Antisnake venom activities of Aloysia citriodora Palau: new applications for a known aromatic plant

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Antisnake venom activities of *Aloysia citriodora* palau: new applications for a known aromatic plant

Abstract

Traditional medicine in Corrientes Province (Argentina) uses preparations from herbs as one form of medication for the treatment of bites from venomous animals in the form of infusions and cataplasms. We evaluated the effect of essential oils and extracts from aerial parts of *A. citriodora* against *Bothrops diporus* venom, yarará chica, in order to prove the traditional antisnake venom properties suggested for this species. Besides, a seasonal and geographical evaluation of the chemical composition of the essential oil was performed in order to assess about its chemical stability. We also demonstrated that *A. citriodora* possesses, in vitro, antisnake venom 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 the plant's potential therapeutic benefits since they attenuate the proteolytic, coagulant and indirect hemolytic activities of *B. diporus* venom.

Keywords: *Aloysia citriodora*, essential oil, antisnake venom activity, *Bothrops diporus*

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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] (Zuloaga and Morrone, 1999). The species under study, *Aloysia citriodora* Palau (Verbenaceae), popularly known in Argentina as cedrón[1] (Zuloaga and Morrone, 1999) is native of South America and widely used in folk medicine to treat digestive disorders as anti-inflammatory, analgesic, antipyretic, herbal tonic and stimulant[2] (Oliva et al., 2010) and sedative[3] (Oksay et al., 2005). Besides, the essential oil has been reported as antimicrobial, antifungal[3,4] (Oksay et al., 2005; Sartoratto et al., 2004) and antioxidant[5] (Stashenko et al., 2003).

The essential 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](Sartoratto et al., 2004; Stashenko et al., 2003; Özek et al., 1996; Ricciardi et al., 2011).

Aloysia citriodora leaves have also been reported as antidote to treat bites from venomous animals in the form of infusions and cataplasms[8,9](Manfred, 1977; Duke et al., 2009). These applications, which are being transmitted from generation to generation, 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, or reject, its use in phytomedicine and, at the same time, will provide people who live far away from health centers, an accessible and safe alternative drug cheaper than the synthesized ones.

In this work, we present the results of an in vitro screening, by SDS-PAGE[10] (Camargo et al., 2011), of the essential oils and extracts from aerial parts of *A. citriodora* against *Bothrops diporus* venom, yarará chica, in order to prove the antisnake venom properties suggested for this species. Besides, a seasonal and geographical evaluation of the chemical composition of the essential oil was performed in order to assess about its chemical stability.

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Materials and methods

Plant material

After an adequate prospection of the plant material, leaves, flowers 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. And samples from Laguna Brava in three different growing stages: fall (I), spring (II) and summer (III) to make a seasonal prospection and evaluate its stability (Herbario CTES, AM Torres and G Ricciardi 9).

Essential oil extraction

The essential oils were obtained by steam distillation of dried aerial parts during 2 hours, using a stainless steel extractor with a capacity of 5L, and those named water oils were obtained by extraction with ethyl ether. Oils were dried with anhydrous sodium sulfate and transferred to glass flasks that were kept at −4ºC until used.

Preparation of plant extracts

Aerial parts from *A. citriodora* were air dried at controlled temperature, powdered and sieved to prepare three extracts: aqueous (maceration in distilled water, 24 hs), alcoholic (ethanol 96º, 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 FID and Shimadzu EZ-Chrom data processor software. Analyses were

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carried out using a Carbowax 20M (Ohio Valley, USA) bonded fused-silica capillary column (25 m × 0.32 mm i.d.), coated with polyethylene glycol (0.25 μm phase thickness) was employed; the oven temperature programme was 40°C for 8 min, rising to 180°C at 3°C/min, then to 230°C at 20°C/min; the injector temperature was 250°C; the detector temperature was 250°C; the carrier gas was hydrogen at 30 kPa; the injection mode was split with a split ratio of 1:30; and the sample volume injected was 0.2 μL.

Gas Cromatography-Mass Spectroscopy

GC-MS analyses were carried out using a Shimadzu QP 5050 apparatus which was equipped with MS reference libraries[11,12] (Adams, 2007; McLafferty and Stauffer, 1991). Analyses were carried out using a BP 20 (SGE, Ringwood, Australia) bonded fused-silica capillary column (25 m × 0.25 mm i.d.), coated with polyethylene glycol (0.25 μm phase thickness) was employed; the oven temperature programme was 40°C for 8 min, rising to 180°C at 3°C/min, then to 230°C at 20°C/min; the injector temperature was 250°C; the carrier gas was hydrogen at 92.6 kPa (55.9 cm/s); the injection mode was split with a split ratio of 1:40; the sample volume injected was 0.2 μL; the interface temperature 250°C; and the acquisition mass range was 40–400 *m/z*.

Identification and quantification of oil components

The components of the oil were identified by comparison of their linear retention indices (LRIs) determined in relation to a homologous series of n-alkanes (C_9–C_{26}) with those of pure standards or as reported in the literature[11,13](Adams, 2007; Davies, 1991). Comparison of fragmentation patterns in the MS with those stored on the GC-MS databases[11,12] (Adams, 2007; McLafferty and Stauffer, 1991) was also performed. 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.

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Screening of the antisnake venom activity of oils and extracts

SDS-PAGE electrophoresis was carried out using a Mini-Protean II Electrophoresis Cell device under denaturing conditions using 10% (w/v) stacking gel solution and 4% (w/v) separating gel solution.

The reagents were prepared as reported by Pilosof and Bartholomai (Pilosof and Bartholomai, (2000)[14]. Electrode TRIS-glycine buffer pH: 8,3; stacking gel buffer pH: 6,8; buffer gel pH: 8,8. Low molecular weight standards of 97,4KDa, 66,2 KDa, 43 KDa, 30 KDa and 14,4 KDa prepared according to the manufacturer´s (Bio-rad) were used; 15 µg of protein was loaded into each gel so as to provide a better visualization of the bands and to maintain the same ratio in the venom/oil-extract samples.

Molecular weight standards, yarará venom (to analyze its protein composition), samples of essential oils and plant extracts (as reference standard for the presence of plant proteins that may interfere with the understanding of results) were included. The supernatant of oils and extracts previously incubated with the yarará venom (30 min at 37 ºC) were analyzed in order to observe the interaction between plant compounds and the protein composition of snake venom.

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 several changes of 7% acetic acid and 30% (v/v) methanol. 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 of *B. diporus* (yarará chica) venom was performed following an adaptation of the SDS-PAGE technique[15,16] (Pardo and Natalucci, 2002; Gay et al., 2004).

Acrylamide solutions, gel buffer, stacking buffer and electrode buffer were as previously described for SDS-PAGE with 10% separating gel solution and 4% stacking gel solution. Casein stock solution

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containing 1g of casein in 100 mL of buffer Tris-HCl 100 mM, pH 8 was prepared. Molecular weight standards (Bio-Rad) and casein standard (0,1 g/10 mL in buffer Tris-HCl 100 mM pH: 8) were employed. Venom + casein solution was used as a standard of complete hydrolysis. Solutions of venom + casein + oil/extract were prepared to observe the possible inhibition of the proteolysis of the casein by the plant extracts/oils. In order to prove this, venom solution was incubated 60´ at 37 °C (0,25 mg/mL in buffer Tris-HCl pH: 8) with the plant extracts. Then, this supernatant was incubated 60' at 37 °C with casein. Solutions of plant extracts/oils (1 mg in 50 µL of buffer Tris-HCl pH: 8) were incubated with casein so as to dismiss the presence of plant proteases. Sample buffer solution (double concentrated) was used with the addition of 4g of urea to improve run results. Gels were stained with Coomassie brilliant blue.

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Inhibition of the indirect hemolytic activity by oils/extracts

Neutralization of *B. diporus* venom enzymes by essential oils and plant extracts was analyzed using an indirect hemolytic assay on agarose-blood–phosphatidylcoline gel plates[17,18] (Gutiérrez et al., 1988; Otero et al., 1995). Essential oils and extracts were reconstituted in appropriate solvents when used. Venom + extract/oil ratio was 1:20.

The different samples of essential oils and extracts were incubated with 1ml of venom dilution (w/w, venom: extract/oil). Then 10 µl of the supernatant was loaded into each well of the Petri dish. Later, the plates were incubated 20 hs at 37 ºC and then the hemolytic halo was measured, comparing it with the MIDH (minimum indirect hemolytic dose that induces a 10 mm diameter halo after 20 hs of incubation). 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[19] (Iovine and Selva, 1985) with a slight modification. 10 µl of saline solution, venom solution or the supernatant of

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venom + extract/oil incubated 30 min at 37 ºC was added to the 0,2 ml of plasma and 0,2 ml $CaCl_2$ 0,025M. With these, we obtained the normal coagulation time (CT), the minimum coagulant dose (MCD) and the capacity of the oil/extract that inhibits the coagulant activity of the venom. A minimum coagulant dose (MCD) was defined as the amount of *B. diporus* venom that clots 0,2 mL plasma in 60 seconds. Ratio tested was 1:20 (50 µg venom: 1000 µg extract/oil). When necessary, higher ratios venom: extract/oil was used.

Results and discussion

Table 1 shows the identified compounds in the volatile oil and their percentages. 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 (Figure 1 and 2). Particularly, the geographical analysis (Figure 1) showed that the essential oil from Paso de la Patria exhibits a slight increase 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**). Figure 2 shows that 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 same variation in sesquiterpenes and oxygenated sesquiterpenes was observed, although the latter ones in smaller percentages.

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 (Figures 1 and 2). Particularly, the geographical analysis (Figure 1) showed that the essential oil from

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Paso de la Patria exhibits a slight increase 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%.

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As the chemical stability for this species has 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. Tables 2, 3 and 4 show the results of the *in vitro* antisnake venom activity of the different samples studied. The results show that they possess similar activity to the other oil samples tested as we expected.

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. No significant differences as regards the season or harvesting location were observed. 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 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 the plant's potential therapeutic benefits since they attenuate the proteolytic, coagulant and indirect hemolytic activities of *B. diporus* venom, supporting the ethnopharmacological use of this Verbenaceae as antivenom.

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Consequently, these results might be considered sufficient for further research in the quest to identify the responsible components for the antisnake venom activity evaluated.

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

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

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

Table 4: Antisnake venom activity of samples from Paso de la Patria

Figure 1: Geographical variation of the identified compounds in summer samples of *A. citriodora* grouped by families

Figure 2: Seasonal variation of the identified compounds in samples of *A. citriodora* from Laguna Brava grouped by families

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

LRI[a]	Identified Compounds[b]	(%)[c]				
		LB I	LB II	LB III	PP III	SLP III
1121	sabinene	1.7	2.3	1.1	1.6	0.6
1160	β-myrcene	0.1	0.1	0.1	0.1	0.1
1202	limonene	10.1	7.7	6.0	8.4	6.3
1239	*trans*-β-ocimene	0.3	0.3	0.2	0.1	0.3
1272	*p*-cimene	0.1	0.1	0.1	0.1	0.1
1332	6-metil-5-hepten-2-one	0.2	0.8	0.4	0.3	0.1
1420	α-thujone	0.2	0.3	0.3	0.6	0.5
1456	limonene oxide *cis*	0.1	0.1	0.0	0.2	0.1
1461	limonene oxide *trans*	0.2	0.1	0.0	0.2	0.1
1479	α-copaene	1.0	0.2	0.3	0.5	0.3
1490	2,2-dimethyl-3,4-octadienal	0.7	0.6	1.0	0.7	0.7
1511	β- bourbonene	0.8	0.3	1.6	0.4	0.9
1545	(*Z*)-isocitral	0.2	0.3	0.8	0.0	0.6
1553	linalool	0.3	0.5	0.6	1.0	0.5
1571	(*E*)-*iso*citral	1.0	0.8	0.6	2.7	0.8
1583	*trans*-β-caryophyllene	3.3	4.6	8.8	0.6	6.4
1638	aromadendrene	0.7	0.4	1.1	0.3	0.9
1659	α-cedrene	0.2	0.4	0.8	0.0	0.5
1688	neral	18.5	22.1	13.1	19.6	14.6
1704	γ-curcumene	1.0	1.8	3.5	0.0	2.7
1718	γ-muurolene	0.2	0.3	1.0	0.0	0.8
1731	β-curcumene	0.0	6.1	3.0	0.0	5.2
1746	geranial	17.7	30.0	22.1	30.8	21.6
1755	bicyclogermacrene	14.0	0.1	0.1	0.0	0.8
1755	geranyl acetate	0.1	0.1	0.1	0.0	0.0
1755	δ-cadinene	0.1	0.1	0.9	0.0	0.1
1765	α-curcumene	4.1	3.1	6.8	4.9	5.9
1778	α-muurolene	0.1	0.0	0.2	0.1	0.2
1804	geranyl propionate	0.2	0.1	0.4	0.2	0.2
1813	(*Z*) + farnesol	0.3	0.2	0.4	0.2	0.5
1886	*epi*-cubebol	0.5	0.4	0.6	0.4	0.5
1952	β-bisabolol	0.7	0.5	0.9	1.3	0.4
1973	caryophyllene oxide	5.8	2.4	8.8	5.4	8.6
1989	(*E*)-nerolidol	1.4	1.4	1.7	2.1	1.1
2037	germacrene-D-4–ol	0.3	0.6	0.6	0.7	0.4
2046	spathulenol	5.2	4.2	4.7	5.3	6.0
2128	germacrene-D-4-ol	0.4	0.3	0.4	0.4	1.1

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Table 1.continuation

2143	β-bisabolol	0.6	0.4	1.0	0.5	1.2
2168	*epi a*-cadinol, -	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 two columns with those from pure standards or reported in the literature, and on comparison of MS with file spectra.

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

LB (Laguna Brava)	**SDS-PAGE (V/E* 1:7)**	**Inhibition of hemolytic activity**	**Inhibition of coagulant activity**	**Inhibition of proteolytic activity**			
Fall		V/E*	HA*	V/E	% Recovery	V/E*	PA*
Aqueous extract	No	1:20	No	1:2.5	9 %	1:120	No
Alcoholic extract	No	1:20	Inhibits	1:2.5	18 %	1:12	Yes
Hexanic extract	No	1:20	Inhibits	1:2.5	18 %	1:120	No
Essential Oil	Yes	1:20	Inhibits	1:10	48 %	1:120	Yes
Water Oil	Yes	1:20	Inhibits	1:10	58 %	1:120	Yes
Spring							
Essential Oil	Yes	1.20	Inhibits	1:10	50 %	1:120	Yes
Water Oil	Yes	1:20	Inhibits	1:10	58 %	1:120	Yes
Summer							
Essential Oil	Yes	1:20	Inhibits	1:10	38 %	1:120	Yes

* V/E: ratio venom (dry weight): oil/extract; HA: hemolytic activity, PA: inhibition of proteolytic activity.

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

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

*V/E: ratio venom (dry weight): oil/extract; PA: inhibition of proteolytic activity

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Table 4: Antisnake venom activity of samples from Paso de la Patria

San Luis del Palmar (SLP)	**SDS-PAGE (V/E* 1:7)**	**Inhibition of coagulant activity**		**Inhibition of proteolytic activity**	
Fall		**V/E***	**% Recovery**	**V/E***	**PA***
Essential oil	Yes	1:9	51	1:120	Si
Water oil	Yes	1:10	92	1:120	Si
Spring					
Essential oil	Yes	1:10	49	1:120	Si
Water oil	Yes	1:10	56	1:120	Si

*V/E: ratio venom (dry weight): oil/extract; PA: inhibition of proteolytic activity

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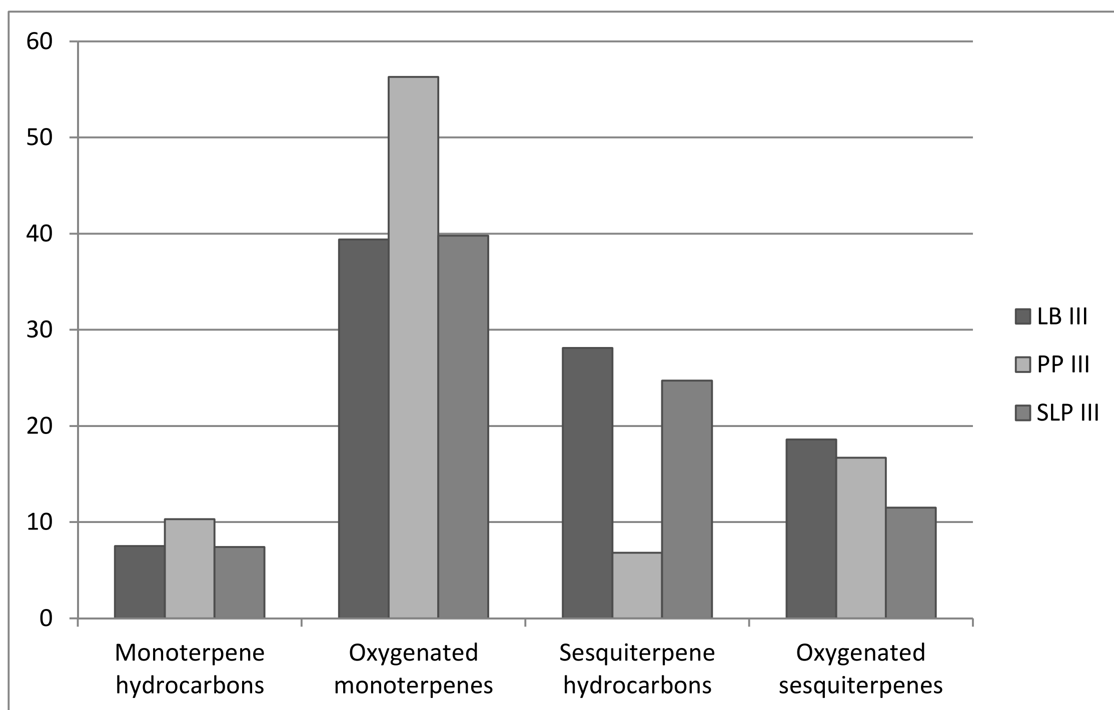
Figure 1: Geographical variation of the identified compounds in summer samples of *A. citriodora* grouped by families



LB, Laguna Brava; PP, Paso de la Patria; SLP, San Luis del Palmar

Samples were collected in the same growing stage (summer, III)

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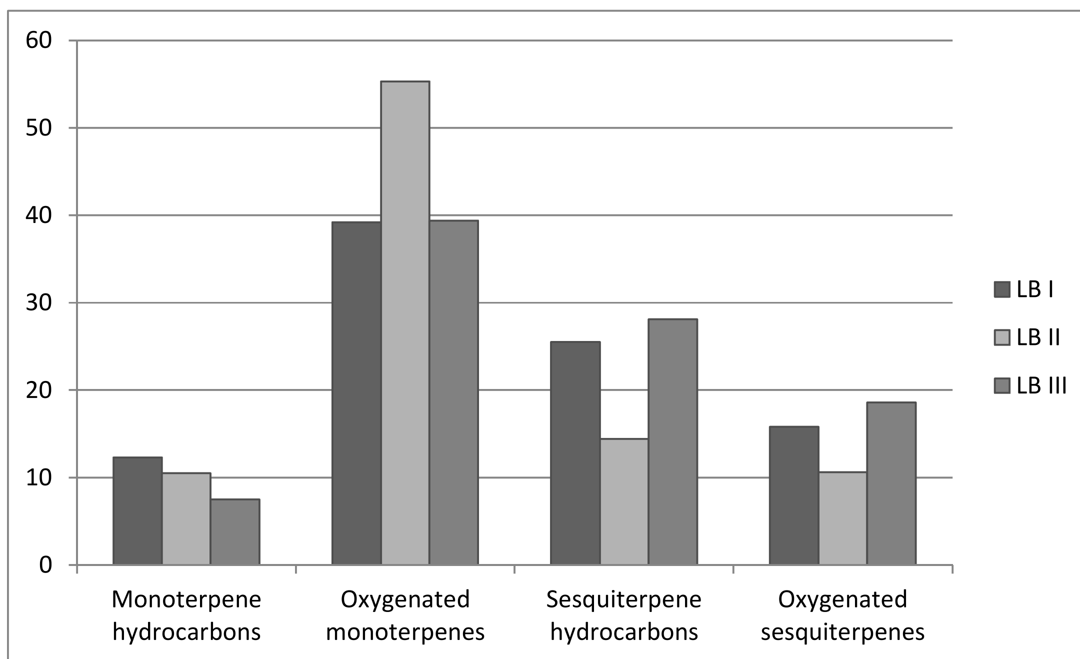
Figure 2: Seasonal variation of the identified compounds in samples of *A. citriodora* from Laguna Brava grouped by families



LB, Laguna Brava; I, fall; II, spring, III, summer

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