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Sedative and antispasmodic effects of *Stevia rebaudiana* and non-competitive inhibition of intestinal contractility by stevioside

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Abstract

Stevia rebaudiana (Asteraceae) is a shrub native from Paraguay whose leaves are used as sweetener due to the presence of stevioside and others. It has also hypoglycemic, hypotensor and diuretic effects. This work studied the effects of both, an aqueous crude extract of *Stevia rebaudiana* (A.c.e) and the stevioside (Stv) in isolated rat thin intestine, and the effects of A.c.e in mice on the open-field-test. The A.c.e of *Stevia rebaudiana* reduced the spontaneous locomotion and exploration of mice, at doses between 40 to 975 mg lyophilized/Kg. In the isolated ileons and duodenums, the A.c.e induced a synergism of the acetylcholine dose-response curve (ACh-DRC) at 0.1-0.3 mg lyophilized/mL and a non-competitive inhibition at higher concentrations (IC50: 1.22±0.16 mg lyoph/ml, n=6). Stv also non-competitively inhibited the ACh-DRC (IC50: 0.84 ± 0.22 mg/ml). Both, A.c.e and Stv inhibited the Ca^{2+}-DRC in 40 mM K^{+}-media in non-competitive way with IC50 of 1.01 ± 0.13 and 1.73 ± 0.47 mg/mL, respectively. Results suggest that *Stevia* extract exhibited sedative effect and important antispasmodic effect due to non-competitive antagonism of acetylcholine and Ca^{2+}-influx to smooth muscle.

Key words: Stevia rebaudiana , stevioside , antispasmodic , Ca2+-blockade , sedative

Introduction

The genus *Stevia* (Asteraceae) is a perennial shrub native from Paraná in Brazil, Paraguay and the Northeast of Argentina. It has been used by Guaraní aborigins as sweetener (1). Nowadays, it is often referred as "the sweet herb of Paraguay", and it is accepted as a natural edulcorant with hypoglycemic properties (2). Extracts of leaves are used as a coadjuvant for the treatment of diabetes.

The leaves of *Stevia rebaudiana* (Bertoni) have a great amount of stevioside, a hydrophilic diterpenoid glycoside which is 300 times sweeter than sugar with a minimal caloric value (3). Gregersen et al. (2) studied the acute effects of stevioside in 12 type-2 diabetic patients included in an acute, paired cross-over study with a meal supplemented with 1 g stevioside in comparison with a control group supplemented with 1 g of maize starch. Stevioside reduced the area under the glucose response curve, but it did not alter the area under the insulin curve, thus, reducing postprandial blood glucose in type 2 diabetic patients (2). Even *Stevia* is used as a dietary supplement in many countries, there were some discrepancies in scientific reports. It was demonstrated that stevioside orally administered to pigs was strongly converted into its aglycon steviol by the bacteria of the colon, which occurs in lower amount in rats (4). In humans, after consuming 750 mg/day, only steviol was present in feces (5) and blood (6). Moreover, it is steviol but not stevioside the one which reduce glucose absorption (7).

Contrarily, it was found that stevioside has itself properties as hypotensor and vasodilator in preclinical and clinical studies (8), as diuretic (9) and bradicardic (10). It has been described that stevioside, as other diterpenic compounds, reduces the vascular resistance by inhibition of calcium influx and release of a vasodilator prostaglandin (11, 12). On the other hand, stevioside showed to be an agonist of peripheral mu-opioid receptors, which contribute to increase glycogen synthesis in liver, without central effects (13). Also, it was described an antiamnesic effect of oral stevioside in rats on the Morris water maze test (14).

In spite of its wide use as a dietary supplement there is only a study on gastrointestinal effects of *Stevia* stems, whose butyl-alcohol fraction of the water extract reduced guinea-pig ileons contractility induced by histamine, acetylcholine and calcium (15). There are not reports about the effects of *Stevia* leaves aqueous extract and of the pure compound stevioside on the gastrointestinal motility and the alert. Then, the aim of this work was to evaluate the effects of the aqueous extract of *Stevia rebaudiana* and its main active principle stevioside on the *in vitro* intestinal contractility of rats and their mechanisms, as well as the effect of the extract on the behavior of mice.

Methods

Plant material: Commercial herboristery samples of *Stevia rebaudiana* leaves were identified in the Museum Carlos Spegazzini (LPE) from the Facultad de Ciencias Exactas, Universidad Nacional de La Plata (UNLP) (voucher LPE-1160). An aqueous crude extract (A.c.e) was prepared by decoction and lyophilization (yield 10%). For the biological protocols, the lyophilized of A.c.e. were diluted and their concentrations expressed in mg lyophilized/ml.

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).

Behavioral effects

Open-field test in mice: The spontaneous locomotion and the exploratory activity of mice were evaluated on the open field, consisted of a 30 x 50 cm white box with walls of 27 cm height divided in 15 squares of 10 cm^2 by black lines. It was placed in a light and sound-attenuated room, and done as previously described (16). Mice were divided in 6 groups, respectively for the following treatments: saline solution (negative control), 1 and 10 mg/kg diazepam (positive control) (Roche, Argentina), and

groups with doses of 40, 100, 450 and 975 mg A.c.e lyophilized/Kg. All drugs were administered by i.p. injections in a volume of 0.1 ml by 10 g of weight. After 30 minutes, each animal was placed in the same corner of the field, and during 5 minutes there were counted the number of crossed lines (CL), rearings (Re), grooming and other signs (16). This rutine was repeated for every mouse at 60, 90 and 120 min of administration and the experiment was repeated until reach a number of experiments between 8 to 9 in each group.

Gastrointestinal effects

Biological preparation and contractile measurements: Sprague-Dawley rats (200-250g) were subjected to a 24 hours fasting with free access to water before experimentation. The animals were full anaesthetized by pentobarbital overdose and then quickly sacrificed by the opening of torax and abdomen. Duodenums and ileons (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 (17, 18, 19). The preparations were equilibrated for at least 45 minutes at 1g of pre-load. Tissues were connected to isometric transducers WPI (USA). The signals of 4 organs were simultaneously amplified by a 4-channels preamplifier (WPI, USA) and acquisited to a computer by Eagle Program.

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 Ca Cl_2

Tyrode-0Ca-40 mM K^+: by adding 0.6 ml KCl 10% to 20 ml Tyrode 0Ca in the chamber.

The DRC were done with acetylcholine bromide (ACh, Sigma, USA), which was diluted in water to obtain solutions of 1, 2, 7, 20, 70, 200, 700 and 1000 µg/mL which were cumulatively added to the chamber. The DRC to Ca were done by preparing $CaCl_2$.anhydro (Mallinckrodt, Germany) at concentrations of 0.882, 1.764, 5.3, 18.5, 53, 185.22 and 530 mg/mL which were cumulatively added to the chamber. The stevioside (Stvd) was a generous gift of Tanki S.A. (Argentina) and diluted in water at 10, 30, 100 and 300 mg/mL. All the solutions were added at 0.2 ml in the chamber with 20 ml of Tyrode, and then there were 1/100 diluted.

***Dose-response curves to acetylcholine*:** Dose-response curves (DRC) to acetylcholine (ACh) were done after a stabilization of intestine portions during about 45 minutes, at least two up to stabilization. Previous tests in the laboratory demonstrated that the DRC of both intestinal portions were not significantly different. The ACh concentrations were cumulatively added to the bath (to reach from 0.01 to 10 g/mL) in the absence (control DRC) and the presence of a unique concentration of either A.c.e or Stv, which was added 5 min before the DRC and remained during it in the bath. A growing order of A.c.e. or Stv concentrations was used for making the several DRC-Ach in each organ.

Dose-response curves to $CaCl_2$: After stabilization during 45 min in Tyrode and testing the muscle response with ACh, the external Ca^{+2} was eliminated with Tyrode-0Ca. To do the DRC of Ca^{2+}, the muscle was depolarized with Tyrode-0Ca-40 mM K^+ and there were cumulatively added the successive aliquots of $CaCl_2$ solutions to reach concentrations from 0.0195 to 17.5 mmol/L. At least two DRC-Ca^{2+} were done as control, while the following DRC were done in the presence of one concentration of the respective A.c.e. or Stv, in growing order of concentrations. These solutions were added 5 min before the depolarization with high-K^+ in Tyrode-0Ca and remained in the bath during the DRC.

***Pharmacological and statistical analysis*:** From the DRC there were calculated the pD_2 of the agonist (as –log EC50, in molar) (20) Van der Brink, 1977).

For the extract and Stv, the pattern of DRC with reduction in the maximal effect (E_{max}) suggested that they act as non-competitive antagonists (20). The inhibitory concentration to 50% (IC50) of extracts was calculated by extrapolating to 50% the individual inhibition curves, which were obtained by plotting E_{max} of the agonist from the respective DRC curves vs. [extract], and expressed as mg lyophilized by mL. Also IC50 from Stvd CDR was calculated and expressed as mg by mL. All results are expressed as media ± SEM. Regression of DRC were done by using Graph Pad Prism 4.0 program.

The comparison of DRC with A.c.e or Stvd versus non-treated DRC (control) from the same preparation were done by two-way ANOVA with the following variables: treatment and log [agonist] (being the agonist either, Ach or Ca^{2+}) followed by "a posteriori" Bonferroni paired tests, always with a level of significance of $p<0.05$. For statistical analysis it was used the program Graph Pad Prisma v. 4.0.

Results

Effects of Stevia rebaudiana on mice in the open field test

Table 1 shows that the A.c.e. of *Stevia rebaudiana* reduced the number of crossed lines (CL) (spontaneous locomotion) at 30 min with the four doses assessed, 40, 100, 450 and 975 mg lyoph/Kg, respectively (*$p<0.05$ vs saline group). It also reduced the number of rearings (Re) but only at the doses of 450 and 975 mg lyoph/Kg, respectively (*$p<0.05$ vs saline group). Both effects were reversed after 30 min, remaining only the effects in CL and Re until 60 min at 975 mg lyoph/Kg. Diazepam at 1 mg/Kg did not change CL nor Re, but at 10 mg/Kg reduced both.

see Table 1.

see Table 2.

Gastrointestinal effects

Figure 1 shows that the A.c.e. of *Stevia rebaudiana* at low concentrations (0.1 and 0.3 mg lyophilized/mL) was synergistic with Ach, but at higher concentrations (1 and 3 mg lyophilized/mL) it inhibited in a non-competitive way the DRC of acetylcholine (ACh-DRC, pD_2 of 5.67 ± 0.10, n=10 for all experiments) to almost complete blockade (5.6 ± 1.4% of the E_{max}) at 3 mg/ml. The IC50 of *Stevia rebaudiana* was 1.22 ± 0.16 mg lyophilized/ml, n=6. Figure 2 shows that stevioside (Stvd) also inhibited the ACh-DRC to 17.4 ± 4.6% E_{max} with an IC50 of 0.84 ± 0.22 mg/ml (n=4).

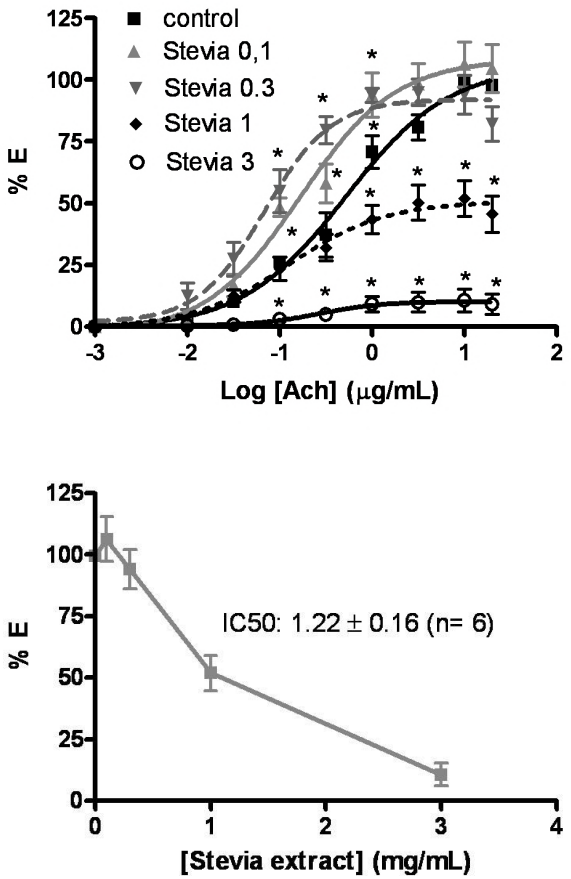

Figure 1: Effects of the aqueous extract of Stevia rebaudiana on the DRC of acetylcholine (pD_2: 5.48 ±0.12, n= 6), and inhibition curve (inset) to extrapolate the IC50 of A.c.e (1.22 ± 0.16 mg lyophilized/mL). Results as mean ± SEM. A.c.e concentrations (mg lyophilized/mL) are in labels. Two-way ANOVA for (a): by treatment: F=173.6, df 4, $p<0.0001$, by concentration: F: 151.3, df 8, $p<0.0001$, post-tests: *$p<0.05$ vs control.

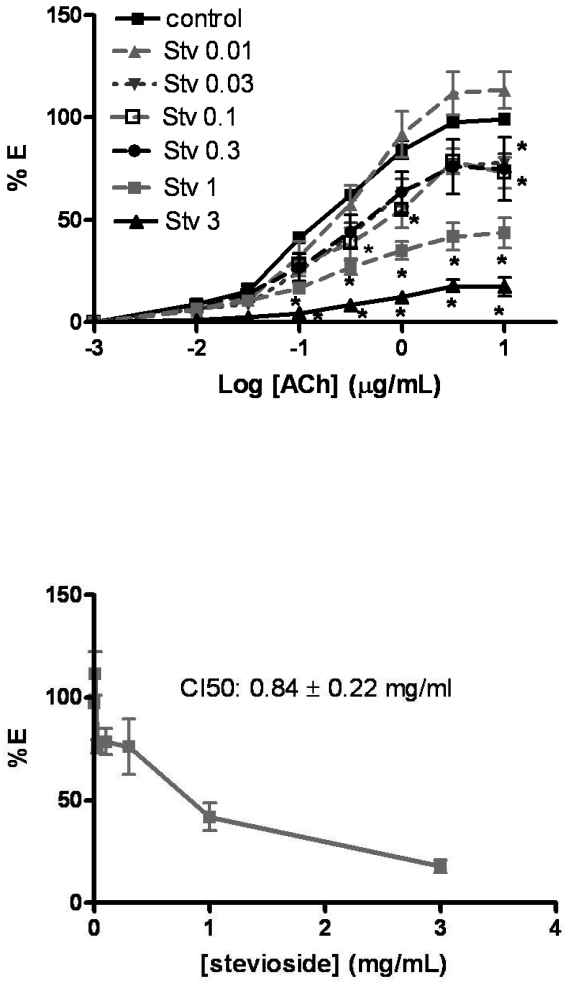

Figure 2: Effects of stevioside (Stv) on DRC of acetylcholine (pD_2: 5.95 ± 0.06, n= 4), and inhibition curve (inset) to extrapolate the IC50 of Stv (0.84 ± 0.22 mg lyophilized/mL). Results as mean ± SEM. Concentrations of stevioside (mg/mL) are in labels. Two-way ANOVA for (a): by treatment: F=62.74, df 6, p<0.0001, by concentration: F: 188.3, df 7, p<0.0001, post-tests: *p<0.05 vs control.

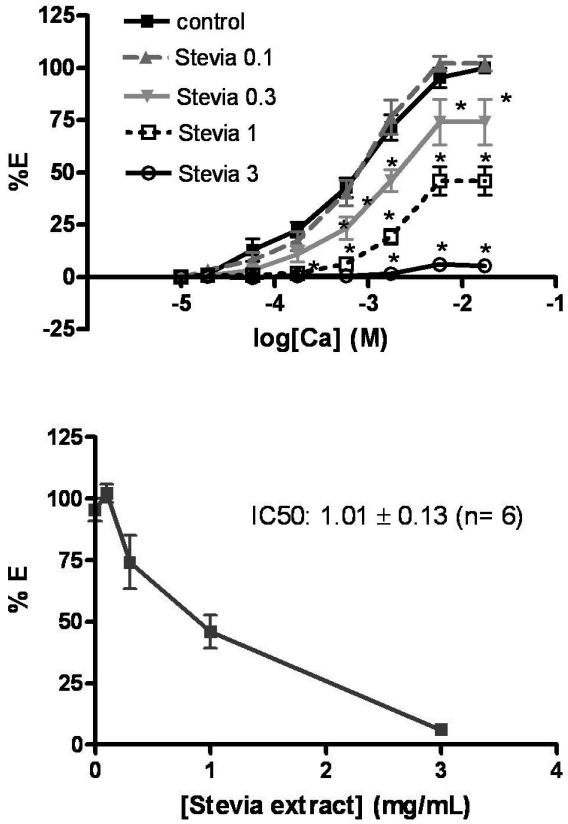

Figure 3: Effects of the aqueous extract of Stevia rebaudiana on the DRC of calcium in high-K Tyrode medium, and inhibition curve (inset) to extrapolate the IC50 of A.c.e (1.01 ± 0.13 mg/mL). Results as mean ± SEM. A.c.e concentrations (mg lyophilized/mL) in labels. Two-way ANOVA for (a): by treatment: F= 148.3, df 4, p<0.0001, by concentration: F: 212.4, df 7, p<0.0001. Post-tests: *p<0.05 vs control.

In order to study the mechanism of the interference with the ACh-DRC, there were evaluated the effects of *Stevia* and stevioside on the Ca^{2+}-DRC (pD_2: 3.44 ± 0.14, n=10 for all experiments). The A.c.e-*Stevia* completely inhibited the Ca^{2+}-DRC in a non-competitive way with an IC50 of 1.01 ± 0.13 mg lyophilized/mL (n=6), while the Stvd also non-competitively blocked the Ca^{2+}-DRC, with an IC50 of 1.73 ± 0.47 mg/mL (n=4).

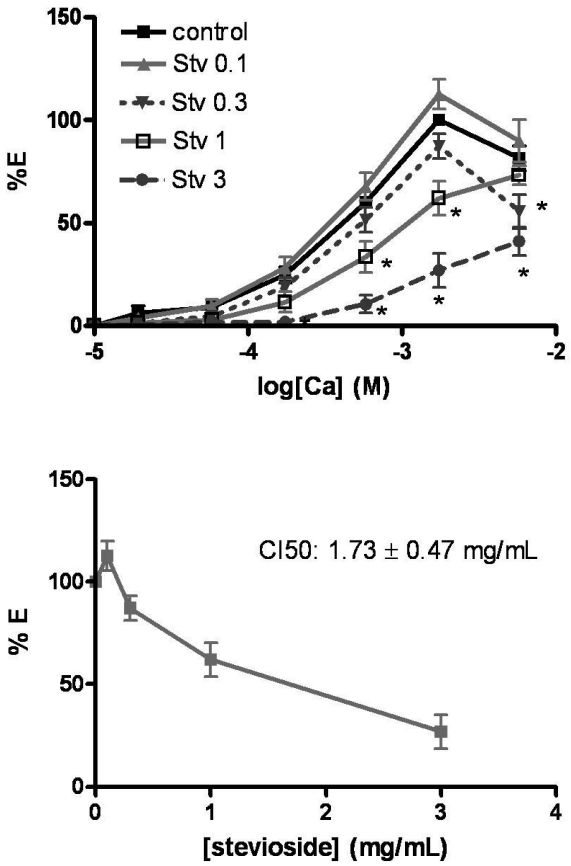

Figure 4: Effects of the stevioside (Stv) on the DRC of calcium in high-K Tyrode medium (pD_2: 3.89 ± 0.04), and inhibition curve (inset) to extrapolate the IC50 of Stv (1.73±0.47 mg/mL). Results as mean ± SEM. Concentrations of stevioside (mg/mL) are in labels. Two-way ANOVA for (a): by treatment: F=50.87, df 3, p<0.0001, by concentration: F: 23.51, df 8, p<0.0001, post-tests: *p<0.05 vs control.

Discussion

The results show that the A.c.e of *Stevia rebaudiana.* has effect as intestinal antispasmodic, which is due to a non-competitive inhibition on the cholinergic contraction at concentrations of 1 to 3 mg lyophilized/mL. This effect agrees with the previously described inhibition of the guinea-pig ileon contractions dependent on acethylcholine, histamine ans calcium reported for the butyl-alcohol fraction of the water extract of *Stevia* stems (15). Our present results add several new findings. First, the demonstration that *Stevia* produces a non-competitive interaction with ACh, which is due to a non-competitive blockade of the calcium influx, both by means of the DRC. Another finding is that stevioside is one of the compounds responsible for these effects on ACh and Ca^{2+}-dependent contractility. Finally, that the leaves of *Stevia*, which are more frequently used as sweetener than the stem, have utility as eupeptic.

Regarding *Stevia rebaudiana* leaves extract, our results show that at low concentrations (0.1 to 0.3 mg lyophilized/mL) *Stevia* produced a synergism with Ach, which can be atributed to a different component of the extract, and could contribute to improve peristaltism as a good eupeptic agent. At higher concentrations (1 to 3 mg lyophilized/mL) the extract of *Stevia rebaudiana* inhibited the ACh-DRC in a non-competitive way, reducing the E_{max} , with an IC50 of 1.22 ± 0.16 mg lyophilized/mL. This reduction in the cholinergic contractility may be explained by the blockade of Ca influx, since the extract also inhibited in a non-competitive way the DRC-Ca in depolarizing media at the same concentration range (IC50 of 1.01 ± 0.13 mg lyophilized/mL) until complete blockade of the E_{max}.

Regarding the pure compound stevioside, the results show that it is a non-competitive inhibitor of both, ACh and Ca^{2+} influx in the thin intestine of rat. The IC50 of the pure substance stevioside (1.73 ± 0.47 mg/mL) on the Ca^{2+}-DRC was a few higher than that on the ACh-DRC (IC50 of 0.84 ± 0.22 mg/mL). Both, A.c.e. and stevioside almost completely inhibited the E_{max} of ACh-DRC, but only the extract did the same with the E_{max} of Ca-DRC. This suggests that stevioside could have another mechanism of inhibition of ACh effect in addition to Ca-influx blockade, even when it is the most important mechanism. Regarding this, it is not a competitive blocker of L-channels as dihydropiridines are, but a non-competitive blocker. This result agrees with that described in the cardiovascular system for *Stevia* and stevioside as hypotensive, diuretic and natriuretic (8, 9, 11, 12), since both vascular and intestinal tones depend on Ca-influx to smooth muscle cells. As in vascular system, the effects of *Stevia* on intestine were directly produced by the stevioside instead of the metabolite steviol. [4,6,7]

The A.c.e of *Stevia* induced a reduction of spontaneous locomotion and exploration of mice in the open-field test. It was of short duration at really high doses of extract in mice (from 40 to 975 mg lyophilized/Kg). The fact that there were needed high i.p. doses to obtain sedation agree with the work which showed that it is difficult for the stevioside to enter the brain (21).

Considering the human dose, the 1 gram stevioside/day used in diabetic patients (2) would produce the plasmatic concentration of about 0.33 mg stevioside/mL. Then, in comparison with our results, it would be expected a slight inhibition of peristaltism at this dose. In contrast, if *Stevia* is used, about 10 grams leaves would be needed to produce the same effect, since they contain about 10% stevioside. But, if consuming 1 gram leaves it will be expected a stimulation of peristaltism, like the effect seen at about 0.1-0.3 mg lyophilized/mL (10% yield of lyophilized *Stevia*). Moreover, there is low possibility that the sedative effects that we observed in mice at 40 to 975 mg lyophilized/Kg were seen in humans, since these doses would be equivalent to about 0.4 to 9.75 grams of *Stevia* leaves, although it is important to consider differences in sensitivity of animal species and human individuals.

In summary, both *Stevia* extract and stevioside produce antispasmodic effects due to Ca^{2+} influx non-competitive inhibition on smooth muscle. Even stevioside convers to steviol in blood, the oral administration may concentrate stevioside in the intestine and then, the antispasmodic effect would occur when they are used as a dietetic supplement or as hypoglycemic coadjuvant. Contrarily, the sedative effect may be less expected at therapeutic doses, depending on the individual sensitivity.

Acknowledgments

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Treatment	30 min	60 min	90 min	120 min
Saline	63.78±7.68	32.78±4.47	27.78±6.86	26.22±3.11
Stevia 40 mg/Kg	19.90±9.31*	25.40±5.82	18.70± 4.29	16.30± 4.59
Stevia 100 mg/Kg	28.50± 11.51*	19.30± 8.07	24.00± 6.35	15.30± 5.47
Stevia 450 mg/Kg	19.67± 7.23*	11.11± 4.92	12.89±5.07	8.22±3.75
Stevia 975 mg/Kg	8.62±5.44*	4.62±3.93*	4.87±4.45	8.00±5.24
Diazepam 1 mg/Kg	45.12±10.09	30.37±12.05	25.25±10.25	27.12± 9.15
Diazepam 10 mg/Kg	10.10±1.29*	10.50±3.61	5.70±1.87	2.70±0.37*

Table 1: Effects of the aqueous extract of Stevia rebaudiana on the locomotion (as number of crossed lines by 5 min) of mice in the open-field test.

Two-way ANOVA: by treatment F= 13, df 6, p<0.0001; by time F= 5.46 df 3, p= 0.0012, n= 9 to 10 by group; *p<0.05 by a posteriori Bonferroni test vs Saline group at the respective time

Treatment	30 min	60 min	90 min	120 min
Saline	10.25±2.33	7.62±2.10	6.62±2.39	5.25±1.29
Stevia 40 mg/Kg	3.50±1.63	5.00±2.13	5.20±1.22	5.00±1.46
Stevia 100 mg/Kg	5.70±3.01	6.30±2.60	8.00±3.00	6.20±2.47
Stevia 450 mg/Kg	1.22±0.49*	2.44±1.97	2.00±1.21	1.67±0.97
Stevia 975 mg/Kg	0.37±0.18*	0.37±0.18*	1.50±1.50	1.50±1.50
Diazepam 1 mg/Kg	11.50±3.83	7.00±2.28	5.12±2.12	9.12±2.99
Diazepam 10 mg/Kg	2.60±0.40*	2.50±0.34	1.70±0.30	0.70±0.26

Table 2: Effects of the aqueous extract of Stevia rebaudiana on the exploration (number of rearings) of mice in the open-field test.

Two-way ANOVA: by treatment F= 9.366, df 6, p<0.0001; by time F= 0.255 df 3, p= 0.8577, n= 9 to 10 by group; *p<0.05 by a posteriori Bonferroni test vs Saline group at the respective time.