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In vitro Anti-snake Venom Activities of *Aloysia citriodora* Palau: New Possibilities for a Known Aromatic Plant

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Abstract: Traditional medicine in Corrientes Province (Argentina) uses herbs preparations in the form of infusions and cataplasms as an alternative medication for the treatment of bites from venomous animals. *Aloysia citriodora* is widely used in folk medicine to treat different disorders, but only few authors cite this Vervenaceae as antivenom against snake bites. In this work, we studied the effect of essential oils and extracts from aerial parts of *A. citriodora* against *Bothrops diporus* venom, yarará chica, so as to evaluate the traditional antisnake venom properties suggested for this species. In addition, a seasonal and geographical evaluation of the chemical composition of the essential oil was performed in order to assess its chemical stability. Results showed that *A. citriodora* possesses *in vitro* antisnake venom activity and that essential oil components could be considered as a part of its active constituents. These are most likely responsible for the plant's potential therapeutic benefits since they attenuate the proteolytic, coagulant and indirect hemolytic activities of *B. diporus* venom. Our results support the ethnopharmacological use of this species as antivenom, and backs the need to continue the research in order to identify the components responsible for the antivenom activity evaluated.

Key words: *Aloysia citriodora*, essential oil, *in vitro* antisnake venom activity, *Bothrops diporus*

Introduction

Since ancient time medicinal plants have been a resource to meet therapeutic needs. In recent years there has been a growing interest in the use of natural products, both for its medicinal properties and its flavor characteristics. Particularly, the Northeast of Argentina is known for its wide diversity of plants, with variations due to edaphological and ecological changes in the environment [1]. *Aloysia citriodora* Palau (Verbenaceae), popularly known in Argentina as cedrón [1] is native of South America and widely used in folk medicine to treat digestive disorders, as anti-inflammatory, analgesic, antipyretic, herbal tonic, stimulant [2] and sedative [3]. Furthermore, the essential oil has been reported as antimicrobial, antifungal [3,4] and antioxidant [5].

The essential oil composition of *A. citriodora* has been studied in many countries, and most of them have citral (neral and geranial) and limonene as main components [4-7]. *A. citriodora* leaves have also been reported as antidote to treat bites

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from venomous animals in the form of infusions and cataplasms [8,9]. These applications, which have been passed down for generations, are currently in wide revalorization as they lack the side effects of synthetic drugs used in traditional medicine. Therefore, the critical examination of the attributed properties would help to confirm its ethobotanical application.

In this work, we present the results of a screening by SDS-PAGE [10] and *in vitro* activities of essential oils and extracts from aerial parts of *A. citriodora* against *Bothrops diporus* venom, yarará chica, in order to evaluate the antisnake venom properties suggested for this species. In addition, a seasonal and geographical evaluation of the chemical composition of the essential oil was performed in order to assess its chemical stability.

Materials and methods

Plant material

After an adequate prospection, around 1500 g of leaves (flowering period, spring-summer) and fresh stems of *A. citriodora* were collected from three different places of Corrientes province (Argentina): Paso de la Patria (PP), Laguna Brava (LB) and San Luis del Palmar (SLP) in the same growing stage (summer, III) to study the geographical factor. Besides, samples from Laguna Brava were collected in three different growing stages: autumn (I), spring (II) and summer (III) to make a seasonal prospection and evaluate its stability. The plants were identified by Prof. Tressens (IBONE/UNNE) and voucher specimens were deposited at the IBONE Herbarium (Id: CTES **9**, A.M. Torres and G. Ricciardi).

Essential oil extraction

The essential oils were obtained by hydrodistillation from dried aerial parts (1.5 kg) during 2 hours, using a macro distillation apparatus equipped with a 2-L flask, and those named hydrolates were obtained by extraction with ethyl ether. The oils were transferred to glass vials after being dried with anhydrous sodium sulfate and kept at -4°C until used.

Preparation of plant extracts

Aerial parts (leaves and stems) from *A. citriodora* were air dried at controlled temperature, powdered and sieved to prepare three extracts according to that reported by Torres *et al*. [11]. aqueous (maceration in distilled water, 24 hs), alcoholic (ethanol 96°, 48 hs) and hexanic (hexane, 48 hs); all were vacuum dried. The three extracts were conveniently stored in desiccators under reduced pressure until use.

Gas chromatography

The composition of the oil was determined by GC using a Shimadzu (Tokyo, Japan) model 14 B gas chromatograph equipped with a FID and Shimadzu EZ-Chrom data processor software following the experimental conditions reported by Torres *et. al*.[11].

Gas cromatography-Mass spectroscopy

GC-MS analyses were carried out using a Shimadzu QP 5050 apparatus which was equipped with MS reference libraries [12,13]. Analyses were carried following the experimental conditions previously reported by Minteguiaga *et al*.[14].

The components of the oil were identified by their linear retention indices (LRIs) using a series of n-alkanes (C_9-C_{26}) and comparing the values obtained with those of pure standards or reported in the literature [13,15]. Fragmentation patterns in the MS were compared with those stored on the GC-MS databases [12,13]. The percentages of each component were reported as raw percentages without standardisation. Repeatability of the measuring system showed variation coefficients under 5 % for all the components reported in Table 1.

Screening of the antisnake venom activity of oils and extracts

SDS-PAGE electrophoresis was carried out using a Mini-Protean IV Electrophoresis Cell device under denaturing conditions using 12 % (w/v) stacking gel solution and 4 % (w/v) separating gel solution. The reagents were prepared as reported by Pilosof and Bartholomai [16] and Ricciardi Verrastro *et al.*[17]. Essential oils from other species (*Cordia curassavica* (Jacq.) Roem. & Schult., *Lippia turbinata* Gris., *Aloysia gratissima* (Gill. et Hook) Tronc. and *Ambrosia tenuifolia* Spreng.) were added as negative controls to assess non-specific binding [18].

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 washed out for 24 h with periodically renewed a 7 % acetic acid and 30 % (v/v) methanol solution. Decreased intensity or disappearance of bands as well as the appearance of bands of different molecular weight in the lanes loaded with venom and extracts/oils were used as reliable indicators of activity.

In vitro activities

Inhibition of proteolytic activity by oils/extracts

The inhibition of proteolytic activity was performed following an adaptation of the SDS-PAGE technique [10].

A. citridora plant extracts /oils solutions (1 mg in 50 mL of buffer Tris-HCl pH: 8) were incubated with casein to dismiss the presence of plant proteases. In particular, we evaluated the extracts (aqueous, alcoholic and hexane) and the essential oils and hydrolates from Laguna Brava at autumn, spring and summer; essential oils and hydrolates from San Luis del Palmar (autumn and spring samples) and from Paso de la Patria (summer). Essential oils from other species (*Cordia curassavica, Lippia turbinate, Aloysia gratissima* and *Ambrosia tenuifolia*) [18] were added as controls to assess non-specific binding. Sample buffer solution (double concentrated) with the addition of 4 g of urea was used to improve run results. Gels were stained with Coomassie brilliant blue.

Inhibition of the indirect hemolytic activity by oils/extracts

Essential oils and plant extracts capability to neutralize *B. diporus* venom enzymes was evaluated through an indirect hemolytic assay on agarose-blood-phosphatidylcoline gel plates [19,20]. Essential oils and extracts were reconstituted in appropriate solvents when used. Venom and extract/oil ratio was 1:20 as previously reported by Ricciardi Verrastro *et al.*[17]. A halo reduction shows an *in vitro* inhibition of the activity of the fosfolipase A_2 of the venom.

Inhibition of the coagulant activity

The neutralization of the coagulant activity was studied by timing citrated plasma recalcification [21] with a slight modification [17].

The amount of *B. diporus* venom that clots 0.2 mL plasma in 60 seconds was defined as the minimum coagulant dose (MCD). Ratio tested was 1:20 (50 µg venom: 1000 µg extract/oil).

Statistical analysis

The geographical and seasonal behavior of the *A. citriodora* volatile metabolism was evaluated by statistical analysis using a dendogram as graphic expression. The analysis was performed using Statistica software (StatSoft, Tulsa, OK, 1984-2005).

Results and discussion

Table 1 shows the percentages of the identified compounds in the volatile oil. The results obtained through the analysis of different groups of identified compounds showed a clear predominance of oxygenated monoterpenes either considering geographical or seasonal variations. Particularly, the geographical analysis showed that the essential oil from Paso de la Patria exhibits a slight increase

Table 1. Major components of the essential oils of *A.citriodora*

No.	LRI[a]	Identified compounds[b]	(%)[c] LB I	LB II	LB III	PP III	SLP III
1	1121	Sabinene	1.7	2.3	1.1	1.6	0.6
2	1160	β-Myrcene	0.1	0.1	0.1	0.1	0.1
3	1202	*d*-Limonene	10.1	7.7	6.0	8.4	6.3
4	1239	*E*-β-Ocimene	0.3	0.3	0.2	0.1	0.3
5	1272	*p*-Cymene	0.1	0.1	0.1	0.1	0.1
6	1332	6-Methyl-5-hepten-2-one	0.2	0.8	0.4	0.3	0.1
7	1420	α-Thujone	0.2	0.3	0.3	0.6	0.5

table 1. (continued).

No.	LRI[a]	Identified compounds[b]	(%)[c] LB I	LB II	LB III	PP III	SLP III
8	1456	Limonene oxide *cis*	0.1	0.1	0.0	0.2	0.1
9	1461	Limonene oxide *trans*	0.2	0.1	0.0	0.2	0.1
10	1479	α-Copaene	1.0	0.2	0.3	0.5	0.3
11	1490	2,2-Dimetil-3,4-octadienal	0.7	0.6	1.0	0.7	0.7
12	1511	β-Bourbonene	0.8	0.3	1.6	0.4	0.9
13	1545	(*Z*)-*iso*citral	0.2	0.3	0.8	0.0	0.6
14	1553	Linalool	0.3	0.5	0.6	1.0	0.5
15	1571	(*E*)-*Iso*citral	1.0	0.8	0.6	2.7	0.8
16	1583	*trans*-β-Caryophyllene	3.3	4.6	8.8	0.6	6.4
17	1638	Aromadendrene	0.7	0.4	1.1	0.3	0.9
18	1659	α-Cedrene	0.2	0.4	0.8	0.0	0.5
19	1688	Neral	18.5	22.1	13.1	19.6	14.6
20	1704	γ-Curcumene	1.0	1.8	3.5	0.0	2.7
21	1718	γ-Muurolene	0.2	0.3	1.0	0.0	0.8
22	1731	β-Curcumene	0.0	6.1	3.0	0.0	5.2
23	1746	Geranial	17.7	30.0	22.1	30.8	21.6
24	1755	Bicyclogermacrene	14.0	0.1	0.1	0.0	0.8
25	1755	Geranyl acetate	0.1	0.1	0.1	0.0	0.0
26	1755	δ-Cadinene	0.1	0.1	0.9	0.0	0.1
27	1765	α-Curcumene	4.1	3.1	6.8	4.9	5.9
28	1778	α-Muurolene	0.1	0.0	0.2	0.1	0.2
29	1804	Geranyl propionate	0.2	0.1	0.4	0.2	0.2
30	1813	Muurol-5-en-β-ol (*cis*)	0.3	0.2	0.4	0.2	0.5
31	1886	*epi*-Cubebol	0.5	0.4	0.6	0.4	0.5
32	1952	β-Bisabolol	0.7	0.5	0.9	1.3	0.4
33	1973	Caryophyllene oxide	5.8	2.4	8.8	5.4	8.6
34	1989	*trans*-Nerolidol	1.4	1.4	1.7	2.1	1.1
35	2037	Germacrene-D-4-ol	0.3	0.6	0.6	0.7	0.4
36	2046	Spathulenol	5.2	4.2	4.7	5.3	6.0
37	2128	(*Z*)-Lanceol	0.4	0.3	0.4	0.4	1.1
38	2143	δ-Cadinol	0.6	0.4	1.0	0.5	1.2
39	2168	*ar*-Turmerol	0.6	0.2	0.5	0.4	1.2
		Grouped compounds					
		Monoterpene hydrocarbons	12.3	10.5	7.5	10.3	7.4
		Oxygenated monoterpenes	39.2	55.3	39.4	56.3	39.8
		Sesquiterpene hydrocarbons	25.5	14.4	28.1	6.8	24.7
		Oxygenated sesquiterpenes	15.8	10.6	18.6	16.7	11.5
		Total	92.8	90.8	93.6	90.1	83.4

[a] The components are reported according their elution order on BP 20

[b] relative proportions of the essential oil constituents were expressed as percentages obtained by peak-area normalisation, all relative response factors being taken as one. For each compound reported, the values were not significantly different between samples ($p < 0.05$)

[c] peak identifications are based on comparison of LRI values on Carbowax 20M with those from pure standards or reported in the literature, and on comparison of MS with file spectra

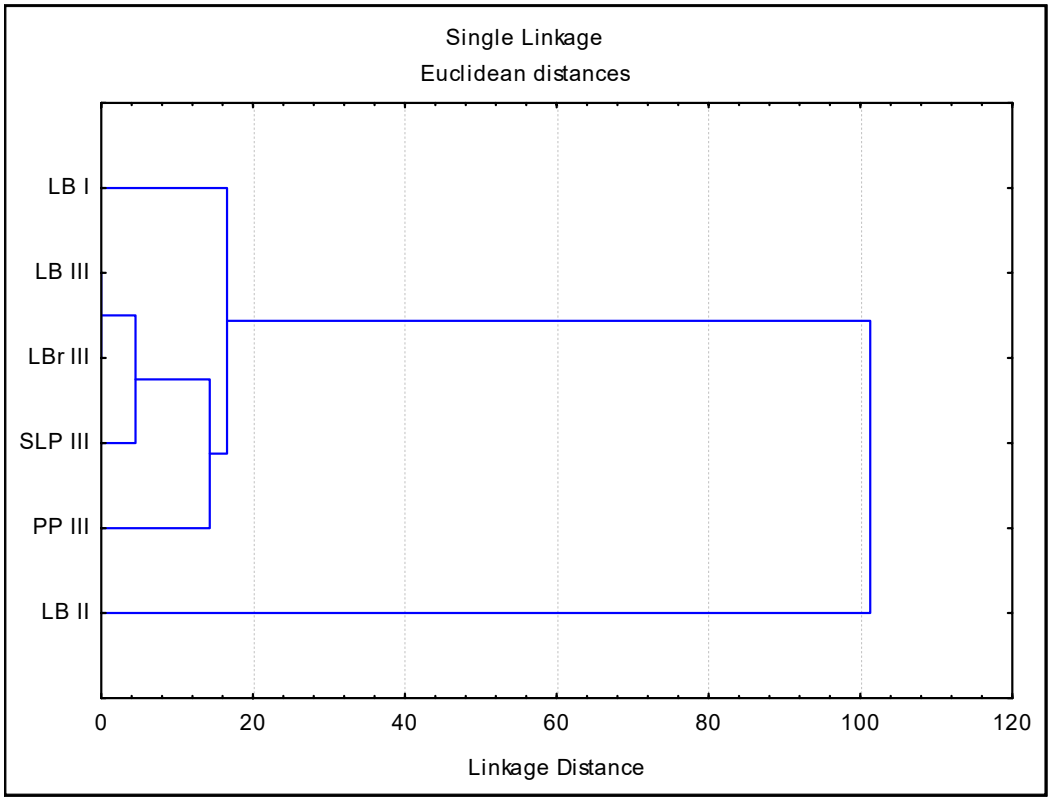
in the monoterpenic fraction, being as well the one with the highest oxygenated monoterpenes fraction. The proportion of sesquiterpene hydrocarbons was found to be quite similar in the oils from Laguna Brava and San Luis del Palmar, contrasting with the low percentage observed in the sample of Paso de la Patria. Regarding to the oxygenated sesquiterpenes fraction, similar proportions were observed in the three samples studied, not being greater than 21 %.

The seasonal study was performed using samples from Laguna Brava in three growing stages (**LBI**, **LBII**, **LBIII**). The growing stage slightly modifies the relative proportion of the grouped components, even though there is an increase in oxygenated monoterpenes. Moreover, we observed a small increase in monoterpenes in the fall sample.

The statistical analysis of the geographical-seasonal behavior for this species showed that the major relative weight on the volatile metabolic expression of *A. citriodora* is given by the time of harvest rather than the location where the samples were collected. Thus we concluded that it is the seasonal factor the one that mainly affects the chemical composition of the essential oil and has to be considered at the time of harvesting. Figure 1 shows a dendrogram where this result is statistically demonstrated using a graphical representation.

Regarding the chemical composition, the results obtained give an account of the chemical stability of this species. So far, qualitative differences in the oil content were not found in the analyzed samples compared with data reported by other authors [4,6,7] that will allow identifying new chemotypes.

As the chemical stability for this species has



LBI: Laguna Brava oil of autumm; LBII: Laguna Brava oil of spring, LBIII Laguna Brava oil of summer, PP III Paso Patria oil of summer, LBrIII: Laguna Brava oil of summer; SLP III: san Luis del Palmar oil of summer

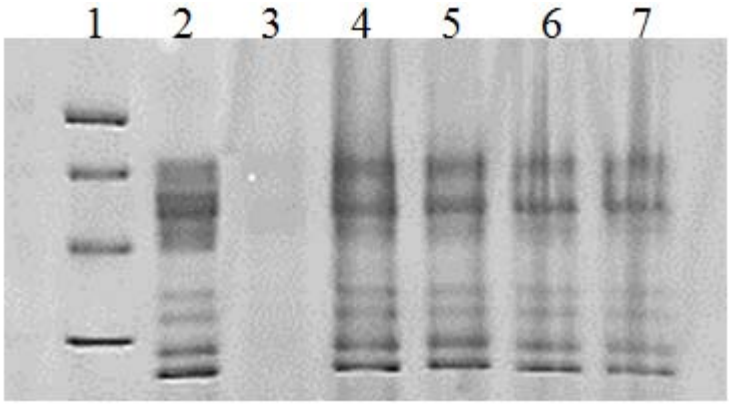
Figure 1. Seasonal and geographical distribution of *A. gratissima* in Corrientes Province. Chemometric analysis

been proved in this study, SDS-PAGE analysis was performed on samples from Paso de la Patria collected in summer (I) as a screening method for antisnake venom activity (Figure 2). Tables 2 to 4 show the results of the *in vitro* antisnake venom activity of the different samples studied. The results obtained show that they possess similar activity to the other oil samples tested as we expected from previous studies [18,22-24].

Figures 3 and 4 show the SDS-PAGE results for the inhibition of proteolytic activity by the extracts and essential oil of *A. citriodora* respectively.

From these data it can be inferred that the essential oils are much more active than plant extracts, as they neutralize the proteolytic, coagulant and indirect hemolytic activity of *B. diporus* venom. To our knowledge, this activity has not been reported by other authors for *A. citriodora* essential oil.

In conclusion, this study demonstrates that *A. citriodora* possesses *in vitro* antisnake venom



1: pattern of PM; 2: *B.diporus* venom (V); 3*: A. citriodora* ed SLII + V; 4: ed *Cordia curassavica* + V; 5: ed *Lippia turbinata* + V; 6: ed *Aloysia gratissima* + V; 7: ed *Ambrosia tenuifolia* + V

Figure 2. SDS-PAGE of essential oils

Table 2. Antisnake venom activity of *A. citriodora* samples from Laguna Brava

Laguna Brava (LB)	SDS-PAGE (V/E* 1:7)	ICA** V/E*	ICA** % Rec	IPA*** (V/E* 1:120)	IHA*V V/E* (1:20)
		Autumm			
Aqueous extract	No	1:2.5	9 %	No	No
Alcoholic extract	No	1:2.5	18 %	Yes	Yes
Hexanic extract	No	1:2.5	18 %	No	Yes
Essential Oil	Yes	1:10	48 %	Yes	Yes
Hydrolate	Yes	1:10	58 %	Yes	Yes
		Spring			
Essential Oil	Yes	1:10	50 %	Yes	Yes
Hydrolate	Yes	1:10	58 %	Yes	Yes
		Summer			
Essential Oil	Yes	1:10	38 %	Yes	Yes

V/E*: ratio venom (dry weight): oil/extract
ICA **: Inhibition of coagulant activity
IPA*** Inhibition of proteolytic activity
IHA*v Inhibition of hemolytic activity, all "Yes" inhibited about 20 %

Table 3. Antisnake venom activity of *A. citriodora* samples from San Luis del Palmar

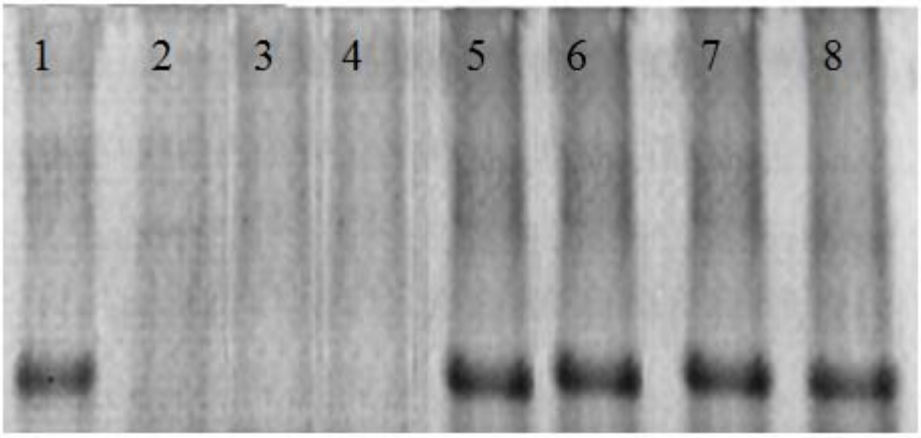
San Luis del Palmar (SLP)	SDS-PAGE (V/E* 1:7)	ICA** V/E*	ICA** % Recovery	IPA*** V/E*	IPA*** PA*
Autumn					
Essential Oil	Yes	1:10	51 %	1:120	Yes
Hydrolate	Yes	1:10	92 %	1:120	Yes
Spring					
Essential Oil	Yes	1:10	49 %	1:120	Yes
Hydrolate	Yes	1:10	56 %	1:120	Yes

V/E*: ratio venom (dry weight): oil
ICA**: Inhibition of coagulant activity
IPA*** Inhibition of proteolytic activity

Table 4. Antisnake venom activity of *A. citriodora* samples from Paso de la Patria

Paso de la Patria (PP)	SDS-PAGE (V/E* 1:7)
Summer	
Essential Oil	Yes
Hydrolate	Yes

V/E*: ratio venom (dry weight)

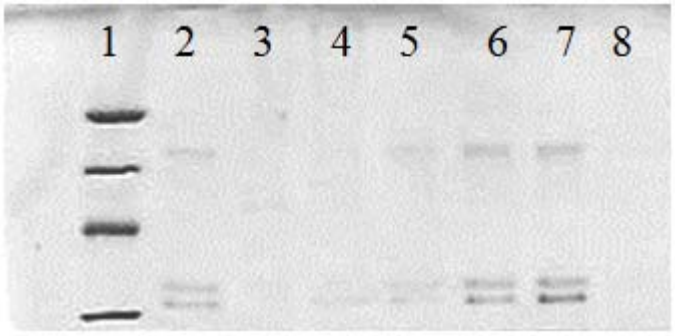


1: Casein (C); 2: Venom (V) + C; 3: Aqueous extract SLP I+C+V; 4: Aqueous extract SLP I+C; 5: Alcoholic extract SLP I+C+V; 6: Alcoholic extract SLP I+C; 7: Hexanic extract SLP I+C+V; 8: Hexanic extract SLP I+C.

Figure 3. Inhibition of proteolytic activity of *B. diporus* venom for extracts (1:120)

activity against *B. diporus* venom and that essential oil components could be considered as a part of its active constituents. They are the most likely responsible for it activities since they attenuate the proteolytic, coagulant and indirect hemolytic activities of *B. diporus* venom, supporting the ethnopharmacological use of this Verbenaceae as antivenom.

Consequently, these results might be considered sufficient for further research in the quest to iden-



1 pattern of PM , 2: Casein (C), 3: venom + casein (V+C); 4: ed *A. citriodora* +C+V; 5: ed *Cordia curassavica* +C+V; 6: ed *Lippia turbinata*+C+V; 7: *Aloysia gratissima*+C+V; 8: *Ambrosia tenuifolia* +C+V

Figure 4. Inhibition of proteolytic activity of *B. diporus* venom for oils (ratio 1:30)

tify the responsible components for the antisnake venom activity evaluated and the most adequate doses actives.

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