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EFFECTS OF THE AQUEOUS EXTRACTS OF PLANTS OF THE GENERA *APODANTHERA* (CUCURBITACEAE) AND *JATROPHA* (EUPHORBIACEAE) ON THE LETHALITY OF THE VENOM OF *BOTHROPS JARARACA* (SERPENTES, VIPERIDAE)

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ABSTRACT

Four plants popularly used as antiophidic in the cerrado and caatiga regions of the Northeastern and Central Brasil were analyzed in order to verify the inhibition of the venom lethal action of the Neotropical viper *Bothrops jararaca* (Serpentes, Viperidae). The plants were *Apodanthera villosa* (Cucurbitaceae), *Apodanthera glaziovii* (Cucurbitaceae), *Jatropha molissima* (Euphorbiaceae) and *Jatropha elliptica* (Euphorbiaceae). The LD_{50} of the venom was 37.1 µg/mouse. The control groups were mouse inoculated with the viper venom (71.2 µg), the experimental groups were mouse inoculated with the viper venom (71.2 µg) and aqueous extract of the plants. The extract of *A. villosa* (1.0 mg) increased the survivor time of the experimental animals compared to control groups.

Keywords: Antiophidic plants, *Apodanthera*, *Jatropha*, caatinga, cerrado.

RESUMO

Foram analisadas quatro plantas popularmente utilizadas como antiofídicas nas regiões de caatinga e cerrado do nordeste e Brasil central, para verificar a inibição da ação letal do veneno da serpente *Bothrops jararaca* (Serpentes, Viperidae). As plantas foram *Apodanthera villosa* (Cucurbitaceae), *Apodanthera glaziovii* (Cucurbitaceae), *Jatropha molissima* (Euphorbiaceae) e *Jatropha elliptica* (Euphorbiaceae). A DL_{50} do veneno foi 37.1 µg/camundongo. O grupo controle foi camundongos inoculados com o veneno de jararaca (71.2 µg), o grupo experimental foi camundongos inoculados com o veneno de jararaca (71.2 µg) e os extratos aquosos das plantas. O extrato de *A. villosa* (1.0 mg) aumentou o tempo de sobrevida dos animais experimentais quando comparados com o grupo controle.

Palavras-chave: Plantas antiofídicas, *Apodanthera*, *Jatropha*, caatinga, cerrado.

INTRODUCTION

The venom of the snakes – the Neotropical vipers – of the genus *Bothrops* (family Viperidae) has a systemic hemorrhagic effect and increases the coagulation time of the blood (Furtado *et al.*, 1991; Gutierréz, 2002). *Bothrops* are responsible for the majority of snake envenomations that occur in Brasil. Treatment with antivenins is effective and recommended, but in many remote regions, where medical services are precarious, envenomation if often treated with local plant-based remedies (Otero *et al.*, 2000; Vilar *et al*., 2004, 2005). Two of the plants recommended for the treatment of envenomation by vipers are known locally as "batata-de-teiú" and "pinhão-bravo" (Braga, 1960; Mors, 1991; Martz,

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1992), although few reliable reports of the antivenin properties of plants with these names are available (Amuí *et al*., 2003). A third plant, referred to as "cabeça-de-negro" is also frequently cited as an inhibitor of viper venom, although the report (Nakagawa *et al*., 1982) does not identify the species, and there are at least ten different plant species known by this name (Silva *et al*., 1998). Given these reports, the objective of the present study was to contribute to the scientific knowledge and therapeutic use of these plants, in particular with regard to their effectiveness against viper venom.

METHODS

Venom: Mixture of lyophilized *Bothrops jararaca* venom provided by the Instituto Butantan, São Paulo State Health Department. The mixture was stored at -20ºC until used.

Animal subjects: Experiments were conducted on non-isogenic Swiss mice weighing 18-22 g supplied by the Central Breeding Laboratory of the Universidade Federal de Sergipe in São Cristóvão.

Plant samples: Four plant species belonging to the Cucurbitaceae and Euphorbiaceae families were analyzed in the present study. The Cucurbitaceae was represented by the herbaceous *Apodanthera villosa* C. Jeffrey, known locally as "batata-de-teiú", and the climber *Apodanthera glaziovii* Glaziou ex Cogniaux, known as "cabeça-de-negro". These two species, together with the bush known as "pinhão-bravo" (*Jatropha molissima* (Pohl) Baill), a member of the Euphorbiaceae, were collected at Curituba (09°41′S, 37°53'W), in the northeastern Brasilian state of Sergipe. A second species of Euphorbiaceae, a herbaceous plant also known as "batata-de-teiú" (*Jatropha elliptica* (Pohl) Muell. Arg.), was collected in Peixe (12º01'S, 48º32'W), in the central Brasilian state of Tocantins. Specimens were identified at the Department of Botany of the Universidade Federal de Goiás in Goiânia, and the Instituto de Botânica of the São Paulo State Environment Department.

Preparation of the aqueous extract: Tubers of *A. villosa, A. glaziovii* and *J. elliptica*, and stems of *J. mollissima* were oven-dried at 40ºC, and ground to dust in a mortar. Raw extracts were prepared via the decoction of 200 grams of the durst for 10 minutes. This extract was filtered and lyophilized.

Lethal dose 50% of the venom: The LD_{50} of the venom was determined by a regression analysis of the number of mice surviving or dying during the 48 hours following inoculation with *Bothrops* venom. The coefficient of regression (y) is given by $y = a + bx$, where x is the logarithm of the dose concentration (Fisher & Yates, 1953). The value of x for 50% mortality is found where $y = 5$. The doses of viper venom were 23.0, 27.6, 33.1, 39.7 and 47.6 µg. The between-dose interval was established based on the LD_{50} of the venom of *B. jararaca* determined by the Instituto Butantan. Starting with the smallest dose of 23.0µg, which is smaller than the venom's LD_{50}, larger doses were determined by a 1.2 dilution factor, the standard ratio for venom studies. For each dosage, six mice were inoculated intraperitoneally with 0.5 ml of the venom dissolved in 0.9% NaCl solution.

Lethal dose 50% of the plant extracts: It was necessary to determine the LD_{50} of the plants in order to establish extract dosages for the tests on the inhibition of the lethality of the venom. Three experiments were conducted on the extracts of each of the species *A. villosa, A. glaziovii* and *J. mollisima.* The LD_{50} values of these plants were obtained from the combined analysis of all experiments, derived from the probits. Extract doses varied from 0.004 to 0.008 g, 0.001 to 0.011 g and 0.0003 to 0.04 g, respectively. Analysis of the 50% lethal dose of *J. elliptica* was

based on a single experiment, with doses varying from 0.01 to 0.03 g

Experiments on the inhibition of lethality: The venom's LD_{50} was doubled to determine the challenge dose (Gutiérrez *et al.*, 1990) used as the criterion for testing the ability of the plants to neutralize the toxin. Lethality was tested in two groups of six mice. In the control group, the mice were inoculated with $2LD_{50}$ of the venom dissolved in 0.9% NaCl solution. In the experimental group, the mice received the same challenge dose as the control group, but with the addition of the plant extracts dissolved in 0.9% NaCl. The solutions of venom plus extract were incubated at 37ºC for 30 minutes prior to inoculation. The doses for each extract were: *A. villosa* 1.00 mg (LD_{50} 0.018 g), *A. glaziovii* 1.00 - 1.48 - 3.0 - 5.0 mg (LD_{50} 0.007 g), *J. mollissima* 1.0 mg (LD_{50} 0.002 g), and *J. elliptica* 0.74 - 1.00 - 1.48 - 5.0 - 10.0 mg (LD_{50} 0,018 g). These doses were determined after establishing the corresponding LD_{50} values, which were done through probitic analysis. Each mouse was inoculated intraperitoneally with 0.5 ml of the solution. To determine survival time, the animals were observed at one-hour intervals during 48 hours.

Data analyses: All probitic values were obtained using the method of Fisher (1949) and Fisher & Yates (1953). In the inhibition of lethality experiments, the first step was to confirm the homogeneity of controls and experimental samples for each dosage of the extract. When considered homogeneous, samples were grouped for final analysis, using one-factor ANOVA and the *t* test for comparisons between the means of two samples (Vanzolini, 1993; Zar, 1996).

RESULTS

A total of six control experiments were carried out, whereas the number of extract tests varied according to the plant species. Regressions and 50% lethal doses were determined for controls and extracts (Table 1). In the case of the control, the regression of lethality on venom dosage was $y = -7.99 + 8.28x$, with a confidence interval of 34.43 ± 40.45 (Table 1). The 50% mortality threshold (LD_{50}) is found at $y = 5$, so in this experiment, the dose of *B. jararaca* venom for 50% mortality of the inoculated mice was estimated as $x = 37.09$ mg. Given this, the challenge dose (2 LD_{50}) used as the criterion for testing the effects of the extracts on the lethality of the venom was 74.18 mg.

Four experiments were conducted using the extract (1.0 mg) of *A. villosa*. As both control and experimental groups were homogeneous ($F_{0.05(1)5,27} = 1.436$, $p>0.05$ and $F_{0.05(1)3,42} = 0.857$, $p>0.05$, Table 2), samples were grouped and compared using the *t* test. Survival time was significantly greater in the experimental group ($t_{0.05(1),67} = 2.084$, $p<0.05$, Table 3), which indicates that the extract of this species was effective in inhibiting the lethality of the viper venom.

The extract (1.0 mg) of *A. glaziovi* was tested in four experiments, the results of which were homogeneous among samples ($F_{0.05(1)2,31} = 2.872$, $p>0.05$, Table 2), and thus grouped for analysis. Doses of 1.48 mg, 3.0 mg, and 5.0 mg were also tested. Differences in survival time were not significant at any dosage, however ($F_{0.05(1)4,63} = 0.544$, $p>0.05$, Tables 4 and 5).

Two experiments were carried out on the extract (1.0 mg) of *J. mollissima*, the results of which were statistically homogeneous ($t_{0.05(2)10} = 0.991$, $p>0.05$, Table 6) and thus grouped for comparison with the control group. As for the previous species, no significant difference was found in survival time ($t_{0.05(1)19} = 0.631$, $p>0.05$, Table 6).

Finally, five experiments were conducted on the extract (1.0 mg) of *J. elliptica*, with doses of 0.74-10.0 mg, which were also homogeneous overall ($F_{0.05(1)4,24} = 1.972$, $p>0.05$, Table 2). Once again, differences between the experimental and control

samples were not significant ($F_{0.05(1)4,39} = 0.91$, $p>0.05$, Tables 7 and 8), contradicting the possible effect of the extract of this plant on the lethality of viper venom.

DISCUSSION

Plants known as "batata-do-teiú" – which means the "potato of the teiú lizard" (*Tupinambis* sp., family Teiidae) – are identified as containing an antivenin based on a legendary lizard which ate the root of the plant and thus became immune to the venom of vipers and rattlesnakes, *Bothrops* and *Crotalus* (Braga, 1960; Mors, 1991). A number of different plant species are known by this name (Martz, 1992), including *Apodanthera villosa*, the extract of which (1.0 mg) was shown in the present study to have a significant retardant effect on the lethality time of the animals inoculated with viper venom. No information is available on the chemical composition of this plant, nor experimental data on its antivenin properties.

The extracts of *Apodanthera glaziovi*, *Jatropha molissima* and *J. elliptica* did not present effects of neutralization on the lethality of *Bothrops jararaca* venom.

There are numerous reports of plants that act on the venom of snakes, in particular the rattlesnakes, genus *Crotalus* (Mors *et al.*, 1989; Pithayanukul *et al*., 2004). Nakagawa *et al*. (1982) pioneering experimental study of plants that affect the lethality of viper venom isolated substances denominated cabenegrin I and II from a plant identified only as "cabeça-de-negro", supposedly Amazonian in origin. Cabenegrins are pterocarpans of the isoflavonoid group, which are produced by plants as a defense against infection by virus or fungi (Costa, 2000). A plant with this name was also mentioned by Borges *et al.* (1996) in their analysis of the product "Específico Pessoa", and concluded that it was ineffective for the treatment of the venom of *Botrops atrox.* This word, "específico" is used generically for remedies in homeopathy or to treat specific problems, and this product "Pessoa" – which is produced from a plant called "cabeça-de-negro", according to its label – is a popular antivenin in northern and northeastern Brasil.

Cabenegrins were mentioned in a second study of plants with antivenin properties (Reyes-Chilpa *et al*., 1994). The extract of the legume *Brongniartia podalyrioides* reduced mortality in mice inoculated with the venom of *Bothrops atrox*, in a similar form to that observed in the case of *A. villosa* in the present study. The active compound isolated from this species was the pterocarpan (-)-edunol, which related structurally to (-)-cabenegrins A-I and A-II.

Few reports are available on the composition of vegetable compounds that reduce the lethality of viper venom. One of the early studies of plants that affect viper venom records the action of the extract of *Mandevilla velutina* (Apocynaceae) on bradicinin, an endogenous polypeptide released into the blood plasma by the enzymatic action of viper venom (Calixto *et al*., 1985). Pereira *et al.* (1994) identify a number of plants and their components as having an effect on the venom of *Bothrops jararaca*: i) triterpenes and steroids of *Periandra mediterranea* (Vell.) Taub (Fabaceae) and *Apuleia leiocarpa* (Vogt) Macbr (Caesalpiniaceae); ii) derivatives of the cafeic acid of *Vernonia condensata* Baker (Compositae) and *Cynara scolymus* L. (Asteraceae); iii) cumarins of *Mikania glomerata* Spreng (Asteraceae) and *Dorstenia brasiliensis* Lam. (Moraceae); iv) flavonoids of *Phyllanthus klotazschianus* M. Arg (Euphorbiaceae), *Citrus sinensis* (L.) Osbeck (Rutaceae), *Apuleia leiocarpa* and *Derris sericea* (H.B.K.) Ducke (Fabaceae); v) lignoflavonoids of *Silybum marianum* Gaertn. (Compositae); vi) cumestans of *Eclipta prostrata*, vii) saponins of *Bredemeyera floribunda* Willd (Polygalaceae).

Mors *et al.* (2000) refer to a variety of substances isolated from plants which interfere in the lethal properties of the venom of *B. jararaca* in mice, such as corticosteroids, triterpenes, phenolic compounds,

hydroxibenzoic acids, chlorogenic acids, curcuminoids, cumarins, flavonoids, pterocarpans, aristolochic acids, tannins and polysaccharides. All these substances belong to the same class of "secondary metabolites", which are capable of interacting with venom receptor and enzymes.

More recently, Ticli *et al.* (2005) analyzed the possible neutralization effects of the methanolic extract of *Cordia verbenacea* (Boraginaceae) on the venom of *Bothrops jararacussu* and its principal phospholipases A_2 (Bothropstoxins I and II). They isolated the plant's active ingredient, identified as rosmarinic acid, and concluded that the pure compound potentializes the action of viper-rattlesnake antivenin by neutralizing the venom's lethal effects.

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TABLES

Table 1. Regression of the probitic value (y) on the logarithm of the concentration (x) of the 50% lethal dose (LD_{50}), control (µg) and extracts (mg).

	y = a+bx	Iy	LD_{50}
Control	– 7.99 + 8.28x	34.43±40.45	37.09
Apodanthera villosa	10.797+3.358x	0.015±0.023	0.018
Apodanthera glaziovii	12.722+3.596x	0.0059±0.0089	0.007
Jatropha mollissima	9.910+1.831x	0.0013±0.003	0.002
Jatropha elliptica	15.691+6.193x	0.013±0.023	0.018
b, regression coefficient	a, constant of regression	Iy, confidence interval of *y*	LD_{50} for $y = 5$

Table 2. ANOVA of survival (hours) of mice and homogeneity of control and experimental samples.

Control/Extract	Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Control	Groups	5	5.721	1.144	1.436ns
	Error	27	21.52	0.7971	
Apodanthera villosa	Groups	3	529.95	176.65	0.857ns
	Error	42	8650.7	205.97	
Apodanthera glaziovii	Groups	2	311.20	155.60	2.872ns
	Error	31	1679.6	54.182	
Jatropha elliptica	Groups	4	2.433	0.6082	1.972ns
	Error	24	7.401	0.3084	

Table 3. Statistics of the frequency distribution of mouse survival (hours), and comparison of the means of the control and sample treated with the extract of *Apodanthera villosa*.

Group	Dose of venom (µg)	Dose of extract (mg)	N	A	m	s	CV	Im	*t*
Control	74.2	-	23	2.26-5.93	3.203±0.182	0.875	27.31	2.825-3.581	2.084 *
Extract	74.2	1.0	46	1.56-48.0	7.609±2.106	14.283	187.7	3.364-11.854	

N, sample size; m, mean±standard error; A, amplitude; s, standard deviation; CV, coefficient of variation; Im, confidence interval of the mean; * significant at 5% level

Table 4. Statistics of the frequency distribution of mouse survival (hours), and comparison of the means of the control and sample treated with extracts of *Apodanthera glaziovii*.

Group	Dose of venom (µg)	Dose of extract (mg)	N	A	m	s	CV	Im
Control	74.2	-	16	2.26-5.93	3.614±0.284	1.138	31.48	3.00-4.221
Extract (1)	74.2	1.00	34	2.05-48.0	4.201±1.332	7.767	184.8	1.489-6.912
Extract (2)	74.2	1.48	6	2.16	2.16±0.0	0.0	0.0	2.16
Extract (3)	74.2	3.00	6	1.35-3.45	2.027±0.3	0..737	36.35	1.253-2.8
Extract (4)	74.2	5.00	6	1.06-1.56	1.297±0.08	0.218	16.80	1.068-1.526

N, sample size; A, amplitude; CV, coefficient of variation; ( ) Experiments 1-4
m, mean±standard error; s, standard deviation; Im, confidence interval of the mean

Table 5. ANOVA of survival (hours) of mice and homogeneity of control and experimental samples of *Apodanthera glaziovii.*

Source of variation	Degree of freedom	Sum of squares	Mean squared	F
Group	4	69.532	17.383	0.544 ns
Error	63	2013.2	31.956	

ns, not significant

Table 6. Statistics of the frequency distribution of mouse survival (hours), control and extract of *Jatropha mollissima*, comparison of the means of the experiments 1 and 2 of the extract and between the control and extract.

Group	Dose of venom (µg)	Dose of extract (mg)	N	A	m	s	CV	Im	*t*
Extract 1	74.2	-	6	2.35-48.0	10.01±7.597	18.608	185.7	-9.51-29.54	0.991ns
Extract 2	74.2	1.0	6	1.55-3.33	2.485±0.254	0.623	25.07	1.83-3.14	
Control	74.2	-	10	2.70-5.13	3.758±0.268	0.849	22.59	3.151-4.365	0.63 ns
Extract	74.2	1.0	11	1.55-48.0	6.517±4.15	13.763	211.1	-2.728-15.763	

N, sample size; A, amplitude; CV, coefficient of variation; ns, not significant
m, mean±standard error; s, standard deviation; Im, confidence interval of the mean

Table 7. Statistics of the frequency distribution of mouse survival (hours), comparison of the means of the control and sample treated with extracts of *Jatropha elliptica.*

Group	Dose of venom (µg)	Dose of extract (mg)	N	A	m	s	CV	Im
Controle	74.2	-	11	2.66-4.93	3.325±0.202	0.670	20.15	2.874-3.775
Extract (1)	74.2	0.74	6	2.25-3.26	2.945±0.144	0.353	11.98	2.574-3.316
Extract (2)	74.2	1.00	6	2.00-3.53	2.842±0.207	0.508	17.87	2.308-3.375
Extract (3)	74.2	1.48	6	1.63-3.38	2.73±0.247	0.605	22.16	2.094-3.366
Extract (4)	74.2	5.00	8	1.48-48.0	7.841±5.74	16.236	207.0	-5.734-1.417
Extract (5)	74.2	10.00	8	1.4-2.68	2.083±0.196	0.555	26.64	1.618-2.547

N, sample size
m, mean±standard error
A, amplitude
s, standard deviation
CV, coefficient of variation
Im, confidence interval of the mean
( ) Experiments 1-5

Table 8. ANOVA of the survival time (hours) of mouse, control and extract of *Jatropha elliptica.*

Source of variation	Degree of freedom	Sum of squares	Mean squared	F
Grupos	5	173.16	34.632	0.727 ns
Erro	39	1855.6	47.580	

ns, not significant