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ANTIOPHIDIAN ACTIVITY OF *BROSIMUM GUIANENSE* (AUBL) HUBER

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ABSTRACT

Snakebites envenomations are a problem public health in worldwide due to the high rates of morbidity and mortality. The *Bothrops* venom causes local tissue damage and inflammation is one of the most important events that occur. At present, effective treatment for snakebites is serum therapy with antivenom, which neutralizes systemic alterations but does not prevent local damage that can cause disabilities. Many plants are used in popular medicine to treat these accidents but few attempts have been made to investigate the scientific validity of these assertions. In Amazon region, indigenous and local people use the macerated bark of *Brosimum guinanensis* applied in the form of cataplasm, on the site of snakebite. This study aimed to analyze the ability of the *Brosimum guianensis* aqueous extract in the neutralization several effects induced by *Bothrops atrox* snake venom to investigate the scientific validity of folk medicine informations by means of controlled experiments. Our results showed that *Brosimum guianensis* aqueous extract was not effective to inhibit oedema, peritonitis, coagulant, myotoxic, phospholipase A2 activity (indirect hemolytic method) induced by *B. atrox venom*, but was able to inhibited significantly hemorrhagic and nociceptive activities. These results support a potential effect of this extract as a compounds source for biotechonological application and synthesis of new drugs with therapeutic purpose.

Keywords: *Bothrops Atrox*, Snake Venom, *Brosimum Guianense*, Medicinal Plant

1. INTRODUCTION

In Brazil, significant snakebites numbers occurs annually and are considered a neglected tropical disease of high impact in the rural areas (Gutiérrez *et al*., 2006; WHO, 2007a). *Bothrops atrox* is ophidian most frequently found in northern Brazil and envenoming caused by this snake are associated with a variety of pathophysiological manifestations, frequently including a severe local tissue damage (Gutiérrez, 2002). In addition, an unknow number of cases end up with permanent sequelar secondary to the tissue-damaging effects of the venom (WHO, 2007b; Warrell, 2010). The use of plants in traditional medicine systems of many cultures has been extensively documented. These plant-based systems continue to play an essential role in health care and the World Health Organization estimates that 80% of the world inhabitants continue to rely mainly on traditional medicine systems for their health care (Gurib-Fakim, 2006; WHO, 2002).

Several vegetal species are popularly known as antiophidian, but only a few species have been

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scientifically investigated and still less have had their active principles isolated and characterized (Veronese *et al*., 2005; Coea and Anderson, 2005; Da Silva *et al*., 2005). The use of plants to treatment snakebites is spread out among Amazon population, that use the macerated bark of *Brosimum guinanensis*, applied in the form of cataplasm, on the site of snakebite.

In the present study was evaluated the ability of the *Brosimum guinanensis* aqueous extract of in the neutralization oedema, peritonitis, nociceptive, coagulant, myotoxic, hemorrhagic and phospholipase A2 activity (indirect hemolytic method) induced by *Bothrops atrox* venom.

2. MATERIALS AND METHODS

2.1. Venom and Antivenom

B. atrox venom was supplied from the Serpentarium at the Toxicology Laboratory, Federal University of Amapá, Brazil. The venom was lyophilized and kept under refrigeration at 4°C. The venom was diluted in Phosphate Buffered Saline (PBS) immediately prior to its use.

2.2. Plant Material

Brosimum guinanensis leaves were collected in Macapá, Amapá, Brazil. The collection place (00º2'41.821"S, 51º5'57.253"W) was marked by a global position measuring (GPS Garmin-modelo nüvi 40). A voucher specimen (460) was deposited at the Herbarium of Federal University of Amapá.

2.3. Preparation of Plant Extract

Dry and worn-out stem leaves were extracted with distilled water, maintained in infusion for 24 h at room temperature and then vacuum filtered. The aqueous Extract (CEE) was lyophilized and stored at-18°C. Before use, it was weighed and dissolved in PBS.

2.4. Animals

All animal care was performed in accordance with the guidelines of the Brazilian College for Animal Experimentation. Male *Swiss webster* mice weighing 20-25 g were used for the experiments and were randomly divided into groups of five animals each. The mice were kept in plastic cages with access to water and food *ad libitum* and were maintained under controlled temperatures (18-20°C) on a 12 h light/dark cycle.

2.5. Groups and Experimental Protocols

The experimental groups consisted of five male mice each administered *B.Atrox* Venom (BAV) alone, *Brosimum Guinanensis* Extract alone (BGE), *B. atrox* venom+*Brosimum guinanensis* extract in concentrations different (BAVBGE), or PBS alone. The venom doses used were selected from previous dose-response experiments, in which it was observed that the venom induced a minimum response for all activities evaluated.

2.6. OEDEMA Induction

The minimum dose was defined as the lowest venom dose required for the formation of 30% paw oedema (Rocha and Furtado, 2007) and was evaluated after subplantar injection of venom, in the right footpad of mice. For inhibition studies, a fixed amount of *Bothrops atrox* venom (0, 20 mg/25 µL PBS) was mixed with *Brosimum guinanensis* extract concentrations different (1, 25; 2,5; 5,0; 7,5 and 10 mg de BGE/Kg/25 µL PBS). Then, the mixture was administered intradermally into the subplantar region in the footpad of mice. Controls animals received only PBS (50 µL), venom (0, 20 mg/Kg/50 µL PBS) or *Brosimum guinanensis* extract (10 mg/Kg/50 µL PBS). The progression of edema was evaluated with a low-pressure pachymeter 0,01mm (Mytutoyo, Japan) in the intervals of 0, 1, 2, 3 e 4 h after injection and was expressed in mm of directly induced oedema.

2.7. Nociception Activity

The method used was a modification of the method previously described by Hunskaar and Hole (1987) modified by (Soares *et al*., 2009; Sousa, 2012). Sample containing 0.05, 0.10 or 0.20 mg kg^{-1} of venom in 50 µL of PBS were injected subcutaneously into the right hind paw for evaluated venom nociceptive effect. The minimum dose was defined as the lowest venom dose required for a statistically significant increase ($p<0.05$) at time (in seconds) spent in licking and biting responses of the injected paw. For inhibition studies, a fixed amount of *Bothrops atrox* venom (0,20 mg/25 µL PBS) was mixed with *Brosimum guinanensis* extract concentrations different (1,25; 2,5; 5,0; 7,5 and 10 mg de BGE/Kg/25 µL PBS). Then, the mixture was administered subcutaneously into the right hind paw of mice. Controls animals received only PBS (50 µL), venom (0, 20 mg/Kg/50 µL PBS) or *B. guinanensis* extract (10 mg/Kg/50 µL PBS). Mice were then put back individually under glass funnel with mirrors behind and also to the side to facilitate observation.

Distinct periods of intensive licking activity were identified and scored separately unless otherwise

stated. The first period (early phase) was recorded 0-5 min and the second period (late phase) was recorded 20-30 min after the injection in the corresponding groups. The time (in seconds) spent in licking and biting responses of the injected paw were taken as an indicator of pain response. The test was performed at ambient temperature of 22-26°C and care was taken to exclude environmental disturbances (high temperature, noise and excessive movement) that might interfere in the study. The animals were individually assessed by only one observer, responsible for all tests. Due to number of animals, tests were performed during two consecutive days.

2.8. Peritonitis Induced by Bothrops Atrox Venom

Peritonitis assays were performed as previously described by (Souza and Ferreira, 1985; Souza, 2006). Initially was administered venom concentrations several (0,05; 0,10; 0,20; 0,30; 0,40 mg/Kg/µL PBS) by intraperitoneal injection in mice. The minimum dose was defined as the lowest venom dose required to induce cellular migration without causing significant local hemorrhage. In the inhibition assays, a fixed amount of *Bothrops atrox* venom (0, 20 mg/25 µL PBS) was mixed with *Brosimum guinanensis* extract concentrations different (1, 25; 2,5; 5,0; 7,5 and 10 mg de BGE Kg/25 µL PBS). Then, the mixture was administered by intraperitoneal injection in mice. Controls animals received only PBS (50 µL), venom (0, 20 mg/ Kg/50µL PBS) or *B. guinanensis* extract (10 mg/Kg/50 µL PBS).

After four hours, the animals were euthanized in a CO_2 chamber and the peritoneal exudates were collected with a plastic Pasteur pipette by abdominal laparoscopy. To facilitate collection, all the animals received an injection of 2.0 mL of heparinized PBS (1 mL/1000 mL de PBS) and their abdomens were massaged to release any cells that had stuck to them. A sample of the peritoneal wash was diluted 1:20 in Türk's solution and the cells were counted in a Neubauer chamber. Peritoneal fluid part was centrifuged at 1000 rpm for 10 min and the supernatant was suspended in 0.4 mL of a solution of albumin in PBS 3%. Differential leukocytes were stained in Instant-Prov and counted under a light microscope, using oil immersion objective. The results were expressed as the total number of cells per peritoneal cavity.

2.9. Coagulant Activity

Firstly was determinate minimum coagulant dose defined as the amount of venom, which clots 200 µL human plasma in 60 s (Theakston and Reid, 1983). For the inhibition tests, several doses *B. guinanensis* extract (26; 52; 104; 208; 416 µg/25 µL PBS), mixed with *Bothrops atrox* venom (20 µg/25µL PBS) were used and added immediately on the citrated human plasma at 37°C. As control assays, PBS (50µL), BAV (20 µg/50 µL PBS) and CEE (416µg/50 µL PBS) were added separately to 200 µL citrated human plasma.

2.10. Hemorrhagic Activity

Hemorrhage was performed as previously described by Kondo *et al*. (1960). Firstly, were administered Intradermally (ID) venom concentrations different on the back of mice to determine a Minimal Hemorrhagic Dose (MHD) defined as that concentration of venom resulting in a 10 mm hemorrhagic spot. After 2 h, the animals were euthanized in a CO_2 chamber. The skin near the injection site was removed and hemorrhagic halo formed was measured in millimeters (mm). Two diameters were achieved for the spot of hemorrhage by measuring the longest diameter of the spot and the diameter perpendicular to the first measurement. In the inhibition assays, a fixed amount of *Bothrops atrox* venom (0, 20 mg/25 µL PBS) was mixed with *Brosimum guinanensis* extract concentrations different (1, 25; 2, 5; 5, 0; 7, 5 and 10 mg de BGE/Kg/25 µL PBS). Then, the mixture was administered by ID injection in mice. Controls animals received only PBS (50 µL), venom (0,20 mg/Kg/50 µL PBS) or *B. guinanensis* extract (10 mg/Kg/50µL PBS).

2.11. Myotoxic Activity

Myotoxic activity was determined using the method of quantification of creatine kinase enzyme (Kaplan and Pesce, 1986). The principle of this method consists in the reaction of creatine phosphate and Adenosine Phosphate (ADP), catalyzed by creatine kinase to form creatine and Adenosine Triphosphate (ATP). Activity was expressed in units/L, one unit corresponding to the production of one micromole of NADH per min. Mice were injected in the gastrocnemius muscle with several concentrations *Bothrops atrox* venom to determined minimum myotoxic dose. 3 h after injection, mice were bled from the orbital plexus with heparinized Pasteur pipettes. After centrifugation, plasma was separated and the CK activity was determinate by using Liquiform CK-NAC Kit (Labtest Diagnostica). In the inhibition assays, a fixed amount of *Bothrops atrox* venom (0, 20 mg/25 µL PBS) was mixed with *Brosimum guinanensis* extract concentrations different (1, 25; 2, 5; 5, 0; 7, 5 and 10 mg de BGE/Kg/25 µL PBS). Then, the mixture was administered by intramuscular injection in mice. Controls animals received only PBS (50 µL), venom (0, 20 mg/Kg/50 µL PBS) or *B. guinanensis* extract (10 mg/Kg/50 µL PBS) by intramuscular route.

2.12. Phospholipase A2 Activity

Phospholipase A2 (PLA2) activity was determined by indirect hemolytic method using agarose, TRIS 20mM, CaCl2 and egg yolk gels as substrate (Guitiérrez *et al*., 1988). After incubation at 37°C for 12 h, was realized measuring translucent halos diameters formed by samples applied. Initially was determined minimum indirect hemolytic dose (DHeM) defined as the amount of *Bothrops atrox* venom able to produce 10 mm halo. Solutions contained *Bothrops atrox* venom (20 µg 25 µL PBS) and *B. guianensis* extract (26; 52; 104; 208 or 416 µg/25 µL) assayed were. PBS (50 µL), venom (20 µg 50 µL PBS) or *B. guianensis* extract (416 µg/50µL PBS) only were used as the control. The experiment was carried out in triplicate.

2.13. Statistical Analysis

The results are presented as the mean±S.E.M. Differences among groups were Analyzed by One-way Analysis of Variance (ANOVA) followed by Tukey-Kramer test. Differences with an associated probability (P value) of less than 5% ($p<0.05$) were considered significant.

3. RESULTS

The venom dose used for oedema induction was 0.20 mg PBBa/25 µL PBS, measured at times 0, 1, 2, 3 and 4 h after treatments. **Figure 1** shows the effect of venom and BGE in the edema formation in mice. At the end 4 h, the results in all test groups were not significantly different from BAV control group. Therefore, BGE was not effective in paw edema reducing.

The subcutaneous injection of 0.20 mg/kg *B. atrox* venom into the right hind paw caused licking and biting responses in injected local as an indicator of pain response. In the first phase of the nociceptive activity (**Fig. 2A**), it was observed that 1:6; 1:12; 1:25; 1:37 and 1:50 BABAVGE groups (35, 20±10,15, p<0, 001; 31, 60±6, 218, p<0, 001; 15, 00±4, 980, p<0, 001; 11, 60±4, 844, p<0, 001; 16, 60±4, 261, p<0, 001, respectively) showed a reduction in the time (in seconds) spent licking and biting the injected paw compared to the BAV group (76,60±5,085). As observed in **Fig. 2B** (second phase), all BGE groups showed effective results in reducing nociceptive activity (55,40±5,036, p<0, 05; 44,00±3,017, p<0,01; 38, 00±2, 608, p<0, 001; 36, 40±11, 36, p<0, 001; 29, 80±7, 378, p<0,00, respectively) compared to the animals administered *B. atrox* venom alone (93, 80±16, 90). The results indicate that BGE has compounds with effective analgesic action in snakebites.

In the inhibition assays, was used *Bothrops atrox* venom (0.20 mg/25 µL PBS) and BGE concentrations different. The influx of leukocytes was analyzed in the peritoneal wash observing cells total count and differential leukocytes.

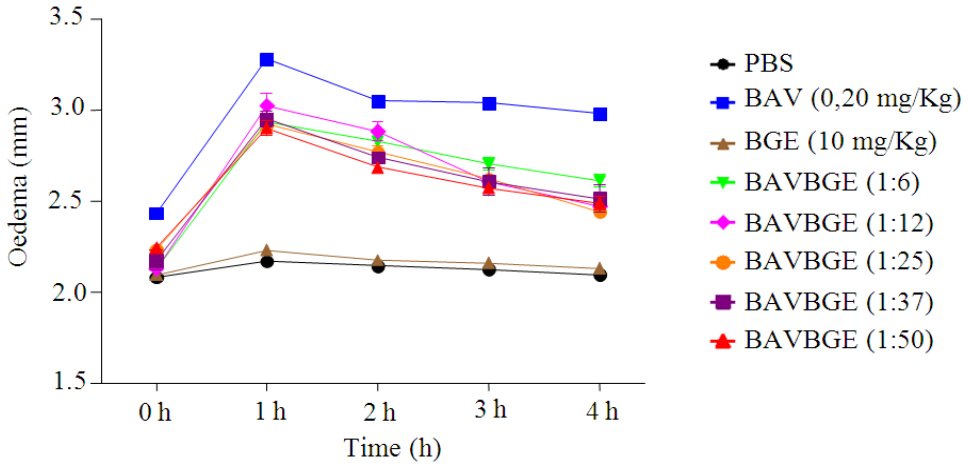


Fig. 1. BGE effect on induced by Bothrops atrox venom administered i.d. in the subplantar region (0,20 mg/25 µL PBS). BAVBGE 1:6 (BAV + 1,25 mg BGE/Kg/25 µL PBS); BAVBGE 1:12 (BAV + 2,5 mg BGE/Kg/25 µL PBS); BAVBGE 1:25 (BAV + 5,0 mg BGE/Kg/25 µL PBS); BAVBGE 1:37 (BAV + 7,5 mg BGE/Kg/25 µL PBS); BAVBGE 1:50 (BAV + 10 mg BGE/Kg/25 µL PBS). The results are presented ± S.E.M. of five animals

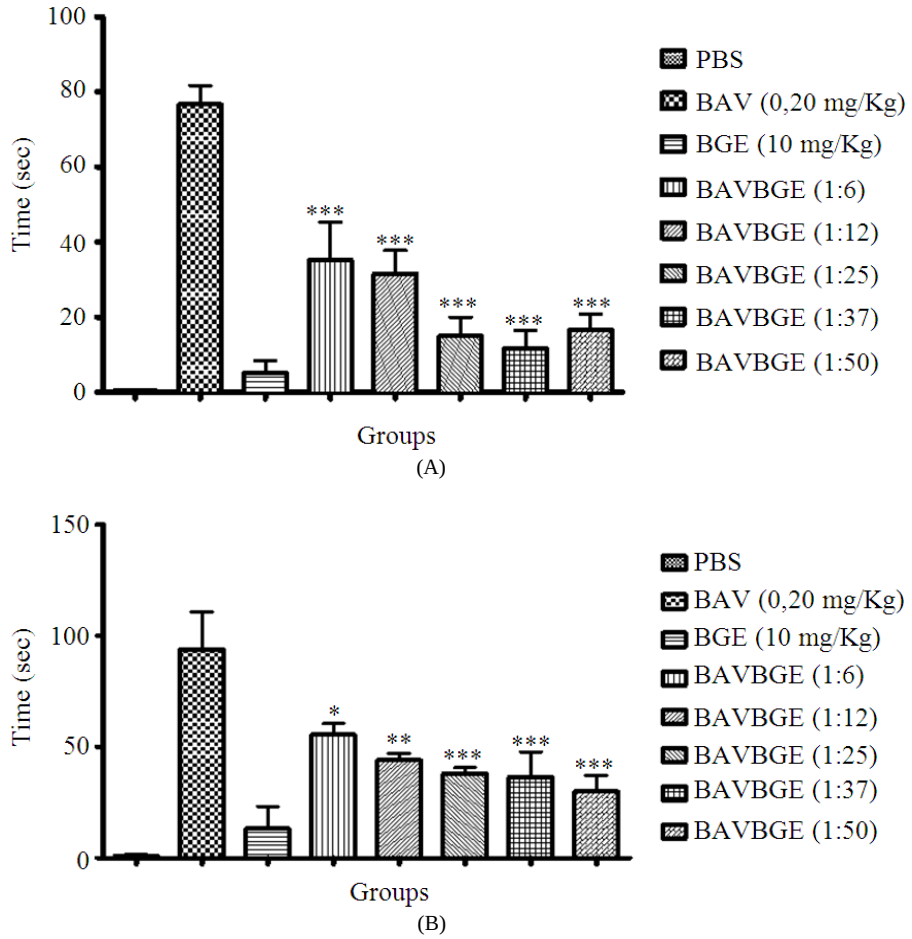
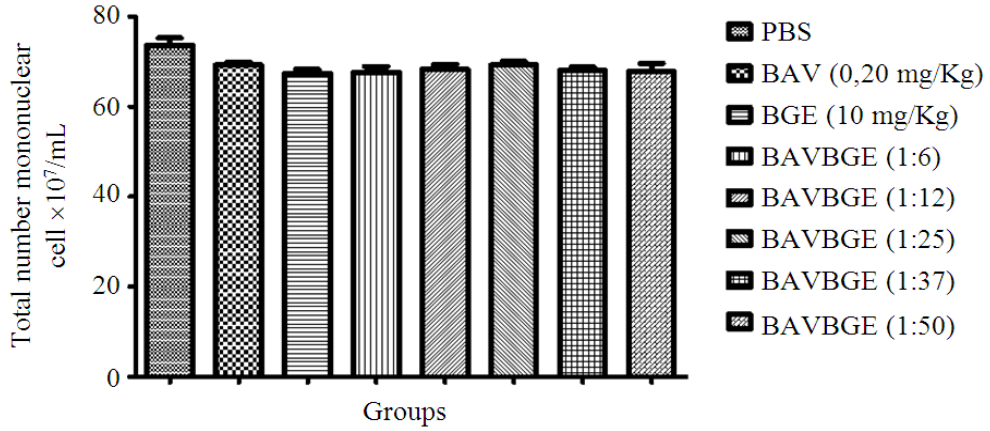


Fig. 2. BGE effect on the nociceptive activity induced by Bothrops atrox venom (0,20 mg/kg) injected subcutaneously into the right hind paw. BAVBGE 1:6 (BAV + 1,25 mg BGE/Kg/25 µL PBS); BAVBGE 1:12 (BAV + 2,5 mg BGE/Kg/25 µL PBS); BAVBGE 1:25 (BAV + 5,0 mg BGE/Kg/25 µL PBS); BAVBGE 1:37 (BAV+7,5 mg BGE/Kg/25 µL PBS); BAVBGE 1:50 (BAV+10 mg BGE/Kg/25 µL PBS). The responses were measured at 5 min (first phase - A) and 20-30 min (second phase - B) after venom or saline solution administration. The results are presentedthe mean ± S.E.M. of five animals. Differences among groups were analysed by one-way Analysis of Variance (ANOVA), followed by the Tukey-Kramer test. Differences with an associated probability (p value) of less than 5% ($p<0.05$) were considered significant. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with BAV group

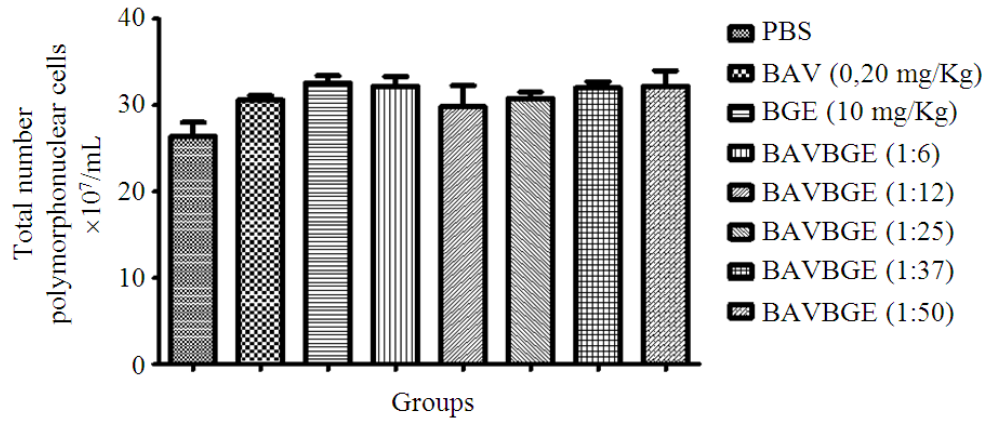
The results showed that all BGE concentrations used in the present study were not effective on the migration of inflammatory cells induced by venom administration (**Fig. 3**).

In vitro studies carried out with human plasma demonstrated that BGE concentrations used in the present study did not inhibit the venom coagulant activity, as shown in **Fig. 4**. BGE effects on hemorrhagic activity induced by *B. atrox* venom was also analysed. As observed in **Fig. 5**, BGE (1:3; 1:6; 1:12; 1:18; 1:25) inhibited significantly the hemorrhage (13, 40±0, 4583, p<0, 05; 13, 50±0, 3536, p<0, 05; 13, 30±0,7842, p<0, 05; 13, 40±0, 3536, p<0, 05, respectively) when compared to the animals administered *B. atrox* venom alone (15, 60±0, 40).

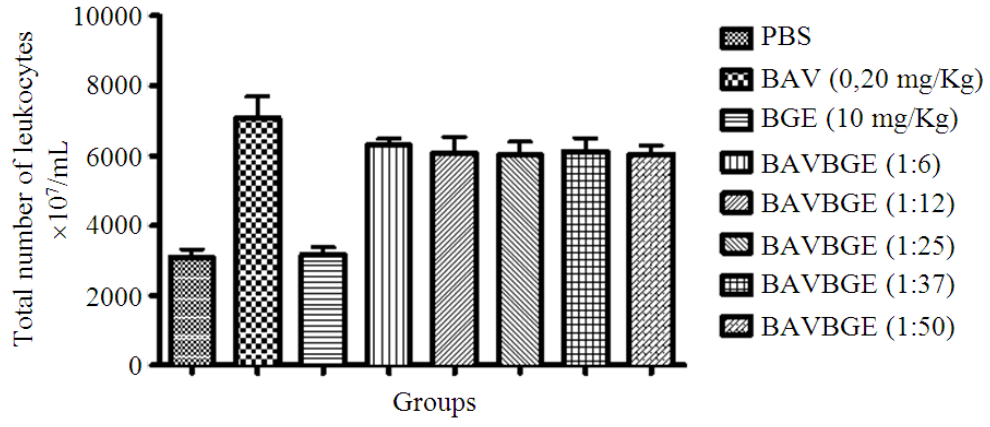
Intramuscular injections of *B. atrox* venom (0.20 mg/25 µL PBS) induced a significant increase on plasma CK activity when compared to PBS control group. The results also showed that PBE was not able to inhibit increase on plasma activity when compared to BAV control group (**Fig. 6**). Indirect hemolytic method, showed that BGE concentrations used in the present study did not inhibit the Phospholipase A2 (PLA2) activity, as shown in **Fig. 7**.



(A)



(B)



(B)

Fig. 3. Peritonitis induced by Bothrops atrox venom and treated with BGE (A, B and C). The mice were injected with 020 mg/kg B. atrox venom by intraperitoneal injection. BAVBGE 1:6 (BAV+1,25 mg BGE/Kg/25 µL PBS); BAVBGE 1:12 (BAV+2,5 mg BGE/Kg/25 µL PBS); BAVBGE 1:25 (BAV + 5,0 mg BGE/Kg/25 µL PBS); BAVBGE 1:37 (BAV + 7,5 mg BGE/Kg/25 µL PBS); BAVBGE 1:50 (BAV+10 mg BGE/Kg/25 µL PBS). The results are presentedas the mean ± S.E.M. of five animals

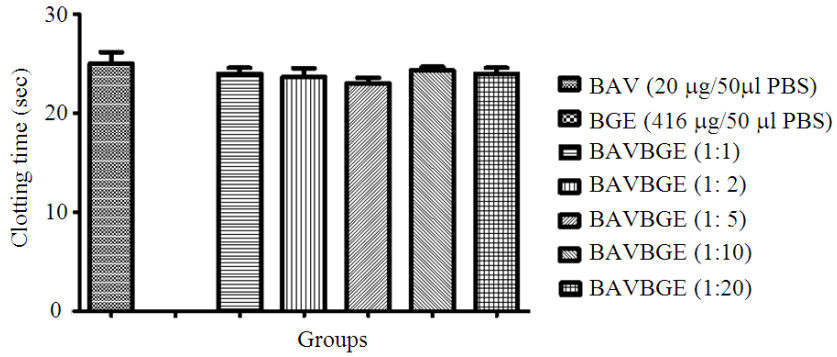


Fig 4. BGE effects on coagulant activity *Bothrops atrox* venom. BAV 20µg/25µL PBS. BAVBGE 1:1 (BAV + 26 µg BGE/25 µL PBS); BAVBGE 1:2 (BAV + 52 µg BGE/25 µL PBS); BAVBGE 1:5 (BAV + 104 µg BGE/25 µL PBS); BAVBGE 1:10 (BAV + 208 µg BGE/25 µL PBS); BAVBGE 1:20 (BAV + 416 µg BGE/25 µL PBS). The experiment was carried out in triplicate

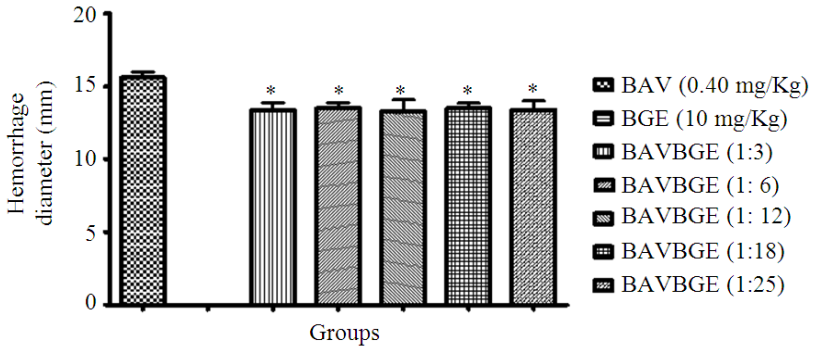


Fig. 5. Hemorrhage induced by Bothrops atrox venom and treated with BGE. BAV (0,20 mg/25 µL PBS) . BAVBGE 1:6 (BAV + 1,25 mg BGE/Kg/25 µL PBS); BAVBGE 1:12 (BAV + 2,5 mg BGE/Kg/25 µL PBS); BAVBGE 1:25 (BAV + 5,0 mg BGE/Kg/25 µL PBS); BAVBGE 1:37 (BAV + 7,5 mg BGE/Kg/25 µL PBS); BAVBGE 1:50 (BAV + 10 mg BGE/Kg/25 µL PBS). The results are presentedas the mean ± S.E.M. of five animals. Differences with an associated probability (p value) of less than 5% ($p<0.05$) were considered significant. *$p<0.05$, compared with BAV group

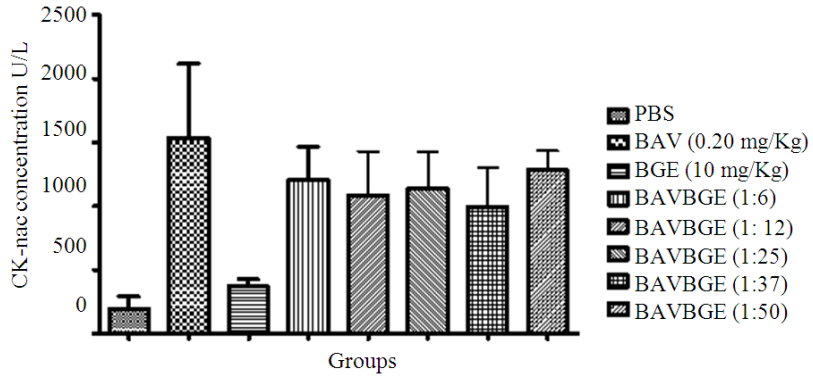


Fig. 6. Myotoxicity induced by Bothrops atrox venom and treated with BGE. BAV (0,20 mg/25 µL PBS) . BAVBGE 1:6 (BAV + 1,25 mg BGE/Kg/25 µL PBS); BAVBGE 1:12 (BAV + 2,5 mg BGE/Kg/25 µL PBS); BAVBGE 1:25 (BAV + 5,0 mg BGE/Kg/25 µL PBS); BAVBGE 1:37 (BAV + 7,5 mg BGE/Kg/25 µL PBS); BAVBGE 1:50 (BAV + 10 mg BGE/Kg/25 µL PBS). The results are presentedas the mean ± S.E.M. of five animals

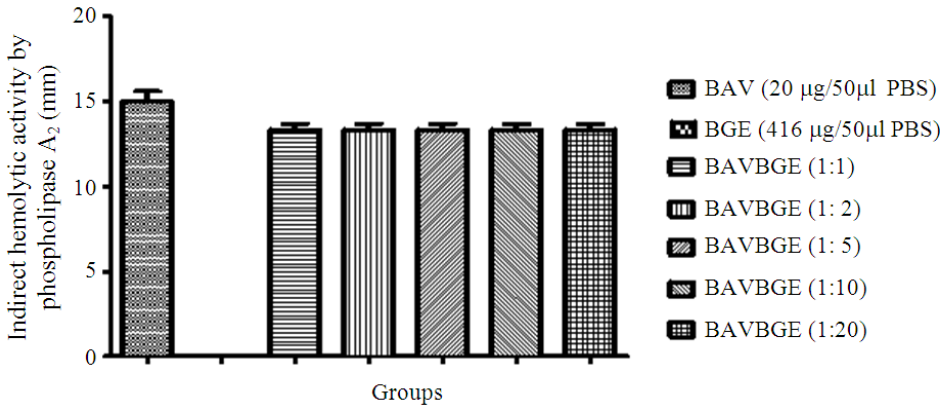


Fig. 7. BGE effects on Phospholipase A2 activity Bothrops atrox venom. BAV 20µg/ 25µL PBS. BAVBGE 1:1 (BAV + 26 µg BGE/25 µL PBS); BAVBGE 1:2 (BAV + 52 µg BGE/25 µL PBS); BAVBGE 1:5 (BAV + 104 µg BGE/25 µL PBS); BAVBGE 1:10 (BAV + 208 µg BGE/25 µL PBS); BAVBGE 1:20 (BAV + 416 µg BGE/25 µL PBS). The experiment was carried out in triplicate

4. DISCUSSION

In many countries, plants have been used for snakebite treatments. In Amazon region, local people use the macerated bark of *B. guinanensis* applied in the form of cataplasm, on the site of snakebite. However, scientific validation of the antiophidian properties is needed. In the present study we analyzed for the first time the ability of the *B. guianensis* aqueous extract in the neutralization several effects induced by *B. atrox venom*.

Our results showed that *B. guianensis* aqueous extract was not effective to inhibit oedema, peritonitis coagulant, myotoxic, indirect hemolytic activities induced by *B. atrox venom*, but was able to inhibited significantly hemorrhagic and nociceptive activities. Soares *et al*. (2005) and coworkers summarized 850 species from 138 families of plants used ethnobotanically and ethnopharmacologically or confirmed by biological assays. Several plant species used by Brazilian folk medicine had been studied against snake venom activities (Melo *et al*., 1994; Da Silva *et al*., 2005; Nishijima *et al*., 2009). De Paula *et al*. (2010) and coworkers evaluated antiophidian properties of 12 Brazilian plants extracts against the hemolytic, coagulant, hemorrhagic and proteolytic effects of *Lachesis muta* venom. Data revealed that most of these aqueous products were capable of inhibiting those activities at different levels, except for *Sapindus saponaria* extract. In contrast, *Stryphnodendron barbatiman* extract completely neutralized all the analyzed biological activities.

5. CONCLUSION

The results of this study indicated that *Brosimum guianensis* extract reduced nociception and hemorrhage produced by *Bothrops atrox* venom in mice, however, was not effective to inhibit oedema, peritonitis, coagulant, myotoxic, phospholipase A2 activity (indirect hemolytic method). Further studies are necessary to isolated and identified yours active components, opening the possibility of synthesis of new compounds and application for therapeutic purpose as supplements to conventional serum therapy in reducing nociception and hemorrhage induced by *B. atrox* venom.

These results also support a potential effect of this extract as a compounds source for biotechonological application and synthesis of new drugs with therapeutic purpose.

6. ACKNOWLEDGEMENT

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