5, and G6. *Bothrops* snake venoms are rich in phospholipases A<sub>2</sub>, which exert a variety of toxic effects such as myonecrosis, hemorrhage, cardiotoxicity, edema, and hydrolysis of membrane phospholipids and the consequent release of platelet-activating factor<sup>26</sup>. Experimental studies have shown that the local inflammatory response induced by *Bothrops* snake venoms is characterized by the exudation of plasma proteins. This fact is attributed to the presence of various toxins in the venom, including metalloproteinases, serine proteases, and phospholipases A<sub>2</sub><sup>20,27</sup>. Furthermore, edema induced by *B. asper* and *B. jararaca* has been shown to increase the levels of prostaglandin E2<sup>28</sup>. The present results seem to confirm these observations, i.e., the inflammatory activity of *B. jararaca* venom was probably triggered by these toxins and the significant anti-inflammatory effects observed in the third hour after intradermal administration of the venom incubated with *M. glomerata* hydroalcoholic extract were due to the inhibition of these inflammation-inducing toxins. Several studies have demonstrated the antiophidic effect of *M. glomerata*, which inhibits the activity of phospholipase A<sub>2</sub> in viperid snake venoms. The inhibition of this enzyme can probably be attributed to the action of coumarin<sup>20,27,29</sup>. Ma<sup>30</sup> studying five types of coumarins isolated from *Angelica gigas* and suggest that these compounds hold great promise for use as potential anti-inflammatory agents. Coumarins have a variety of bioactivities including anticoagulant, antimicrobial, vasodilatador, anti-inflammatory<sup>31</sup>.

In the present study, administration of the venom incubated with *M. glomerata* promoted significant inhibitory effects, especially at the proportion of 1:4, i.e., the results showed a progressive inhibition of the hemorrhagic halo in parallel to the

increase in the proportion of *M. glomerata*. In addition, the most significant results were observed in the third hour after intradermal administration of the venom incubated with the extract.

Local and systemic hemorrhage is an evident sign of envenomation by snakes of the family Viperidae and the hemorrhagic activity of *Bothrops* venom is attributed to metalloproteinases, the major component of venom. The hemostatic mechanisms of various toxins have been characterized, including those of metalloproteinases which are directly or indirectly involved in the hemorrhagic activity of snake venoms<sup>32</sup>. The local hemorrhage observed in *Bothrops* envenomation is the result of extensive damage to tissues and vessel walls caused by metalloproteinases, which degrade type IV collagen, leading to capillary collapse<sup>33</sup>.

Several studies have reported the inhibition of the hemorrhagic effects of snake venoms by plant extracts<sup>34</sup>. In the study of Maiorano<sup>20</sup> the hemorrhagic activity of the venom of various *Bothrops* species was significantly inhibited by *M. glomerata* extract in mice. The authors concluded that the extract could be used as an alternative treatment to serotherapy. In the present study, comparison of the size of the hemorrhagic halo induced by the intradermal administration of *B. jararaca* snake venom incubated with *M. glomerata* extract showed the antihemorrhagic activity of the extract. This finding suggests that the soluble fraction of *M. glomerata* extract blocks metalloproteinases and consequently inhibits the hemorrhage triggered by these enzymes suggesting that the compounds presents in the soluble fraction, associated at conditions of pre-incubation used in experimental procedures favored the inhibition of hemorrhage.

Further studies investigating in detail the isolated chemical components of *M. glomerata* with a potential local neutralizing effect on *B. jararaca* venom are needed to better understand the mechanisms of action involved in the antihemorrhagic activity of this plant.

## Conclusion

*B. jararaca* snake venom incubated with *M. glomerata* extract showed the antihemorrhagic activity of the extract.

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