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# The spasmolytic effect of *Aloysia citriodora*, Palau (South American cedrón) is partially due to its vitexin but not isovitexin on rat duodenums

María Inés Ragone [a], Mariana Sella [a], Paula Conforti [b], María G. Volonté [b], Alicia E. Consolini [a,*]

[a] *Cátedra de Farmacología, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 47 y 115 (1900), La Plata, Argentina*
[b] *Cátedra de Control de Calidad de Medicamentos, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Argentina*

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**Abstract**

The spasmolytic effects of an acqueous extract of cedrón (AEC) were studied on rat isolated duodenums. This plant *(Aloysia citriodora* Palau, Verbenaceae) is widely used for gastrointestinal disorders and as eupeptic in South America. AEC non-competitively inhibited the dose–response curve (DRC) of Ach (IC50 of 1.34 ± 0.49 mg lyophilized/mL) and the DRC of $Ca^{2+}$ in high-$[K^{2-}]_o$ (IC50 of 2.64 ± 0.23 mg/mL). AEC potentiated the non-competitive inhibition of either 30 μmol/L W-7 (a calmodulin blocker) and 5–15 μmol/L papaverine on the $Ca^{2+}$-DRC. Also, AEC relaxed the contracture produced by high-$[K^+]_o$ (IC50 of 2.6 ± 0.2 mg/mL) until 81.0 ± 3.2% of the maximal effect of papaverine and 78.1 ± 5.0% of the quercetin, the most selective inhibitor of PDE. The AEC relaxation was non-competitively inhibited by 10–30 μmol/L methylene blue and competitively antagonized by 40 mmol/L TEA. The relaxation of 1 mg/mL AEC was inhibited by hypoxia, but not that of 2 mg/mL. Two flavonoids were identified by HPLC in the AEC: vitexin and isovitexin. Vitexin non-competitively inhibited the Ach-DRC ($pD_2'$ of 5.7 ± 0.4) but significantly run leftward the DRC of $Ca^{2+}$. Isovitexin did not significantly inhibit the DRC of Ach nor $Ca^{2+}$. The results suggest that the spasmolytic effect of AEC could be mostly associated to the increase in cGMP (target shared with the PDE inhibitors) and the activation of $K^+$-channels. At low concentrations, AEC also inhibits the aerobic metabolism. The flavonoid vitexin is partially responsible for the effect, since it non-competitively inhibits Ach but not the $Ca^{2+}$ influx. Isovitexin was devoid of activity on duodenums.


*Keywords:* *Aloysia citriodora* (Palau); Verbenaceae; Rat duodenum; Antispasmodic; Vitexin; Isovitexin

## 1. Introduction

Many plants are used in folk medicine to treat gastrointestinal disorders, such as espasmo or indigestion. *Aloysia citriodora* Palau, Verbenaceae (syn. *Aloysia triphylla* (*L'Hér.*)) is a plant originally from South America popularly known as "cedrón" or "hierba Luisa". The leaves are prepared as an aromatic infusion commonly used as dietary supplement or eupeptic tea; also as decoction for abdominal pain or against nausea and dizziness (Soraru and Bandoni, 1978; Alonso, 2004). In Cuba is used as expectorant and against insomnia, in Guatemala as antiasmatic and antiparasite, in Paraguay is used against cardiac palpitations (Alonso and Desmarchelier, 2005). In spite of its wide use and the regulations for cultivation, until now there were no studies about its pharmacological properties except some reports about antimicrobial activity of the essential oil and depressant effect of the leaves infusion (Alonso and Desmarchelier, 2005). A little is known about its active principles, except the presence of a flavonoid, luteolin-7-diglucuronide (Skaltsa and Shammas, 1988; Carnat et al., 1995), the phenolic compound verbascoside and the composition of the essential oil (Zygadlo et al., 1994; Carnat et al., 1999; Alonso and Desmarchelier, 2005).

At a taxonomic level "cedrón" is related to the genus *Lippia* (Pascual et al., 2001), which is widely used in the North hemisphere. It has been reported to exhibit an antiespasmodic activity. Based on both criteria, ethnopharmacological and philogenetic, this work studied the effects of an acqueos crude extract of "cedrón" (AEC) on the isolated rat duodenum to elucidate the

* Corresponding author. Tel.: +54 221 423 5333x42.
*E-mail address:* dinamia@biol.unlp.edu.ar (A.E. Consolini).

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mechanism of action. Also, two flavonoids vitexin and isovitexin were identified in the extract, and their spasmolytic effects were studied.

Rat duodenum was a useful model to study the gastrointestinal effects of the extract and drugs. It has muscarinic receptors and, as well as the ileon, let to study the antispasmodic effects over the smooth muscle or the cholinergic stimulation (Karamenderes and Apaydin, 2003; Emendörfer et al., 2005).

## 2. Materials and methods

### 2.1. Plant material and extracts preparation

*Aloysia citriodora* Palau (Verbenacea) was provided by a local herboristery, and the plant was authenticated by Prof. Dra. Etile Spegazzini. The voucher specimen (herbarium number LPE 1039) of the plant was kept in the Herbarium Museum of Botany and Pharmacognosy, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Argentina.

Aqueous extract of "cedrón" (AEC) was prepared by boiling 30 g dried leaves in 200 mL distilled water for 20 min, as the ethomedicinal use. After filtration the decoction was lyophilized, obtaining a 15% (w/w) yield of the dried leaves. The lyophilized extract was diluted in distilled water and Tyrode solution for in vitro tests the day of each experiment. With this procedure, the essential oil is not included in the lyophilized sample.

### 2.2. Solutions and drugs

The solutions used had the following composition: Tyrode (Tyr): 150 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM $PO_4H_2Na$, 1.8 mM $CaCl_2$, bubbled with air (pH 8.2).

Tyrode-0Ca: by eliminating $CaCl_2$.

Tyrode-0Ca-80 mM $K^+$: by replacing 77.3 mM $Na^+$ by $K^+$, and eliminating $CaCl_2$.

Tyrode-glucose free hypoxic (Tyr-0 glu-$N_2$) was prepared by replacing glucose by sacarose to keep osmolarity constant, and bubbled with 95% $N_2$–5% $CO_2$.

The drugs employed in biological tests were: papaverine hydrochloride (Merck, Germany), acetylcholine bromide (Sigma, USA), *N*-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide (W-7 from Sigma), methylene blue (Parafarm, Argentina), tetraethylammonium chloride (Sigma) and the flavonoids quercetin (Sigma), vitexin (Extrasynthese, France) and isovitexin (Extrasynthese). The flavonoids were initially dissolved in dimethylsulphoxide (DMSO) at 30 μg/mL for vitexin and isovitexin and at 200 μM for quercetin, and then diluted with water to obtain the lower concentrations. A DRC control was done with DMSO at 1% in the Tyrode medium, which was not different from the DRC without vehicle. The standard drugs used to identify components by HPLC were: isovitexin (Extrasynthese) and vitexin (Extrasynthese) in solutions at 20 μg/mL; chlorogenic acid (Extrasynthese) at 29.5 μg/mL; hyperoside (Extrasynthese) at 26 μg/mL; vitexin 2″-rhamnoside (Extrasynthese) at 30 μg/mL; orientin (Extrasynthese) at 30 μg/mL; homoorientin (Extrasynthese) at 22.5 μg/mL; quercetin 3-*O*-β-glucopyranoside (Extrasynthese) at 35 μg/mL; rutin (Extrasynthese) at 26 μg/mL and quercetin (Sigma) at 300 μg/mL, all prepared with the respective mobile phase.

### 2.3. Animals

The research was conducted in accordance with the internationally accepted principles for the laboratory animal use and care as was established by US guidelines (NIH publication #85-23 revised in 1985).

#### 2.3.1. Biological preparation and contractile measurements

Sprague–Dawley rats (200–250 g) were subjected to a 24 h fasting with free access to water before experimentation. The animals were anaesthetized by inhalation with sulfuric ether and then quickly sacrificed by the opening of torax and abdomen, in order to avoid a relaxant effect of any other anesthetic on the intestinal tissue.

Duodenums (about 2 cm long) were prepared and mounted in organ baths of 20 mL containing Tyrode solution at 37 °C constantly oxygenated with air (pH 8.2) as in other works (Emendörfer et al., 2005). The preparations were equilibrated for at least 45 min at 1 g of pre-load. Tissues were connected to either, an isotonic force transducer Letica TRO 015 (PanLab, Spain) or an isometric one Power Lab MLT0201 (Power Lab, AD Instruments). Both types of response were statistically compared in each protocol, without significant differences between results. Because of that, both types of data were mixed. The signals were acquisited in a computer, respectively, by a National Instruments PC-516 NI-Daq SW with Virtual Bench logger and by Chart 4 of Power Lab program.

### 2.4. Assays

#### 2.4.1. Dose–response curves to acetylcholine

Dose–response curves (DRC) to acetylcholine (Ach) were done for the rats duodenums after a stabilization of 45 min. Ach doses were cumulatively added to the bath (to reach from 0.01 to 10 μg/mL) in the absence and the presence of AEC after 10 min of contact with 0.1, 0.2, 0.6, 1, 2 or 6 mg/mL of lyophilized. The same protocol was carried out with the flavonoids vitexin and isovitexin (at 0.3, 1, 3, 10 and 30 μg/mL).

#### 2.4.2. Dose–response curves to $CaCl_2$

After the stabilization during 45 min in Tyrode, the external $Ca^{2+}$ was eliminated with Tyrode-0Ca and then, muscle depolarized with Tyrode-0Ca-80 mM $K^+$ isoosmotic. DRC of $Ca^{2+}$ were obtained by cumulatively adding $CaCl_2$ to reach concentrations from 0.0195 to 17.5 mmol/L. AEC at final concentrations of 0.1, 0.2, 0.6, 1, 2 or 6 mg/mL were added to the bath 10 min before the respective dose–response curve of $CaCl_2$. The same protocol was carried out with the flavonoids vitexin and isovitexin at 20 μg/mL; with 30 μmol/L W-7 (a calmodulin antagonist) without and with 1 mg/mL AEC; with papaverine (5, 15, 30

and 45 μmol/L); with AEC (1 mg/mL) plus papaverine (5 and 15 μmol/L).

*2.4.3. Dose–relaxation curves*

Tissues were contracted by depolarization with 0.6 mL ClK 10% (80 mM hyperosmotic in the Tyrode) until obtaining a tonic response. At this point, doses of AEC (0.6, 1, 2 and 3 mg/mL) were cumulatively added to construct a dose–relaxation curve. Finally, papaverine 15, 30 and 45 μmol/L or quercetin 300 μmol/L were added to completely relax the muscle.

In other experiments, muscles under tonic contracture obtained by depolarization with 80 mM ClK were exposed to the relaxation DRC of quercetin (3 and 400 μmol/L), or to the relaxation DRC of AEC (0.6, 1, 2 and 3 mg/mL) in the absence and in the presence of 10 or 30 μmol/L methylene blue (an inhibitor of guanylate cyclase).

In another group of muscles, the tonic contracture was obtained by adding 10 μg/mL acetylcholine (Ach). Then, the relaxation curves of AEC (0.6, 1, 2 and 3 mg/mL) were obtained in the absence and in the presence of 40 mmol/L tetraethylamonium (TEA, a non-selective $K^+$-channels blocker).

*2.4.4. Effects of AEC under hypoxia*

After obtaining a contracture with 80 mM $K^+$ in normal Tyrode, the medium was replaced by a glucose-free Tyrode bubbled by 95% $N_2$–5% $CO_2$ (Tyr-0 glu-$N_2$). After 5 min, another contracture was developed by raising $[K^+]_o$ to 80 mM. This protocol was repeated in each muscle, by adding AEC 1 or 2 mg/mL in Tyr-0 glu-$N_2$ media during 5 min before the depolarization. Changes to normal Tyr and aerobic contractures were inserted between the hypoxic ones to confirm the viability of muscles, which were reproducible. The effects of AEC under hypoxia were expressed as percentage of the aerobic control contraction, in both phasic and tonic responses.

*2.5. HPLC analysis*

The acqueous crude extract was analyzed by HPLC to obtain the "finger-print" and compare it with some standard herbal components.

As previously described, it was used a Konik KNK 500 G chromatographer (Konik, Barcelona, Spain), with sample injector of fixed loop for 20 μL (Rheodyne, Cotati, CA, USA) (see other details in Consolini et al., 2006). The analysis was performed by using two chromatographic systems, both with a Lichrocart RP 18 reverse phase column of 250 mm × 4 mm i.d. with a particle size of 5 μm, at room laboratory temperature (25 °C) and with an isocratic elution mode.

There were used two systems of mobile phase, as follows: in system A it was composed by isopropanol: tetrahydrofuran: water (5:15:85) at pH 4.33; in system B it was water:tetrahydrofuran:isopropanol:acetonitrile (88:8:1.6:2.4) with 0.05% phosphoric acid in the aqueous phase at pH 2.61. In both systems, flow rate was 1.0 mL/min and the wavelength for detection was 336 nm. Samples were prepared from the lyophilized extract with mobile phase at a concentration of 700 μg/mL, then filtered and injected for triplicate into the HPLC system. The following compounds were used as standards: chlorogenic acid, homoorientin, isovitexin, vitexin 2″-rhamnoside, orientin, rutin, vitexin, quercetin, quercetin 3-*O*-β-glucopyranoside and hyperoside, all prepared with the mobile phase, respectively, in each systems A and B. The retention times ($R_t$) of those compounds were: in system A 3.1, 8.7, 10.6, 12, 14.6, 17, 20, 24.77, 25.6 and 26.8 min, respectively; in system B 12.3, 28, 43.7, 50.5, 44.3, 14.73, 60, 34.47, 32.3 and 29.4 min, respectively.

*2.6. Statistical analysis*

From the DRC there were calculated the $pD_2$ of the agonist (as $-\log EC50$, in molar) and the affinity ($pD_2'$) of the pure non-competitive antagonists. $pD_2' = -\log[B'] + \log[(E_A - E_{AB'max})/(E_A - E_{AB'}) - 1]$, where $B'$ is the concentration of the non-competitive antagonist, in molar, and $E_A$, $E_{AB'}$ and $E_{AB'max}$ and are the maximal effects of the agonist, respectively, in the absence (A) and in the presence of the antagonist ($B'$) and the maximal concentration of it ($B'$max) (Van der Brink, 1977; Kenakin, 1984). For the extract, the IC50 was calculated by extrapolation from the respective DRC curves at maximal effect of the agonist, and expressed as mg lyophilized by mL. All results are expressed as media ± ESM. Regression of DRC and statistics were done by using Graph Pad Prism 4.0 program, and by applying one-way ANOVA test for multiple comparisons followed by Tukey's a posteriori tests. Paired *t*-test for comparison of paired results was also done. In all tests it was considered a significance of $p < 0.05$.

## 3. Results

*3.1. Pharmacology of the extract*

The extract of *Aloysia citriodora* (AEC) reduced the maximal effect of the Ach DRC on a dose-dependent way (Fig. 1a), suggesting a non-competitive antagonism over the cholinergic contraction. The extrapolated IC50 of AEC was 1.34 ± 0.49 mg/mL. In order to elucidate whether the non-competitive antagonism of AEC was associated to inhibition of $Ca^{2+}$ influx to the smooth muscle, curves of $Ca^{2+}$ were done on depolarizing medium. Fig. 1b shows that AEC was also a non-competitive inhibitor of $Ca^{2+}$ influx. The extrapolated IC50 was 2.64 ± 0.23 mg/mL ($p = 0.03$ versus that on Ach DRC).

Since AEC seems to interfere with a cellular process between $Ca^{2+}$ influx and the contraction, it was assayed whether AEC blocks the calmodulin-Ca-binding. Curves of $Ca^{2+}$ were done in the absence and presence of either, 1 mg/mL AEC or 30 μmol/L W-7 (a Ca-calmodulin-inhibitor) plus 1 mg/mL AEC. Fig. 2a shows that W-7 potentiated the non-competitive inhibition produced by AEC on the DRC of $Ca^{2+}$. On the other side, to evaluate whether AEC acts by a mechanism similar to papaverine (one of the most active relaxant of smooth muscle) their effects were compared. Papaverine non-competitively inhibited the $Ca^{2+}$ curve, and completely blocked the response at 30–45 μmol/L, with a $pD_2'$ of 5.05 ± 0.4 (IC50: 4.7 ± 0.9 μg/mL). Fig. 2b

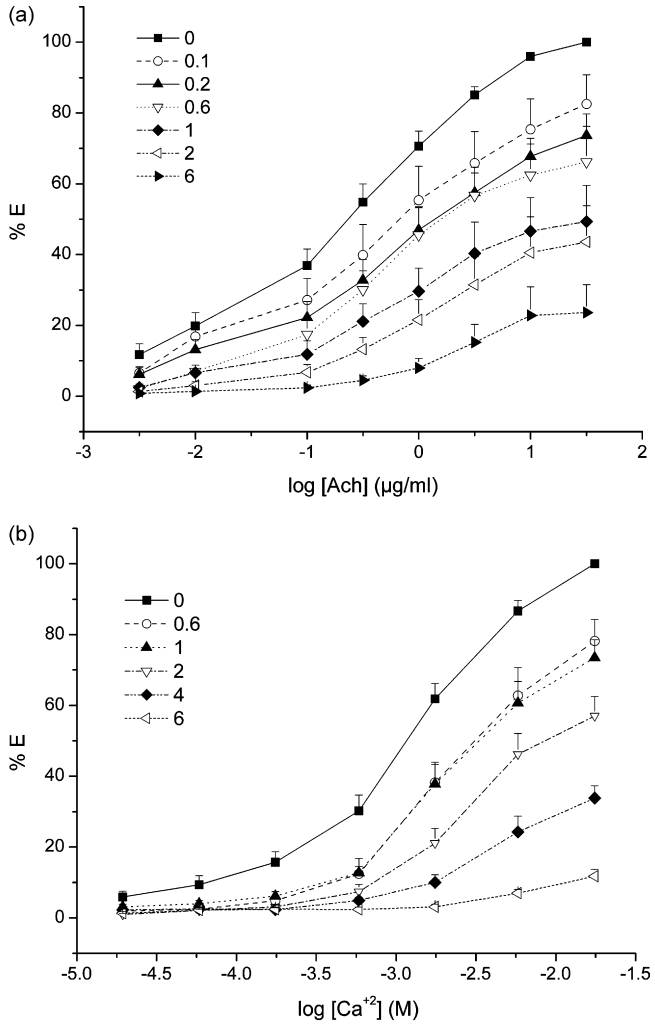


Fig. 1. Effects of *Aloysia citriodora* extract on the dose–response curves of: (a) acetylcholine ($pD_2$: $5.77 \pm 0.13$, $n = 12$) and (b) $CaCl_2$ ($pD_2$: $2.79 \pm 0.10$, $n = 10$). Results are presented as mean values and S.E.M. Dose extract (mg/mL) in labels. See the IC50 in the text.

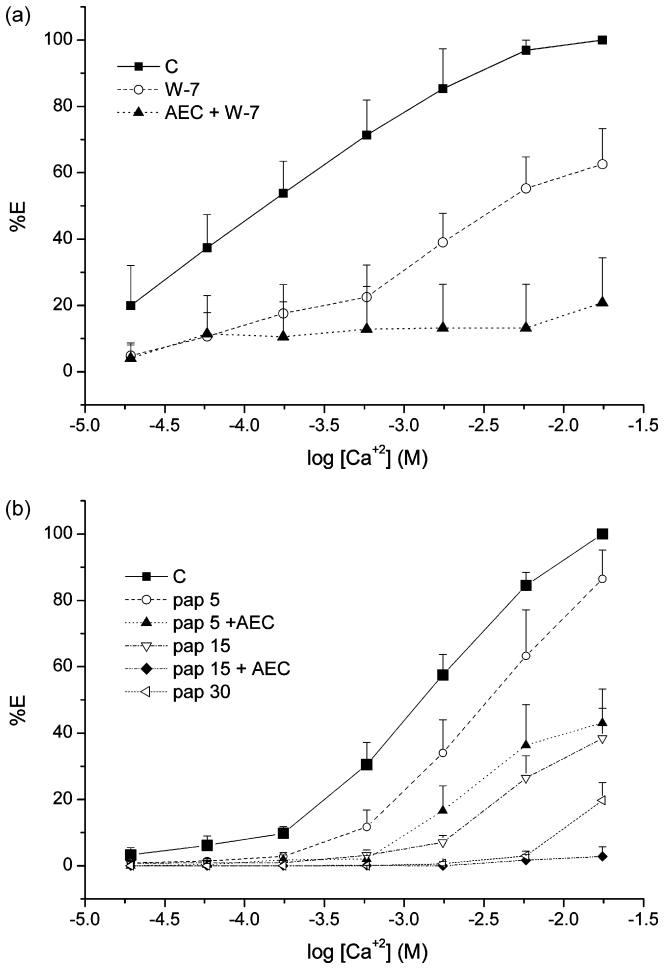


Fig. 2. Effects of *Aloysia citriodora* extract (AEC) at 1 mg/mL on the DRC of $CaCl_2$ in the presence of: (a) 30 µmol/L W-7 ($n = 7$) and (b) 5 and 15 µmol/L papaverine ($n = 8$), comparing with the effects of the individual drugs. Results are presented as mean values and S.E.M. Note that AEC potentiated the effect of both drugs.

shows that AEC potentiated the non-competitive effect of papaverine. To further explore an inhibition of the phosphodiesterase (PDE), we compared the relaxant effects of AEC on the tonic contracture upon high-$K^+$ with the maximal relaxation produced by papaverine (a non-selective PDE inhibitor) and the flavonoid quercetin (a selective PDE inhibitor) (Beretz et al., 1978; Ko et al., 2002). Table 1 shows the IC50 and the maximal relaxation of AEC regarding the maximal relaxation of muscle produced by either, 45 µmol/L papaverine or 300 µmol/L quercetin. It can be noted that there were not significative differences between the IC50, and that AEC produced almost the same relaxation (about 80%) than papaverine and quercetine. Moreover, Fig. 3a shows that the relaxation curve of AEC was non-competitively blocked by an inhibitor of the guanylate cyclase, methylene blue at 10 and 30 µmol/L (Fig. 3a, $pD_2'$ of $4.36 \pm 0.39$), suggesting that the extract increase the GC activity.

Table 1
Parameters of the relaxation curves of *Aloysia citriodora* extract (AEC) on the tonic contracture induced by high-$K^+$ and finally relaxed with either, papaverine or quercetin

| | I.C.50 | % of Maximal relaxation |
|---|---|---|
| $DRC_{AEC}$ followed by 45 µM papaverine | $2.57 \pm 0.24$ | $81.0 \pm 3.2\%$ |
| $DRC_{AEC}$ followed by 300 µM quercetin | $3.07 \pm 0.74$ | $78.1 \pm 5.0\%$ |
| $p$ | NS | NS |

I.C.50: inhibitory concentration 50%.

On the other side, since the IC50 of AEC was lower on the Ach-DRC than on the $Ca^{2+}$ DRC in high-$K^+$ media, it was hypothetized that AEC would activate $K^+$-channels upon the agonist, which would not be active in high-$K^+$ media. Then, after developing the tonic contracture with Ach, the relaxation curve of AEC was done before and after adding 40 mmol/L tetraethylammonium (TEA), a non-selective $K^+$ channels. Fig. 3b shows that TEA run rightward the DRC of AEC (EC50 from $2.998 \pm 0.281$ to $5.495 \pm 0.759$ mg/mL, $n = 6$, paired $t$-test $p = 0.0219$), suggesting a competitive inhibition ($pA_2$ of $1.197 \pm 0.169$ for TEA).

Since it has been found that the relaxant effects of papaverine on ileon of guinea-pig were in part due to inhibition of the aerobic metabolism (Kaneda et al., 1998) we assayed whether

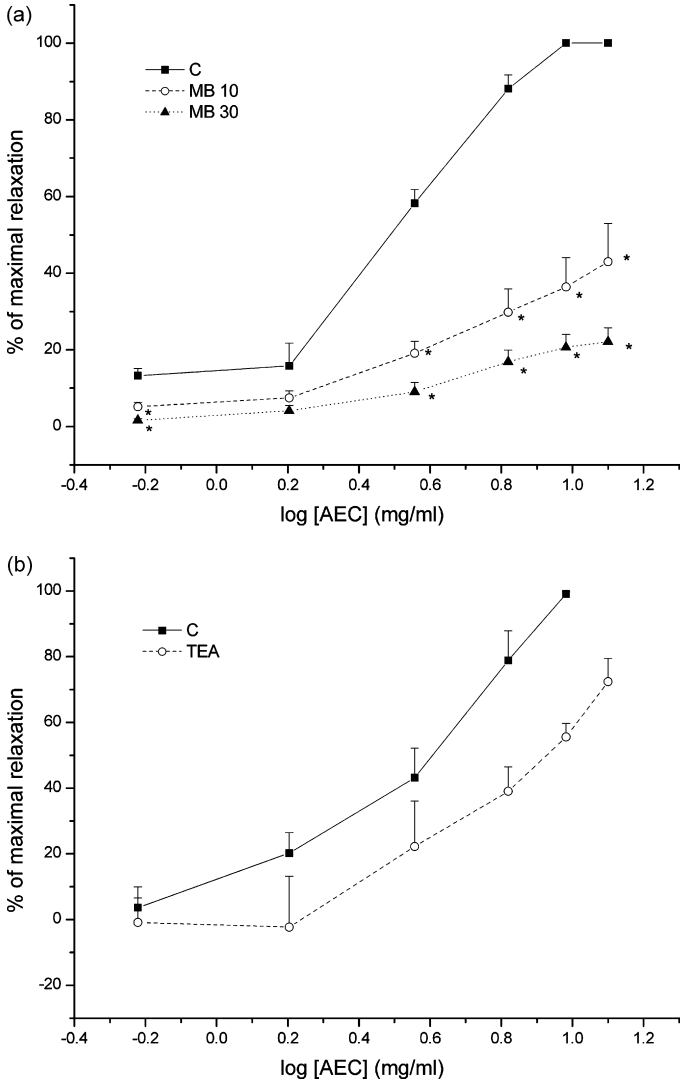


Fig. 3. Relaxant effects of AEC on the tonic contractures obtained by: (a) 80 mM $K^+$ in the absence (C) and in the presence of 10 and 30 μM methylene blue (MB) (*$p < 0.05$ vs. C, $pD_2'$ 4.36 ± 0.39); (b) 10 μg/mL acethylcholine in the absence (C) and in the presence of 40 mM tetraethylammonium (TEA) (note the competitive antagonism and see parameters in the text).

AEC shares that mechanism on duodenum. Then, a protocol was done where the muscles were first contracted by 80 mM $K^+$ in the oxygenated Tyrode (Tyr), and then in a glucose-free hypoxic Tyrode (Tyr-0 glu-$N_2$). Hypoxia significantly reduced ($p < 0.001$) both, the tonic (to 29.7 ± 5.1% of the aerobic) and the phasic (to 71.8 ± 6.3% of the aerobic) contractions. Fig. 4 shows that 1 mg/mL AEC did not significantly reduce neither the phasic (58.7 ± 6.7% of the aerobic, $n = 8$) nor the tonic (18.5 ± 3.6% of the aerobic) contractions in Tyr-0 glu-$N_2$. Nevertheless, at 2 mg/mL the AEC significantly decreased the phasic response until 38.2 ± 5.9% and the tonic one to 9.9 ± 1.0% of the aerobic ($p < 0.001$ versus the respective untreated hypoxic contractions).

### 3.2. Identification of components in Aloysia citriodora

The qualitative evaluation of *Aloysia citriodora* was made by comparing the retention times of standards and the extracts under

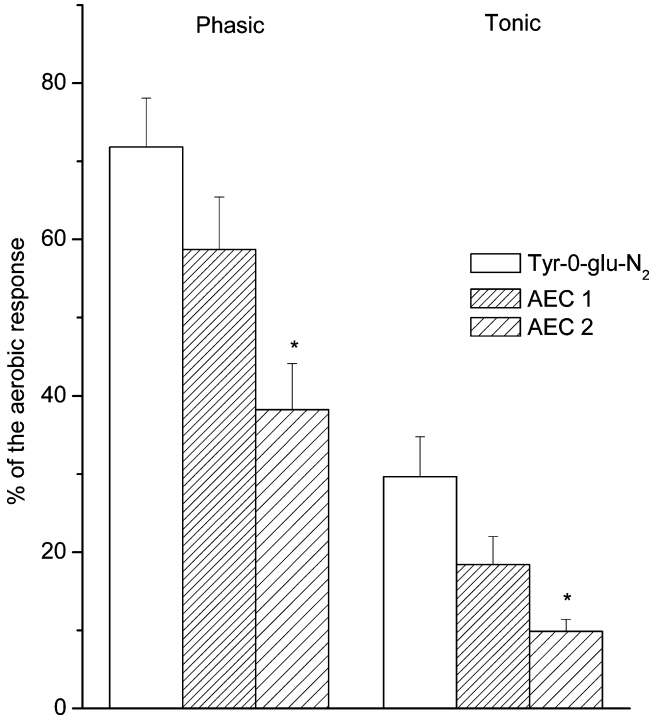


Fig. 4. Effects of glucose-free hypoxia (Tyr-0 glu-$N_2$) and AEC (1 and 2 mg/mL) on the phasic and tonic contractions induced by 80 mM $K^+$, as a percentage of the respective aerobic contraction. Results are presented as mean values and S.E.M. For each type of contraction ANOVA test was $p < 0.0001$ ($F = 7.254$ and 7.143 for phasic and tonic, respectively), *$p < 0.01$ vs. the respective hypoxic contraction without AEC by Tukey test ($n = 16$ in each bar).

the same chromatographic conditions. The chromatograms showed a good separation profile with a total running time for the assay within 40 min for the system A and 60 min for B. Only vitexin (retention times of 20 min in system A and 60 min in system B) and isovitexin (retention times of 10.6 min in A and 43.7 min in B) were identified in the extracts. Fig. 5 shows the chromatographic profile of the standards vitexin and isovitexin and the ACE run in both systems, A and B.

### 3.3. Are the effects of the AEC due to vitexin and isovitexin?

The flavonoid vitexin produced a non-competitive antagonism on the Ach dose–response curve, as it is shown on Fig. 6a. The affinity calculated as $pD_2'$ was 5.7 ± 0.4 ($n = 5$) and the maximal inhibition obtained at 30 μg/mL vitexin was 48.6 ± 12.0% of the $E_{max}$ of Ach. On the DRC of $Cl_2Ca$, 20 μg/mL vitexin induced a leftward shifting (the $pD_2$ of Ach increased from 2.5 ± 0.1 to 2.8 ± 0.2, $n = 4$, $p = 0.0176$ by paired *t*-test; Fig. 6b).

The stereoisomer isovitexin did not significantly inhibit the DRC of Ach at 30 μg/mL (81.4 ± 9.5% of $E_{max}$ of Ach, NS) neither modify the $Ca^{2+}$-DRC at 20 μg/mL ($pD_2$ of 3.25 ± 0.11 versus 3.21 ± 0.13, $n = 4$, NS) (Fig. 6c and d).

## 4. Discussion

This is the first work that describes the spasmolytic effect of *Aloysia citriodora Palau* popularly known as “cedrón” o “hierba

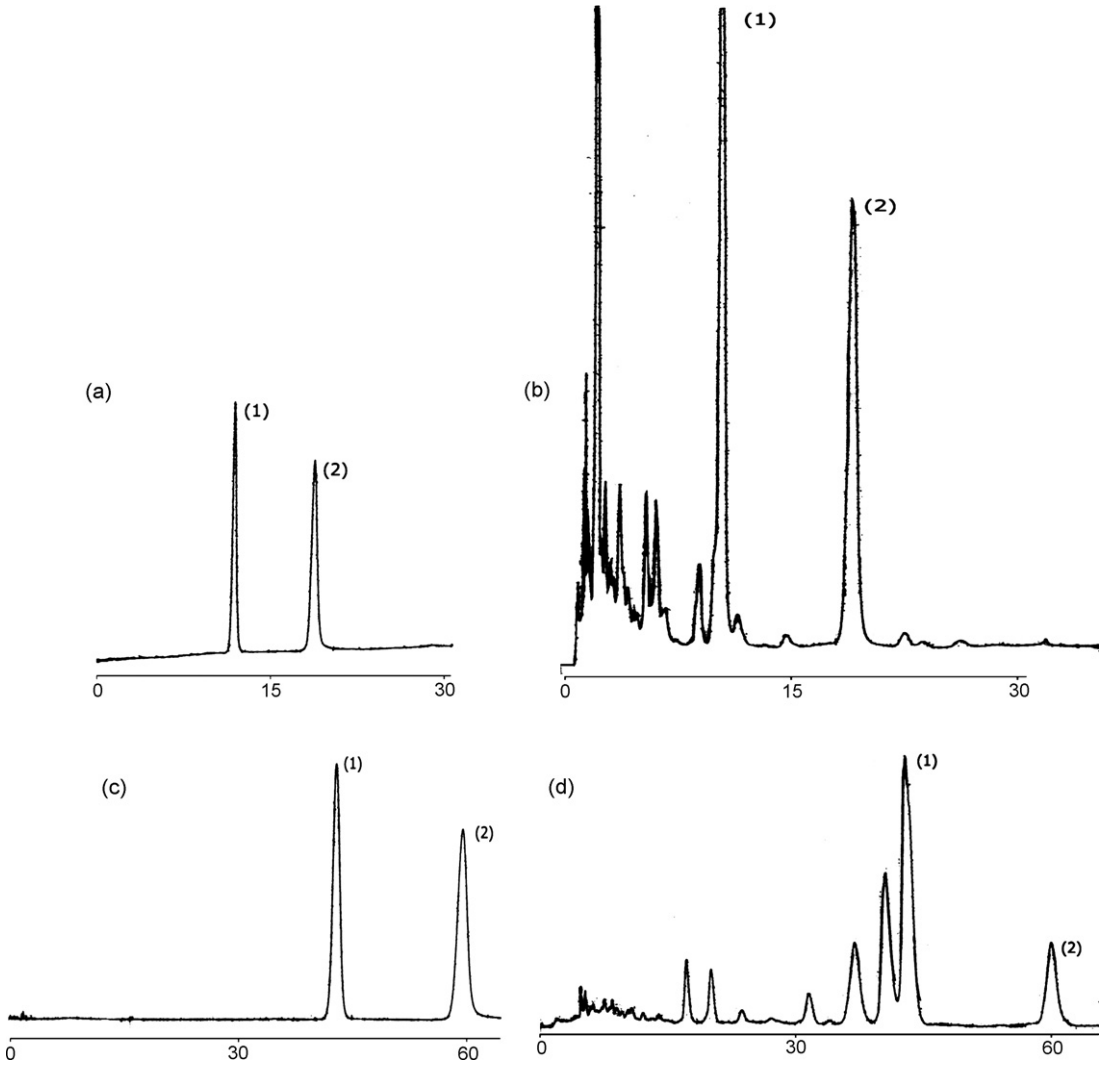


Fig. 5. HPLC traces of the standard solutions of isovitexin (1) and vitexin (2) in system A (a) and system B (c), in comparison with the HPLC traces of the acqueous extract of *Aloysia citriodora* in system A (b) and system B (d). Retention time scale in minutes. For the experimental conditions, see the HPLC analysis in Section 2.

Luisa". The acqueous extract (AEC) directly relaxed the intestinal smooth muscle and non-competitively inhibited the DRC of Ach, suggesting an intracellular site for reducing the contraction. The extract cannot be an antagonist of $Ca^{2+}$ influx since it inhibited the DRC of $Ca^{2+}$ on high $[K^+]_o$ in a non-competitive way. Looking for the origin of that effect, the calmodulin antagonist W-7 (Silver et al., 1984; Asano, 1989) produced and additive effect with AEC 1 mg/mL on the $Ca^{2+}$-DRC, which suggests that AEC and W-7 would act on different sites. Similarly, papaverine potentiated the non-competitive inhibition of AEC on $Ca^{2+}$ curves. Papaverine is the most classical relaxant of smooth muscle (Sánchez de Rojas et al., 1995; Karamenderes and Apaydin, 2003) and the extract produced about an 81% of the maximal relaxation of papaverine on high-$K^+$ medium, suggesting a common target. The mechanism of action of papaverine has differences between visceral and arterial muscles. While in aorta papaverine mostly increased the cAMP and cGMP levels dependent on the PDE inhibition, in the guinea-pig ileon the relaxation was mainly related to the inhibition of mitochondrial respiration (Kaneda et al., 1998). This mechanism of papaverine was also the most important in other spontaneously depolarized smooth muscles as guinea-pig urinary bladder (Shimizu et al., 2000a) and rat uterus (Shimizu et al., 2000b). Thus, we assayed AEC on rat duodenums upon a hypoxic-free glucose medium (Nasu and Nishikawa, 2000), founding that the tonic contraction was more sensitive than the phasic, as it was reported (Nakagawa et al., 1976). In ileal muscle the phasic contraction depends on the endogenous glycogen while the tonic depends on aerobic metabolism (Nasu and Nishikawa, 2000). Fig. 4 shows that at low concentrations (1 mg/mL) AEC lost its capacity to relax either the phasic or the tonic hypoxic responses, suggesting that the effect of AEC should be associated to metabolic inhibition. Nevertheless, at 2 mg/mL AEC significantly inhibited both types of contraction, showing another mechanism, as previously discussed.

Several plants are non-competitive antagonists of Ach in duodenal or ileal smooth muscles (Sánchez de Rojas et al., 1995; Karamenderes and Apaydin, 2003; Camara et al., 2003; Emendörfer et al., 2005), as well as flavonoids as quercetin (Di Carlo et al., 1999). The presence of quercetin could not

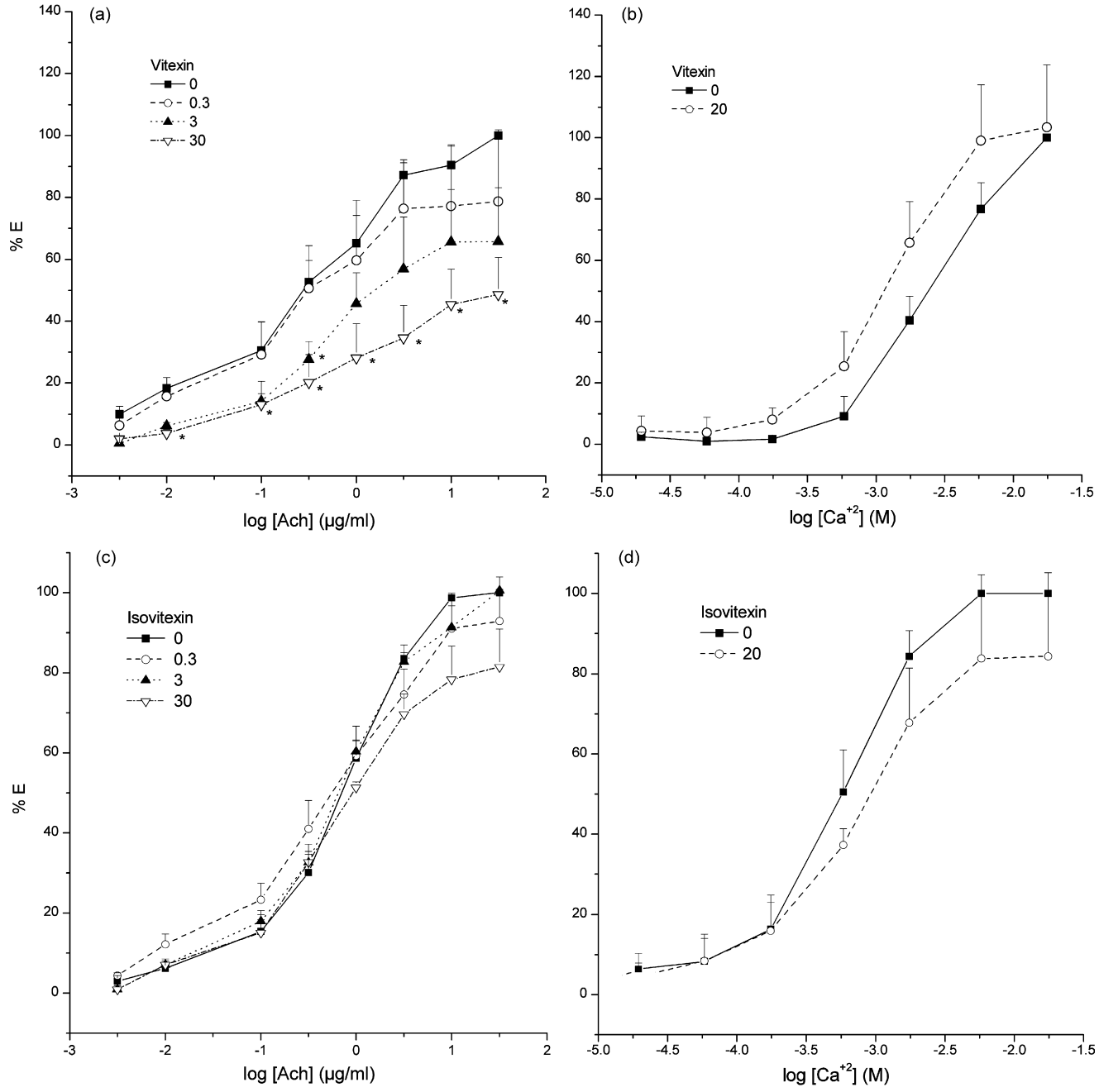


Fig. 6. Effects of vitexin and isovitexin on: (a and c): DRC of Ach ($n = 5$, $^*p < 0.05$ vs. control; see $pD_2'$ of vitexin in the text); (b and d): DRC of $Ca^{2+}$ ($pD_2$: $2.5 \pm 0.1$ vs. $2.8 \pm 0.2$, before and after vitexin, $n = 4$, $p = 0.0176$ by paired $t$-test; and $3.25 \pm 0.11$ vs. $3.21 \pm 0.13$, for isovitexin, $n = 4$, NS).

be demonstrated in the AEC by HPLC, but we used quercetin as a tool to more specifically block the phosphodiesterase of cAMP/cGMP (Beretz et al., 1978). On the high-$K^+$ contracture quercetin relaxed the muscle with a $pD_2$ of $4.33 \pm 0.09$ which agrees with the IC50 described on guinea-pig ileon (Di Carlo et al., 1999) and is near to that from its derivative 3-*O*-methyl-quercetin on guinea-pig trachea (Ko et al., 2002). The relaxation of AEC reached about 80% of the maximal relaxation of quercetin, as well as for papaverine (Table 1), suggesting that both drugs and AEC would share the same mechanism of action. Finally, the non-competitive blockade of methylene blue (non-selective inhibitor of guanylate cyclase, Mayer et al., 1993) over the relaxation of AEC (Fig. 3a) strongly suggested that the extract increased cGMP, the target shared with quercetine and papaverine as PDE inhibitors.

Moreover, Fig. 3b shows that the non-selective inhibitor of the $K^+$-channels, TEA, competitively antagonized the relaxation curve of AEC developed on the cholinergic contraction, suggesting that AEC also activates $K^+$-channels. As it is known, a $K^+$-channel opener hyperpolarizes and relax smooth muscles exposed to agonists but not to high-$K^+$ depolarization (Karaki et al., 1997).

Since the extract was prepared as decoction, the antispasmodic effects cannot be attributed to the essential oil, as other

suggested (Alonso, 2004), but to fixed compounds. Vitexin and isovitexin were identified in the AEC with standards. These flavonoids have been described in other plants as the genus *Passiflora* (Muller et al., 2005), *Ochrocarpus* and *Arnebia* (Prabhakar et al., 1981). Only an early report qualitatively described the effects of vitexin in vitro and in vivo as hypotensive, anti-inflammatory and antispasmodic (Prabhakar et al., 1981). As Fig. 6 shows, vitexin non-competitively inhibited the DRC of Ach on rat duodenums with an affinity ($pD_2'$ of $5.7 \pm 0.4$) higher than the $pD_2'$ reported for 3-methyl-*O*-quercetin (4.68), one of the most active flavonoids on ileon (Hammad and Abdalla, 1997). Nevertheless, the 6-C-glucopyranosil-apigenin (isovitexin) lacked the cholinergic inhibitory effect of the 8-C-isomer (vitexin), suggesting that the position of the sugar in the flavonoid determines its activity. Moreover, none of the studied flavonoids inhibited the DRC of $Ca^{2+}$ as the extract did. Instead of that, vitexin increased $Ca^{2+}$ affinity. This suggests that these flavonoids are not the only responsibles for the antispasmodic effect of *Aloysia citriodora*. Regarding the presence of verbascoside in this plant (Carnat et al., 1999) there are several reports about its effects as antitumoral (Kunvari et al., 1999), antioxidative (Liu et al., 2003) and cardiac inotropic and chronotropic (Pennacchio et al., 1999), but none on smooth muscle. Further studies will be necessary to identify other components responsible for the antispasmodic effect of "cedrón".

In summary, the antispasmodic effect of *Aloysia citriodora* can be explained by a non-competitive inhibition of $Ca^{2+}$ influx, which may be mostly associated to a target shared with papaverine and quercetin, which is sensitive to methylene blue, denoting an increase in cGMP. Other mechanism of AEC was the activation of $K^+$-channels, which could be a consequence of increasing cGMP, and drives to hyperpolarization and relaxation. Finally, at low concentrations, AEC inhibited the aerobic metabolism. Vitexin, but not isovitexin, does contribute to the effect of cedrón: the non-competitive inhibition of Ach but not on $Ca^{2+}$ influx, suggesting that other components are implied in the spasmolytic effect.

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