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PII: S0378-8741(17)31795-6
DOI: http://dx.doi.org/10.1016/j.jep.2017.09.015
Reference: JEP11027

To appear in: *Journal of Ethnopharmacology*

Received date: 6 May 2017
Revised date: 5 September 2017
Accepted date: 14 September 2017

Cite this article as: Bárbara Ricciardi Verrastro, Ana Maria Torres, Gabriela Ricciardi, Pamela Teibler, Silvana Maruñak, Chiara Barnaba, Roberto Larcher, Giorgio Nicolini and Eduardo Dellacassa, The effects of *Cissampelos pareira* extract on envenomation induced by *Bothropsdiporus* snake venom, *Journal of Ethnopharmacology,* http://dx.doi.org/10.1016/j.jep.2017.09.015

The effects of *Cissampelos pareira* extract on envenomation induced by *Bothropsdiporus* snake venom

Bárbara Ricciardi Verrastro[a], Ana Maria Torres[a], Gabriela Ricciardi[a], Pamela Teibler[b], Silvana Maruñak[b], Chiara Barnaba[c], Roberto Larcher[c], Giorgio Nicolini[c], Eduardo Dellacassa[d]

[a]Facultad de Ciencias Exactas y Naturales y Agrimensura, Universidad Nacional del Nordeste, Av. Libertad 5470; 3400, Corrientes, Argentina.

[b]Facultad de Veterinaria, Universidad Nacional del Nordeste, Sargento Cabral 2139, 3400-Corrientes, Argentina.

[c]Facultad de Química, Universidad de la República, Av General Flores 2124, 11800-Montevideo, Uruguay.

[d]Centro Trasferimento Tecnologico, Fondazione Edmund Mach di San Michele all'Adige, Via E. Mach 1, 38010-S, Michele all'Adige (TN), Italy.

Correspondence to: Departamento de Química Orgánica, Facultad de Química, Universidad de la República, Gral Flores 2124, 11800-Montevideo, Uruguay.

E-mail addresses: barb_ricciardi@hotmail.com (B. Ricciardi), amtorres@exa.unne.edu.ar (A.M. Torres), gricciardi@exa.unne.edu.ar (G. Ricciardi), serviciofarmacia@vet.unne.ed.ar (P. Teibler), patmed@vet.unne.edu.ar (S. Maruñak), chiara.barnaba@fmach.it (C. Barnaba), roberto.larcher@fmach.it (R. Larcher), giorgio.nicolini@fmach.it (G. Nicolini), edellac@fq.edu.uy (E. Dellacassa).

Contributions to the study: phytochemistry and chromatography (B. Ricciardi, G. Ricciardi, E. Dellacassa), *in vitro* experiments (A.M. Torres), *in vivo* experiments (P. Teibler, S. Maruñak), MS experiments (C. Barnaba, E. Dellacassa), spectra interpretation (R. Larcher, G. Nicolini).

Ethnopharmacological relevance

Ophidian accidents are a serious public health problem in Argentina; the *Bothrops* species is responsible for 97% of these accidents, and in particular, *B. diporus* is responsible for 80% of them. In the northeast of the country (Corrientes Provinces), *Cissampelos pareira* L. (Menispermaceae) is commonly used against the venom of *B. diporus;* its use is described in almost all ethnobotanical literature from countries where the plant grows,.

Aim of the study

In this study, the *in vitro* and *in vivo* antivenom activities of *C. pareira* extracts were evaluated against *B. diporus* venom, with a particular focus on the local effects associated with envenoming. The seasonal influence on the chemical composition of the active extracts was also studied, in order determine the associated range of variability and its influence on the antivenom activity.

Materials and Methods

This research was conducted using aerial parts (leaves, flowers, tender stems) and roots of *Cissampelos pareira* collected from two different phytogeographic regions of Corrientes (Argentina); Paso de la Patria and Lomas de Vallejos. In addition, to perform a seasonal analysis and to evaluate the metabolic stability, material was collected at three different growth stages. *In vivo* and *in vitro* anti-snake venom activities were tested, and a bio-guided chromatographic separation was performed in order to determine the active chemicals involved. The fractions obtained were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the chemical profile of the most active constituent was analyzed by ultra high-performance liquid chromatography coupled to quadrupole/high-resolution mass spectrometry (Q-Orbitrap). (UHPLC-MS).

Results

The alcoholic extract was found to be the most active The bio-guided fractionation allowed selection one fraction to be analyzed by UHPLC-MS in order to identify the components responsible for the activities found; this identified five possible flavonoids.

Conclusions

. Our studies of the activity of *C. pareira* against the venom of *B. diporus* have confirmed that this species possesses inhibitory effects in both *in vitro* and *in vivo* models.

Moreover, the present data demonstrate that certain flavonoids may mitigate some of the venom-induced local tissue damage.

Keywords: *Bothrops diporus*; Ophidian accidents; *Cissampelos pareira*; Snake antivenom; UHPLC-MS (Q-Orbitrap); Flavonoids.

1. Introduction

Cissampelos pareira L. (Menispermaceae) is commonly known as *ka'apeva, ka'á-peva, ysypó-morotí, caápebá, zarza, pareirabrava,* or *mil hombres*. Its popular uses are mentioned in almost all ethnobotanical literature from countries where the plant grows, including South America, Asia, and Africa (Semwal et al., 2014; Singh et al., 2010). As per traditional knowledge, it is used as a carminative, a febrifuge, for the alleviation of liver disorders, and for constipation, menstrual pain, colic, and rheumatism (Arora et al., 2012; Semwal et al., 2014).

Ethnopharmacological surveys have established that *Cissampelos pareira* decoctions containing leaves and roots, as well as aqueous or alcoholic infusions, are traditionally used for ophidian antivenom in Paraguay (González Torres, 2005; Jolis, 1789; Manfred, 2014; Montenegro, 2007), India and Pakistan (Chakraborty and Bhattacharjee, 2006; Dey and De, 2013; Jabeen et al., 2009; Kadel and Jain, 2008; Katewa and Galav, 2006; Sankaranarayanan et al., 2010), and Mexico (Ramos Hernandez et al., 2007). Its use also extends to the Amazon (Ecuador and Peru) and Central America (Barranco Pérez, 2010; Giovannini et al., 2017).

Some of its pharmacological properties have been scientifically investigated; specifically, anti-inflammatory, antispasmodic, and muscle and uterine relaxant properties in rats and rabbits (Feng et al., 1962), and diuretic and curare mimetic effects (Basu, 1970). Further, the activities of some of its isolated components have been investigated, including tetrandrine (analgesic, antipyretic, anti-inflammatory, cardioactive, and hypotensive effects), pareirubines A and B (antileukemic), alkaloids (febrifuges and curarizers), berberine (hypotensive, antimicrobial, and antifungal effects) (Sanchez-Medina et al., 2001), and cissampelin (muscle relaxant) (Semwal et al., 2014). In particular, in Costa Rica it has been shown that a 10% aqueous infusion of the whole plant has anti-hemorrhagic and anti-proteolytic activity against *B. asper* venom (Badilla et al., 2008). Snakebites are seriously under-reported worldwide. This is especially true in countries where agricultural activities are predominant, since this is one of the occupations most often affected by snakebites. Moreover, studies show not only a higher incidence in men but also a reasonably high incidence in children. This may be related to the fact that in rural areas of many under developed countries, where snakebites represent a major health issue, children

take part in agricultural activities or are attacked due to their innate curiosity (Chippaux, 1998).

Generally, local effects of a snakebite occur in the first 10 to 30 minutes; there may be numbness around the bite with bleeding, or a purpuric rash, and/or necrosis or gangrene (Cavazos et al., 2012). These local reactions are not effectively neutralized by conventional antivenom serum therapy, as revealed by animal models and clinical studies (Avila-Aguero et al., 2001; Lomonte et al., 1994). In severe cases, local effects of envenoming may lead to permanent tissue loss, disability, or amputation (Gutierrez, 2002).

In Argentina, ophidian accidents are a serious public health problem. *Bothrops* is responsible for 97% of accidents and, in particular, *B. diporus* accounts for 80% of these (Boletín Epidemiológico Periódico, 2009). As a consequence of this situation, and given the context of the use of *Cissampelos* sp. for the treatment of snakebites in traditional medicine, in this study, we investigated the antivenom activity of *C. pareira* against the *B. diporus* species from northeast Argentina.

In order to understand the limits of variability associated with a bioactive natural product, such as the plant extracts prepared in this work, we studied the chemical fingerprint of these plant extracts, and its association with the factors influencing such variability.

2. Material and methods

2.1. Venom

Bothrops diporus venom was obtained by personnel of Corrientes Serpentario, Argentina. Captured specimens were milked, resulting in a representative pool of snakes; the venom was then dried under vacuum. All experimental procedures were performed in accordance with the guidelines of the institutional animal care and use committee of the Universidad Nacional del Nordeste (UNNE), in accordance with the legislation on animal care. *In vivo* studies were developed following protocol 056, and were approved by the Ethics and Biosafety Committee of the Faculty of Veterinary Sciences of UNNE.

2.2. Plant Material

Leaves, flowers, tender stems (A), and roots (B)of *Cissampelos pareira* were collected from two different phytogeographic regions of Corrientes (Argentina); Paso de la

Patria (PP, San Cosme Department, 27°22'58.6'S, 58°34'56.4'W, 65 masl) and Lomas de Vallejos (LV, General Paz Department, 27°45'37.8'S, 57°55'56.6"W, 59masl). In addition, to perform a seasonal analysis and to evaluate the metabolic stability, material was collected at three different growth stages: autumn (I, May 2013), spring (II, November 2013), and summer (III, February 2014). The species was identified by Prof. Tressens (Instituto de Botánica del Nordeste (IBONE/UNNE), and specimens were deposited in the IBONE herbarium (CTES 17 Torres, CTES 19 AM and B. Ricciardi).

2.3. Extract preparation

The plant material, divided into aerial parts (leaves, flowers, and tender stems) and roots, was dried by aeration at approximately 66°C and humidity within 10-15%; the material was turned over during the drying process. Three extracts were prepared: (1) aqueous (maceration in distilled water, 24 h), (2) ethanolic (48 h), and (3) hexane (48 h). Next, all extracts were filtered and evaporated under reduced pressure. Extracts were kept in a refrigerator in closed containers until use.

2.4. Screening of antivenom activity

2.4.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis

The protein composition of snake venom was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Mini-Protean IV Electrophoresis Cell equipment. SDS-PAGE was performed on a slab according to the method of Laemmli (1970), with 4% (w/v) stacking gel (pH 6.8; Tris 6%, SDS 0.4%) and 12% (w/v) buffer gel (pH 8.8; Tris 18.2%, SDS 0.4%). The solutions for resolving gels and stacking gels for Tris-Glycine-SDS-Polyacrylamide Gel Electrophoresis were prepared as previously reported (Pardo and Natalucci, 2002; Pilosof and Bartholomai, 2000). Gels were stained for 3-4 h at room temperature with 0.25% (w/v) Coomasie brilliant blue R in 9.2% (v/v) acetic acid and 55.4% (v/v) methanol, and were then destained for 24 h with several changes of 7% acetic acid and 30% (v/v) methanol (Camargo et al., 2011).

Modification of the pattern of bands obtained from the standard venom compared to the extracts is considered an indicator of antivenom activity.

2.5. In vitro activities

2.5.1. Inhibition of proteolytic activity

The neutralization of the proteolytic activity of *B. diporus* venom was performed following an adaptation of the SDS-PAGE technique, as previously reported by Torres et al. (2014).

Venom + casein solution (1 g/100 mL in 100 mM Tris-HCl buffer; pH 8) was used as the complete hydrolysis standard. Solutions of venom + casein + plant extract were prepared in order to observe the inhibition of proteolysis by the plant extracts. The venom solution was pre-incubated for 60 min at 37°C (0.25 mg/mL in Tris-HCl buffer; pH 8) with the extract solution (venom:extract ratio of 1:30). They were then incubated with casein (10 mg/mL) for 60 min at 37°C. Solutions of plant extracts were incubated with casein to discard the presence of plant proteases. Urea(4M) was added to the sample buffer solution to improve resolution.

2.5.2. Inhibition of indirect hemolytic activity

The ability of plant extracts to neutralize the enzymes of *B. diporus* venom was evaluated by an indirect hemolysis assay on blood phosphatidylcholine agar plates (Gutiérrez et al., 1988; Otero et al., 1995) using a ratio venom:extract of 1:20.

Plant extracts (1500 g in 0.2 mL solvent: water, alcohol, or hexane) were incubated with 1 mL of venom solution (50 g/mL, minimum indirect hemolytic dose (MIHD) of venom produces a 10 mm halo diameter after 20 h of incubation). The ratio of venom:extract was 1:20. Next, 10 µL of these solutions were incorporated into each wells of agar. The plates were incubated for 20 h at 37°C, and the hemolysis halo was measured and compared with the MIHD. Reductions in the diameter indicated inhibition of phospholipase A_2 and its *in vitro* activity.

2.5.3. Inhibition of coagulant activity

A minimum coagulant dose (MCD) was defined as the amount of *Bothrops* venom which clots 0.2 mL of plasma in 60 sec. The method used was that described by Iovine and Selva (1985) with a slight modification, by which 0.2 mL of plasma and 0.2 mL of 0.025M $CaCl_2$ were added to 10 µL of saline solution, venom solution, or supernatant from the incubation of the venom + extract for 30 min at 37°C. Inhibition of coagulant activity was expressed by the normal coagulation time restitution percentage after addition of extract incubated with venom.

2.6. Bio-guided fractionation

2.6.1. Fractionation

According to the results obtained in section 2.5, the ethanolic extract of the aerial parts collected in summer from Paso de la Patria (A_2 III PP) resulted in the most active components, and was fractionated by introducing 500 mg on a chromatographic column (24 x 400 mm, silica gel flash 60 0.04-0.063mm, MN) connected to a CX-1000 air pump. A solvent eluotropic series of increasing polarity (toluene 100; toluene:ethyl acetate 50:50; ethyl acetate 100; ethyl acetate:methanol 60:40; ethyl acetate:methanol 40:60; methanol 100) was used. The fractions were collected in the test tubes, grouped by their composition profile on thin layer chromatography (toluene:ethyl acetate 9:1, visualization at UV 254/365 nm and by spraying with anisaldehyde in sulfuric acid), and evaporated under reduced pressure. Then, the grouped fractions were analyzed by SDS-PAGE to evaluate their alexiteric activity.

2.6.2. In vitro study of fractions

The fractions obtained were analyzed by SDS-PAGE under the same conditions as described in section 2.4. Next, *in vitro* tests of the inhibitory capacities on venom activities were carried out, as described in sections 2.5.1, 2.5.2, and 2.5.3.

2.7. Ultra high-performance liquid chromatography coupled to quadrupole/high-resolution mass spectrometry (Q-Orbitrap) (UHPLC-MS)

The active fraction (fraction F_6) was analyzed by UHPLC-MS. Chromatographic separation was carried out using a Thermo Ultimate R3000 UHPLC (Thermo Scientific, Sunnyvale, CA, USA), equipped with a Rheodyne 6-port automated switching valve used for on-line clean-up, adopting the method recently proposed by Barnaba et al. (2015). Identification of phenolic compounds in the active fraction was performed using a Q-Exactive TM hybrid quadrupole-orbitrap mass spectrometer (HQ-OMS, Thermo Scientific, Bremen, Germany) equipped with heated electrospray ionization (HESI-II). Mass spectra were acquired in negative ion mode through full MS-data dependent MS/MS analysis (full MS-dd MS/MS), recording full mass spectra at a mass resolving power of 140,000 full width at half-maximum (FWHM), and data-dependent mass spectra at 17,500 FWHM. The mass spectrometer operated as reported by Barnaba et al. (2016).

2.8. In vivo studies.

2.8.1. Inhibition of lethal activity

The inhibition of lethal activity was performed according to the Spearman-Karber method (World Health Organization, 1981) using groups of four CF1 mice (18-20 g) injected intraperitoneally, and recording the results after 48 h. Literature values of LD_{50} for *Bothrops diporus* were used; 38.18 μg per mouse (Maruñak et al., 2010). The reagents were sterilized with filters (0.2 μm) and the solutions were prepared under laminar flow hood. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as described by the European Community guidelines (EEC Directive of 1986; 86/609/EEC) and the US guidelines (NIH publication #85-23, revised in 1985).

Working groups were as follows: Group 1: challenge dose $4LD_{50}$ of venom in 0.12 M NaCl, 0.04 M phosphate buffer, pH 7.2 (PBS); Group 2: 5 mg A_2 III PP in PEG 400:ethanol:PBS (10:10:80) with 153 μg of venom incubated for 30 min at 37°C; Group 3: extract control group, 3 mg and 5 mg A_2 III PP in PEG 400:ethanol:PBS (10:10:80) and 3 mg F_6 in PEG 400:ethanol:PBS (10:10:80); Group 4: 5 mg F_6 in PEG 400:ethanol:PBS (10:10:80) with 153 μg of venom incubated for 30 min at 37°C; Group 5: 3 mg F_6 in PEG 400:ethanol:PBS (10:10:80) with 153 μg of venom incubated for 30 min at 37°C. Injection volume, 0.5 mL.

3. Results

The polyacrylamide gel electrophoresis for *C. pareira* extracts collected in summer in the area of Paso de la Patria is shown in Figure 1; the bands corresponding to the molecular weight pattern proteins of the venom and studied extracts can been seen.

The variation in the protein profile of venom compared to the plant extract is chosen as an indication of alexiteric activity; as can be seen in row A_2, the venom bands were completely erased. SDS-PAGE (Table 1) results indicated that the ethanolic extract of the aerial parts, collected in summer in Paso de la Patria, had the most active constituents.

The alexiteric activity was enhanced in summer, particularly in the ethanolic extract; this is likely related to the presence of more polar compounds, suggesting that the time of plant collection has important effects on the alexiteric activity of *C. pareira*.

When the results were evaluated from an edaphological point of view, the extracts from Paso de la Patria showed higher activity than those of Lomas de Vallejo. In addition, variation in activity was also found between the extracts from the different parts of the plants, that is, the leaves were more active in Paso de la Patria and the roots in Lomas de Vallejos were more active.

In order to investigate its proteolytic activity, an extract is considered active when it inhibits the proteolysis produced by venom, and the bands corresponding to casein remain intact. Figure 2 shows that the ethanolic extract of leaves produced the most active inhibition of the proteolysis of the venom; this activity was verified for all the ethanolic extracts from all the regions and seasons studied (Table 2). The inhibition of indirect hemolytic activity of venom was found to be concentration dependent (Table 3), and higher activity was found in Paso de la Patria extracts collected in spring, while those from Lomas de Vallejo did not present any activity.

Regarding the inhibition of blood coagulant activity, neither the extracts nor the solvents modified the coagulation time (Torres, 2012). The results of coagulant activity of extracts when incubated with venom are shown in Table 2, where the main activity was found in spring and summer.

Ethanolic extract obtained from aerial parts collected in summer from the Paso de la Patria region (11% w/w) was responsible for all inhibition activities, and thus was fractionated by flash chromatography in order to identify the active fraction. Six fractions were obtained and analyzed by SDS-PAGE in order to monitor the antivenom activity. Fractions 4, 5, and 6 (25, 38, and 22% w/w, respectively) completely erased the protein profile of venom on SDS-PAGE, and were thus the most active (Figure 3), whereas *in vitro* activities were 100% for all fractions (Table 4).

In order to evaluate the lethal activity and acute toxicity of the ethanolic extract and fraction F_6, five groups of four mice each were prepared: Group 1, venom control group (100% lethality); Group 2, 5 mg A_2 III PP + venom (0% lethality, 100% protection); Group 3, extract control group (0% lethality); Group 4, 5 mg F_6 + venom (0% lethality, 100% protection); and Group 5, 3 mg F_6 + venom (25% lethality, 75% protection).

At all concentrations tested, the plant extracts showed no acute toxicity and, in addition, we verified that the extract and the active fraction provided very good protection *in vivo*.

The structural elucidation of flavonoids was carried out by UPLC-MS. The following flavonoids were identified: quercetin 3-O-sophoroside [quercetin 3-O-β-D-glucosyl-(1→2)-β-D-glucoside] (1), naringenin 7-O-β-D-glucoside (2), eriodictyol-7-O-beta-D-glucoside (3), galangin-7-glucoside (4), and baicalein-7-O-glucoside (oroxin A) (5) (Figure 4). Phenolic compounds, especially complex polyphenols such as some tannins, can bind to proteins acting directly upon venom constituents, thus avoiding the reaction on receptors, or provoking competitive blocking of receptors (Soares et al., 2005). However, only quercetin has been previously identified in *C. pareira* (Amresh et al., 2007a). These findings support the traditional use of the ethanolic extracts of this plant in the treatment of snake venom attacks at rural areas.

4. Discussion

For most *Bothrops* species, bites lesions will vary from minor to life threatening. Whether life threatening or of no clinical significance, bites are invariably painful and are usually accompanied by local swelling and inflammation, bruising, blistering, necrosis, and abscess formation.

Medicinal plants and plant-based natural products have been reported to possess antivenom properties; laboratory assays correlate with ethnopharmacological studies (Soares et al., 2005). Natural inhibitors of snake venoms, particularly polyphenols, have been studied in the search for the most efficient wound treatments (Al Asmari et al., 2016; Caro et al., 2017; De Moura et al., 2015; Emmanuel et al., 2014; Gomes et al., 2016; Nirmal et al., 2008; Omale et al., 2012; Pithayanukul et al., 2004; Sánchez and Rodríguez-Acosta, 2008; Urs et al., 2014; Vale et al., 2011). Amresh et al. (2007a, 2007b, 2007c) suggest that internal use of *C. pareira* root preparations may counteract the inflammatory response following snakebites. The aerial parts from this species also show anti-inflammatory and analgesic effects in animal models of pain and inflammation, when administered orally; this may explain their use for treatment of some symptoms of snakebites. It is also interesting that a 70% ethanol extract of *C. pareira* leaves was found

to be effective against anxiety-like behaviors (Thakur and Rana, 2013), explaining why the leaves are often used for snakebites, in order to calm the victims.

The present study found that the aqueous extracts of both root and aerial parts of *C. pareira* do not inhibit the proteolytic activity of *B. diporus* venom, as previously reported by Badilla et al. (2008) using aqueous root extracts on *Bothrops asper* venom. Similarly, and in agreement with the work of Saravia-Otten et al. (2015), we found no anti Phospholipases A2 (PLA_2) activity in the ethanolic extract of *C. pareira* roots. However, the ethanolic extract of *C. pareira* aerial parts, collected in spring and summer, was very active, suggesting the aerial parts should be used for plant based drugs, instead of the entire plant.

Ethanolic extract activity was observed for plants collected from Paso de la Patria in all seasons. This is in agreement with the results of Barranco Pérez et al. (2010) who investigated leaves and root liquor. Our study found that the activity of *C. pareira* varied according to the part of the plant used, the vegetative state, and the place of harvest; as such, the aerial parts collected in summer in Paso de la Patria are a source of active metabolites against *B diporus.*

Although many medicinal plants traditionally used against snakebites have been investigated pharmacologically, there is a large number yet to be evaluated. *C. pareira* has been reported as one of the antiophidian ethnomedicinal plants used in India (Dey and De, 2013) but, to our knowledge, ours is the first study where phytochemical constituents were identified as responsible for the antisnake venom activity.

We isolated and characterized a flavonoid enriched fraction responsible for the antivenom activity: quercetin 3-O-sophoroside [quercetin 3-O-β - D-glucosyl-(1,2)-β -D-glucoside], naringenin 7-O-β -D-glucoside, eriodictyol-7-Obeta-D-glucoside, galangin-7-glucoside, and baicalein-7-O-glucoside (oroxin A). Although the mechanisms of action of these active compounds have not been elucidated, their activity could be attributed to their ability to bind to biological polymers. Flavonoids can form hydrogen bonds with proteins due to the proximity and coplanarity between the carbon 5 phenolic hydroxyl and the pyrronic carbonyl (Grassman et al., 1956; Jin et al., 1990). This explains the ability of flavonoids to act as inhibitors of inflammatory, hepatotoxic, hypertensive, and allergic processes and, more importantly, as enzymatic inhibitors. In addition, flavonoids have the

ability to act as metal binders (chelating agents), particularly with zinc, which has the potential to affect the functionality of metalloproteinases present in snake venom (Badilla Baltodano et al., 2006; Houghton, 1993; Mors et al., 2000).

This study supports the preparation of a protocol for extraction and fractionation of the plant species. Once its pharmacological and/or toxic properties (toxicokinetic and toxicodynamic mechanisms) have been validated, this fraction, or the isolated active compounds, could be applied as an alternative to the conventional treatment of snakebite accidents by *B. diporus*; this would provide a possible solution to people living in rural areas, away from health care sites.

5. Conclusion

The current study scientifically explains the ethnobotanical use of *C. pareira* against venom of *B. diporus* in our region, by identifying the compounds responsible for the alexiteric activity.

The findings suggest that collection of this species should be performed in spring-summer. After an adequate investigation of the drug-toxicological characteristics, the current study suggests that an ethanolic extract composed of aerial parts of the plant has potential to form a phytopharmacological product that could be used for first aid in cases of snakebite accidents.

Glossary

Ultra high-performance liquid chromatography coupled to quadrupole/high-resolution mass spectrometry (Q-Orbitrap). (UHPLC-MS); Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Author contributions

All authors participated in the design (B. Ricciardi Verrastro, G. Ricciardi, C. Barnaba, P. Teibler, S. Maruñak,), interpretation of the studies (A.M. Torres, R. Larcher, G. Nicolini) analysis of the data (A.M. Torres, E. Dellacassa) and review of the manuscript (, E. Dellacassa. All authors read and approved the final manuscript.

Acknowledgements

The authors are thankful to the Secretaría General de Ciencia y Técnica (UNNE) for financial support and to Prof. S. Tressens (IBONE/UNNE) for technical assistance.

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Figure 1: Polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) for Paso de la Patria collected in summer (PP III) extracts. Venom:extract ratio 1:10.

Figure 2: Polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) with casein for Paso de la Patria collected in summer (PP III) extracts. Venom:extract ratio 1:30.

Figure 3: Polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) of fractions of the active extract; ethanolic extract from Paso de la Patria collected in summer (A_2 III PP).

Figure 4: Flavonoids identified by quadrupole/high-resolution mass spectrometry (UHPLC-MS/Q-Orbitrap).

Table 1. Screening of antisnake venom activity of *C. pareira* extracts by sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE.

	AUTUMN		SPRING		SUMMER	
Paso de la Patria	**Aerial parts**	**Roots**	**Aerial parts**	**Roots**	**Aerial parts**	**Roots**
Aqueous extract	-	-	++	+	-	-
Ethanolic extract	++	+	+++	+	++++	++
Hexane extract	-	-	+	+	-	-
Lomas de Vallejos						
Aqueous extract	-	+++	-	-	-	++
Ethanolic extract	-	+++	+	+++	-	+++
Hexane extract	-	++	+	-	++	+++

Venom:extract ratio 1:10

(-), No activity; (+), little activity; (++), moderately active; (+++), very active.

Table 2. Screening of hemolytic, coagulant, and proteolytic activities of the aqueous, ethanolic, and hexanic extracts.

Activity			Neutralization of hemolytic activity 1:30 (V:E) % Restitution		Neutralization of coagulant activity 1:15 (V:E) %Restitution		Neutralization of proteolytic activity 1:30 (V:E)	
Area			**LV**	**PP**	**LV**	**PP**	**LV**	**PP**
AUTUMN								
Extract	A	1	21	0	6	7	(-)	(-)
	R		8	0	8	0	(-)	(-)
	A	2	12	0	**41**	**43**	(+)	**(+++)**
	R		12	5	6	6	**(+++)**	**(+++)**
	A	3	17	20	7	**70**	(-)	**(+++)**
	R		8	0	5	**80**	(-)	**(+++)**
SPRING								
Extract	A	1	4	0	**100**	13	(-)	(-)
	R		0	0	17	15	(-)	(-)
	A	2	19	**100**	**40**	**43**	**(+)**	(++)
	R		0	20	22	22	**(+++)**	(++)
	A	3	23	0	11	16	(+)	(+)
	R		8	0	11	29	(+)	(+)
SUMMER								
Extract	A	1	0	0	**49**	**40**	(-)	(++)
	R		10	0	**35**	**43**	(-)	(-)
	A	2	0	**35**	**66**	**94**	**(+++)**	**(+++)**
	R		0	0	22	**39**	**(+++)**	**(+++)**
	A	3	0	0	**43**	18	(-)	(-)
	R		0	0	27	28	(-)	(+)

V:E, venom:extract ratio; LV, Lomas de Vallejos; PP, Paso de la Patria; A, aerial parts; R, roots; 1, aqueous extracts; 2, ethanolic extracts; 3, hexanic extracts. (-), No activity; (+), little activity; (++), moderately active; (+++), very active.

Table 3. Inhibition of indirect hemolytic activity at different ratios (VenomExtract) of the active alcoholic extracts of aerial parts (A_2) of *C. pareira*.

SPRING (A_2 II)									
	Paso de la Patria					**Lomas de Vallejos**			
V:E ratio	X_1	X_2	X_p	%Restitution	V:E ratio	X_1	X_2	X_p	%Restitution
1:5	3	3	3	(+) 74%	-	-	-	-	-
1:10	2	2	2	(+) 83%	-	-	-	-	-
1:20	0	0	0	(+) 100%	-	-	-	-	-
1:30	0	0	0	(+) 100%	1:30	10	11	10.5	(-)
1:40	0	0	0	(+) 100%	-	-	-	-	-
1:50	0	0	0	(+) 100%	1:50	11	12	11.5	(-)
V	11	12	11,5		V	11	12	11.5	
SUMMER (A_2 III)									
	Paso de la Patria					**Lomas de Vallejos**			
V:E ratio	X_1	X_2	X_p	%Restitution	V:E ratio	X_1	X_2	X_p	%Restitution
1:5	8	7	7.5	(-) 25%	1:5	10	10	10	(-)
1:10	7	6	6.5	(+) 35%	1:10	10	10	10	(-)
1:20	6	6	6	(+) 40%	1:20	10	10	10	(-)
1:30	6	7	6.5	(+) 35%	1:30	10	10	10	(-)
1:40	4	4	4	(+) 60%	1:40	10	10	10	(-)
1:50	4	4	4	(+) 60%	1:50	10	10	10	(-)
V	10	10	10		V	10	10	10	

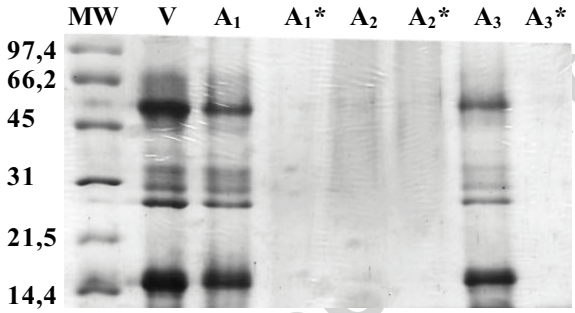
(-), No inhibitory activity; %Restitution > 30% was considered (+); X_1, X_2: diameter in mm of hemolysis halo of ethanolic extract pre-incubated with venom or venom alone (V); X_p: average of X_1 and X_2; %Hemolysis = (X_p extracts x 100)/X_p V. %Restitution = 100 - %Hemolysis.

Table 4. Inhibition of *in vitro* activities of venom by A_2 III PP fraction, as obtained by flash chromatography.

Inhibited activity	F_1	F_2	F_3	F_4	F_5	F_6
Proteolysis	(++)	(+++)	(++)	**(++++)**	**(++++)**	**(++++)**
Indirect hemolysis	(-)	(-)	(-)	**100%**	**100%**	**100%**
Coagulation	**34%**	(-)	(-)	**100%**	**100%**	**100%**

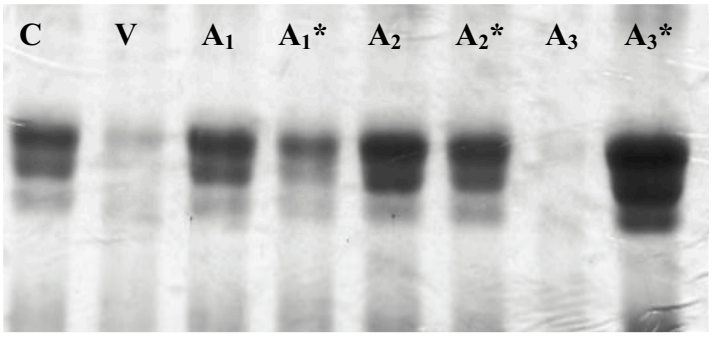
F_1, fraction 1; F_2, fraction 2; F_3, fraction 3; F_4, fraction 4; F_5, fraction 5; F_6, fraction 6. Fractions were obtained by column chromatography from the ethanolic extract of aerial parts collected in summer in the area of Paso de la Patria (A_2 III PP); (-), no inhibition activity; (++), moderate inhibition activity; (+++), strong inhibition activity; (++++), total inhibition activity.

Figure 1



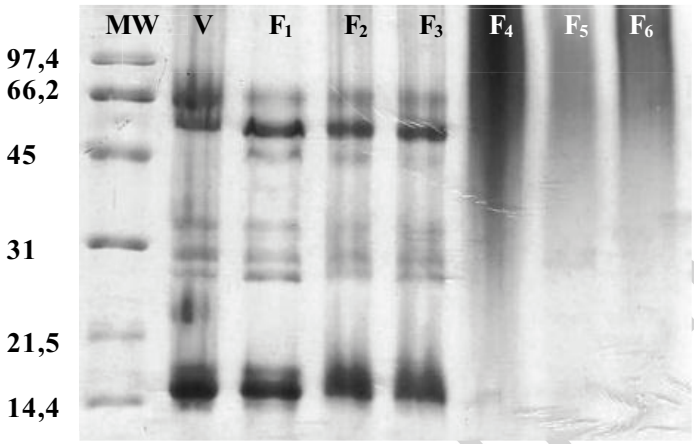
MW: molecular weight pattern; **V:** venom; **A_1:** aqueous extract of aerial parts + venom; **A_1*:** aqueous extract of aerial parts; **A_2:** ethanolic extract of aerial parts + venom; **A_2*:** ethanolic extract of aerial parts; **A_3:** hexanic extract of aerial parts + venom; **A_3*:**hexanic extract of aerial parts.

Figure 2



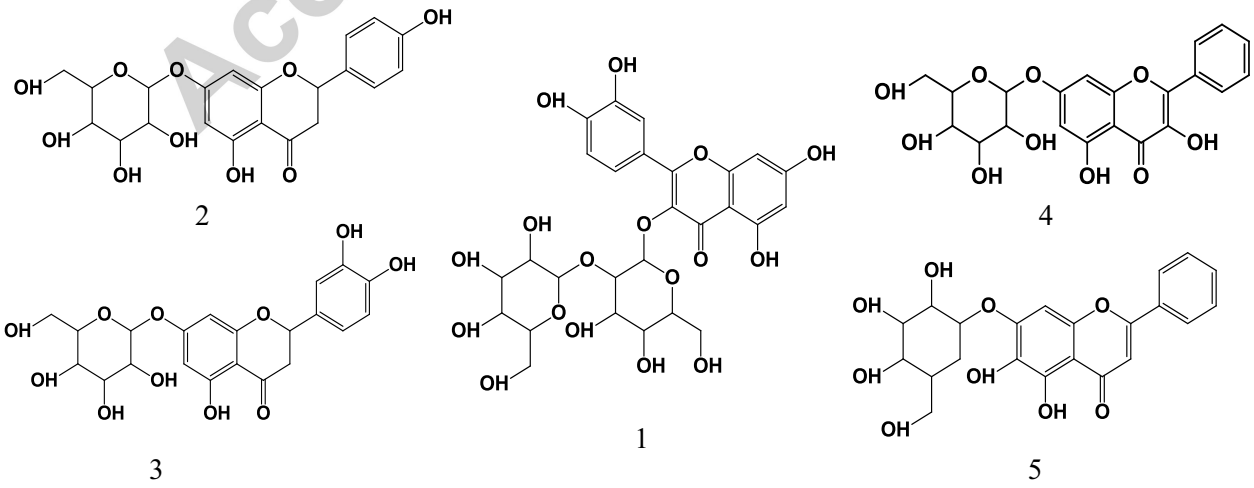
C: casein; **V:** venom + casein; **PA_1:** aqueous extract of aerial parts + venom+ casein; **A_1*:** aqueous extract of aerial parts + casein; **A_2:** ethanolic extract of aerial parts + venom + casein; **A_2*:** ethanolic extract of aerial parts + casein; **A_3:** hexane extract of aerial parts + venom+ casein; **A_3*:** hexane extract of aerial parts + casein.

Figure 3



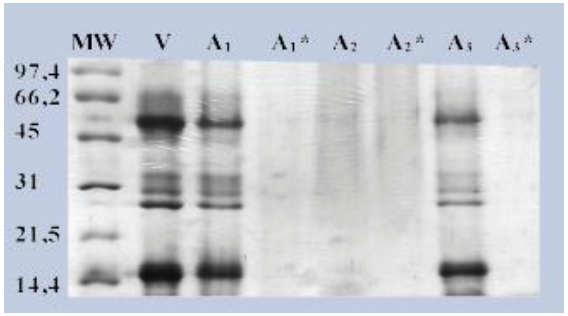
MW: molecular weight standards; **V:** venom; **F_1:** fraction 1 + venom;**F_2:** fraction 2 + venom; **F_3:** fraction 3 + venom; **F_4:** fraction 4 + venom; **F_5:** fraction 5 + venom; **F_6:** fraction 6 + venom

Figure 4



Bothrops diporus venom

Cissampelos pareira L. extracts



SDS-PAGE screening of antivenom activity

Anti snake venom activity

Plant material collection and extracts preparation

↓

In vitro inhibition of *Bothrops diporus* venom activities

↓

Selection of the most active extract

↓

Bioguided fractionation of the active extract

↓

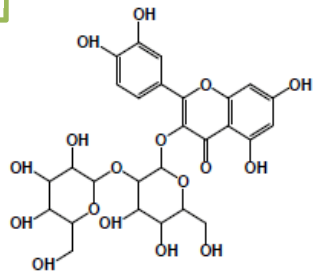
In vitro evaluation of the venom activities inhibition

↓

Selection of the most active fraction

↓

Identification of bioactive components
Inhibition of letal dosis *in vivo* test



bioactive compound