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Antispasmodic effects of *Mikania micrantha* Kunth and dual gastrointestinal effect of *Mikania cordifolia* (L.F.) Willd (asteraceae) on isolated rat thin intestine

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Summary

Gastrointestinal effects and the phytochemical profile of two species of the genus *Mikania* Willd. (Asteraceae) were studied. *Mikania micrantha* Kunth. and *Mikania cordifolia* (L. f.) Willd., known as "guaco" were collected from the jungle of Punta Lara and Martín García Island, ecosystem of Buenos Aires, Argentina. The aqueous extracts of both (AE-M.m and AE-M.c, respectively, prepared by decoction at 20% and lyophilized) were added to isolated rat intestines, before evoking contraction by dose-response curves (DRC) of acethylcholine (ACh). The AE-M.m inhibited in a non-competitive way the ACh-DRC (IC50: 0.54±0.05 mg/mL), as well as the Calcium-DRC (Ca-DRC) in high-[K^+]. The AE-M.c produced a dual effect on the ACh-DRC with synergism at low [ACh] and antagonism at high [ACh] (IC50: 2.04±0.06 mg/mL), and was a partial agonist (EC50: 4.17 mg/mL) blocked by atropine. The preliminary phytochemical tests showed the presence of flavonoids in both, AE-M.m and AE-M.c, but alkaloids only in AE-M.c. The TLC detected flavonoids (with EthylAcetate/MeOH/H_2O 100:13:10 and EthylAcetate/FormicAcid/ AcOH/H_2O 100:11:11:26) and alkaloids (with tholuene/AcOEt/diethylamine 70:20:10).

Conclusions: results validate the popular use of M.m and M.c as eupeptic, with antispasmodic effect associated to a non-competitive Ca^{2+}-influx blockade typical of flavonoids. Also, M.c has an additional prokinetic effect due to muscarinic agonism.

KEY WORDS: *MIKANIA MICRANTHA, MIKANIA CORDIFOLIA,* ANTISPASMODIC, PROKINETIC, CA-ANTAGONIST, RAT INTESTINE, FLAVONOIDS, ALKALOIDS

Introduction

The genus *Mikania* Willd., family *Asteraceae*, tribu *Eupatorieae*, includes near 430 species of perennial creeping climber and is distributed mostly in Brasil, Argentina, Paraguay, Uruguay, Bolivia, Perú, Venezuela and Colombia (1,2,3). In Argentina there is a Natural Reservation on the Rio de La Plata river in Buenos Aires province, Punta Lara and Martín García Island, in which two plants of this genus *M. cordifolia* (L. f.) Willd. and *M. micrantha* Kunth spontaneously grow (4). They are popularly known as "guaco", and it is recognized that have several medicinal properties (4,5,6).

Mikania cordifolia has been used to treat respiratory illnesses (7) and it was used by the aborigines from American jungles to treat snake bites from ancient times (8). Also, it was cited as antirheumatic, antiinflammatory, analgesic and used as bath for venereal illnesses (9). *M. micrantha* was used as alexiteric, antirheumatic, antispasmodic, diaforetic, antiinflammatory, expectorant and broncodilator, with frequent and popular use in Brasil (10,11).

Both species are herbal creeper, while *M. cordifolia* has six ribs pubescents stems and *M. micrantha* has lobulated and glabrous stems. The leaves are opposite, peciolated, heart-shaped and pubescent for *M. cordifolia* but glabrous for *M. micrantha*. They have white flowers associated in chapters and fruits as cypselas with pappus.

In Brasil there are also other species of *Mikania* also popularly known as "guacos" (*M. glomerata* and *M. laevigata*) which are used to treat respiratory diseases in traditional medicine. Both species are included in the Brazilian Farmacopea, and used in several commercial medicines with other phytotherapic products. In the brazilian species *M. glomerata* was described the presence of cumarins as well as an antispasmodic effect (12).

The purpose of this work was to study whether *M. micrantha* and *M. cordifolia* have antispasmodic effect and which types of active principles have.

Materials and methods

Plant material

Stems and leaves of *Mikania cordifolia* (L. f.) Willd were collected in the Martín García Island (12-XII-2009, *Colares & Martínez 54* LPAG), and *M. micrantha* Kunth was collected in the Natural Reservation of Punta Lara (22-XI-2008, *Colares 50* LPAG), both in the Province of Buenos Aires. Their samples were kept in the Herbarium LPAG, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata (UNLP), Argentina.

A- Pharmacological studies

Extracts preparation

The plant material was dried at 40°C. Aqueous extracts (AE) were prepared by boiling 20 grams dried leaves in 100 mL distilled water for 20 min, as a decoction. After filtration the respective decoction was lyophilized, obtaining a 7% 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 (to final concentrations of 0.3, 1 and 3 mg/ml). With this procedure, the essential oil is not included in the lyophilized sample.

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), and was approbed by the local ethical comitee.

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 ileums (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 (13,14,15). The preparations were equilibrated for at least 45 minutes at 1g of pre-load. Tissues were connected to an isometric transducer 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 drugs employed in biological tests were: Acetylcholine bromide (ACh, Sigma, USA) and atropine sulphate (Sigma, USA), which were diluted in water.

Protocols

Dose-response curves to acetylcholine:

Dose-response curves (DRC) to acetylcholine (ACh) were done for the rat duodenums and ileums after a stabilization of 45 minutes, at least two up to stabilization. Previous tests in the lab 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 the respective AE, which was added 5 min before the DRC and remained during it in the bath. A growing order of AE concentrations was used for making the several DRC-ACh in each organ.

Dose-response curves to M. cordifolia extract

In a different group of muscles, two DRC to ACh were done as previously described and then a DRC of *Mikania cordifolia* extract was done by cumulatively adding to the bath the following concentrations: 0.1, 0.3, 1, 3, 5, 7, 10 mg/mL, first in the absence and then in the presence of a unique concentration of the antagonist atropine at 0.1 µg/mL added 5 min before the respective DRC.

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 AE, in growing order. The AE was 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) (16). For the extracts, the pattern of DRC suggested whether they act as competitive agonist, or non-competitive antagonist (16,17). The inhibitory concentration to 50% (IC50) of extracts was calculated by extrapolating to 50% the individual inhibition curves, which were obtained by plotting the maximal effect (E_{max}) of the agonist from the respective DRC curves vs. [extract], and expressed as mg lyophilized by mL. All results are expressed as media ± SEM. Regression of DRC and statistics were done by using Graph Pad Prism 4.0 program, and by applying two-

way ANOVA test for multiple comparisons (treatments and concentrations) followed by *a posteriori* Bonferroni tests. In all tests it was considered a significance of $p<0.05$.

B- Phytochemical studies

Preliminary chemical tests

The aqueous extract of the plants was lyophilized and yielded 6.23% for *M. cordifolia and* 5.89% for *M. micrantha (as* g of extract from 100 g crude drug). Samples of the aqueous extract of both species of *Mikania* were used to evidence the presence of phytochemical groups, as was previously described (18).

a) Characterization of flavonoids: with the Shinoda reaction, the reaction with 2% $FeCl_3$ in EtOH and visible observation; reaction with 2% $AlCl_3$ in EtOH and visible and UV366 observation; reaction with 2% boric acid in EtOH and visible and UV366 observation; and reaction with 1% KOH in EtOH and visible and UV366 observation.

b) Characterization of alkaloids: it was done with Dragendorff reactive (iodine-potassium bismutite), Mayer-Valser reactive (aqueous solution of potassium tetraiodomercurate), Bouchardat reactive (aqueous solution of iodine in potassium iodure), Marmé reactive (cadmium iodure in aqueous solution of iodum in potassium iodure); reaction with 1% picric acid; reaction with 1% tannic acid; reaction with 10% sulphuric acid.

TLC analysis

As previously described (19) the presence of flavonoids and alkaloids in both species of *Mikania* was evaluated by thin-layer chromatography (TLC), on silica gel 60 GF 254 0.25 mm (Merck, Germany) and three systems of mobile phase: 1) EtOAc/MetH/AcH/H_2O (100:11:11:26) and 2) EtOAc/MeOH/H_2O (100:13:10) for flavonoids, and 3) tholuene/AcOEt/diethylamine (70:20:10) for alkaloids. As standards there were used 1% methanolic solution of kaemferol (Sigma) and rutin (Sigma) for flavonoids and pylocarpine clorhidrate (Merck) for alkaloids. The detection of bands of flavonoids was performed under UV light (254 and 366 nm) with and without ammonia clouds, and with natural product spray (1% methanol solution of 2-amino ethyl-diphenil-boric acid) and it was observed under visible and UV light, and with Dragendorf reactive and visible observation. The lyophilized 20% aqueous extracts of aerial parts of both species of *Mikania* were run.

Results

A- Pharmacological studies

Effects of Mikania micranta

The extract of *M. micranta* (AE-M.m) reduced the maximal effect (Emax) of the ACh- DRC on a dose-dependent way up to total blockade (Fig. 1), suggesting a complete non-competitive antagonism on the cholinergic contraction. The pD_2 of ACh was 6.66 ± 0.28 and the extrapolated IC50 of AE-M.m was 0.54 ± 0.05 mg lyophilized/mL (Fig. 1inset).

In order to evaluate whether the non-competitive antagonism of A.E-M.m was associated to inhibition of Ca^{2+} influx to the smooth muscle, the DRC of Ca^{+2} were done on high-K^+-medium to activate the channels by depolarization. Figure 2 shows that A.E-M.m also produced a non-competitive inhibition of Ca^{+2} influx up to complete blockade. The extrapolated IC50 was 0.51 0.06 mg/mL, which is similar to the IC50 of A.E-M.m. on the ACh-DRC.

see Fig. 1

see Fig. 2

Effects of Mikania cordifolia

The extract of *Mikania cordifolia* (AE-M.c) caused a dual effect on the ACh DRC: at low [ACh]o it developed an agonistic effect, while at high [ACh]o it was inhibited (Fig. 3). The extrapolated IC50 of

AE-M.c was 2.04 ± 0.06 mg lyophilized/mL (Fig. 3 inset).

see Fig. 3

In order to evaluate whether *Mikania cordifolia* acts as a muscarinic agonist, the DRC of AE-M.c was done after the ACh-DRC for comparison in the same muscle. Fig. 4 shows that AE-M.c developed a dose-dependent contraction as a partial agonist, with Emax of 59.1 ± 8.8% of the maximal effect of ACh and EC50 of 4168 ± 1.94 µg/mL. This EC50 resulted considerably higher than that of ACh (0.14 ± 0.016 µg/mL). In the presence of atropine 0.1 µg/mL the effect of *M. cordifolia* was completely inhibited in the assessed range of [AE-M.c], suggesting that the effect was due to a muscarinic agonism (Fig. 4).

see Fig. 4

B. Phytochemical tests

The Table 1 shows the preliminary chemical tests, in which the Shinoda reactive and those with 2% $FeCl_3$, 2% $AlCl_3$, 2% boric acid and 1% KOH gave positive in different degrees for both species of *Mikania*. The general reactions for alkaloids were also positive with Dragendorff reactive, picric acid and tannic acid for the extract of *M. cordifolia* (Table 1).

see Table 1.

TLC results

Table 2 shows the results obtained with TLC for detecting the presence of flavonoids in the systems of higher resolution. In the system 1, AcOEt/AcOH/MetH/H_2O (100:11:11:26), the aqueous extract of *M. cordifolia* yielded 9 bands after UV366 and natural products reaction, 7 of which had R_f between 0 and 0.51, and three of them (R_f 0.28, 0.35 and 0.44) developed blue-clair fluorescence, as well as those at R_f 0.91 and 0.96. The aqueous extract of *M. micrantha* exhibited 8 bands in the system 1, with R_f and colour fluorescence in a different pattern to that of *M. cordifolia*. The TLC exhibited 4 main bands in the first half, 3 of which were similar to those of *M. cordifolia*. In the second half, there were 2 bands of blue fluorescence which were not present in *M. cordifolia*, as well as the common bands at R_f 0.91 and 0.96 (Table 2). The extracts did not exhibit any band similar to those found with the reference substances rutin and kaemferol.

In the system 2 (EtOAc/MeOH/H_2O 100:13:10), the aqueous extract of *M. cordifolia* developed 9 bands, from which the main were two nearer to the origin with strong orange fluorescence, and another with R_f 0.80 and blue fluorescence at UV366 after reaction with natural products (Table 2). In this system, *M. micrantha* exhibits a different pattern with 10 bands, 9 of which were in the first 2/3 of the plate. One of the first 4 bands (R_f 0.15) with yellow-green fluorescence at UV 366, and another band with blue fluorescence at R_f 0.51 were not present in *M. cordifolia*, while the 4 bands between R_f 0.55 to 0.76 did not appear in *M. micrantha* (Table 2).

The TLC for evaluating the presence of alkaloids were run with the system 3 (tholuene/AcOEt/diethylamine 70:20:10), with pylocarpine as reference substance. The TLC of the aqueous extract of *M. micrantha* did not exhibit any substance able to react with Dragendorf reactive, while *M. cordifolia* showed an only band at Rf ~0.30) while the standard of pylocarpine gave a Rf of 0.20.

Discussion

Results showed that aqueous crude extracts of *Mikania micrantha* and *Mikania cordifolia* have effect on the gastrointestinal system, but with differences among them, as well as in the preliminar phytochemical profile. The AE-M.m was antispasmodic with IC50 of 0.54 mg/mL, while the reduction of Emax indicated a non-competitive inhibition of the ACh effect (Fig. 1). This inhibition can be associated to the blockade of calcium L-channels, since the AE-M.m also inhibited in a complete non-competitive manner the DRC-Ca^{2+} with a similar IC50 (0.51 mg/mL) (Fig. 2).

On the other hand, *Mikania cordifolia* exhibited a

dual effect with properties as partial agonist of the muscarinic effect and non-competitive antagonist (Fig. 3). It may be due to the presence of different active principles in the extract. The agonistic effect was evidenced by the "running to the right" of its DRC in the presence of atropine (Fig. 4). It was a partial agonist, since it reached an Emax of only about 57% of the ACh maximal effect, and had a lower potency than ACh (about 1/29700). At higher [ACh]o, the AE-M.c. induced a non-competitive antagonism to ACh, as well as the AE-M.m. did. Nevertheless, this effect appears at 4 times higher concentration in AE-M.c. than in AE-M.m., since the IC50 for AE-M.c. was about 2 mg/mL. Then, *Mikania micrantha* would be 4 times more potent than *Mikania cordifolia* as antispasmodic. But, at least two compounds have gastrointestinal activity in the AE of *M. cordifolia*, with opposite effects. They could modulate and regulate the intestinal peristaltism depending on the basal cholinergic degree of stimulation: at low cholinergic tone as a partial agonist, but at high cholinergic stimulation as a non-competitive antagonist.

see Table 2.

In the phytochemical profile, the positive reactions of Shinoda and ethanolic solutions of 2% Cl_3Al, Cl_3Fe, H_3BO_3 and KOH suggested the presence of flavonoids, mostly as heterosides since they were found in the aqueous crude extract. This presence agrees with that reported by Carollo for this genus (2008). The patterns of colour and fluorescence at UV366 agree with that expected to flavonoids with at least two adjacents phenolic –OH (positive with Cl_3Al, Cl_3Fe, KOH) and the presence of C=O in 4 position associated with an –OH in C5 of the flavonoid nucleous (reaction with H_3BO_3) (Domínguez, 1973).

Nevertheless, the TLC profiles were different between both species. According to Wagner and Bladt (20) the bands with blue-clair fluorescence developed by *M. micrantha* at Rf 0.28, 0.35 and 0.44, as well as those at Rf 0.91 and 0.96 (in system 1) are typical of extracts with flavonoids containing phenol-carboxylic acids, such as caffeic and chlorogenic acids or caffeoilkinic derivatives. Some of these blue bands also appeared in the A.E. of *M. cordifolia* (Rf 0.28, 0.44 and 0.96 in system 1), but it has other blue bands at 0.60 and 0.77, and a different pattern in system 2 (Rf 0.74 in EA-M.c vs 0.51 in EA-M.m), suggesting also the presence of flavonoids but as different compounds. The presence of flavonoids in both species could be associated to their common effects as non-competitive antagonists of ACh and Ca^{2+}, since these activities were frequently found in flavonoids (21,22,23,14).

Only in *M. cordifolia* the phytochemical tests with Dragendorff reactive and picric and tannic acids were positive, which suggest the presence of alkaloids. This type of compounds could explain the agonistic effect of the AE-M.c since this activity was only found in natural products as alkaloids, such as pylocarpine and muscarine. Nevertheless, there was not found the presence of pylocarpine by TLC.

In summary, our results are in agreement with those found for *M. glomerata* in Brazil (12) regarding the antispasmodic effects of both species of *Mikania*. Nevertheless, the differences in pharmacological mechanisms seen in the argentinian species presently and previously described (24), give them important therapeutic properties. Our results suggest the possibility of *M. cordifolia* to act as prokinetic or antispasmodic depending on the cholinergic tone. This property could be applied in the common bowel irritant syndrome, and then has a great importance in phytotherapy.

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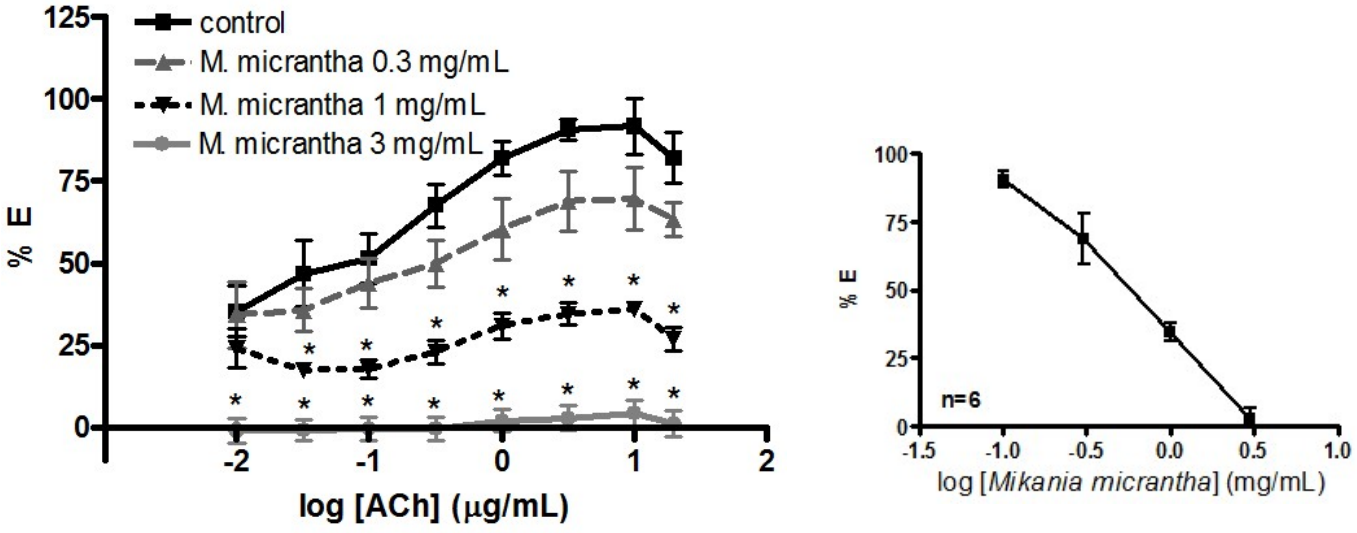

Figure 1: Effects of the aqueous extract of *Mikania micrantha* on the dose-response curves of acetylcholine (pD_2: 6.66±0.28), and inhibition curve (inset) to extrapolate the IC50 of EA-M.m (0.54±0.05 mg/mL). Results as mean±SEM. Concentrations of AE (mg lyophilized/mL) in labels. Two-way ANOVA for (a): by treatment: F=193.8, df 3, p<0.0001, by concentration: F: 13.06, df 7, p<0.0001, post-tests: $^*p$<0.05 vs control

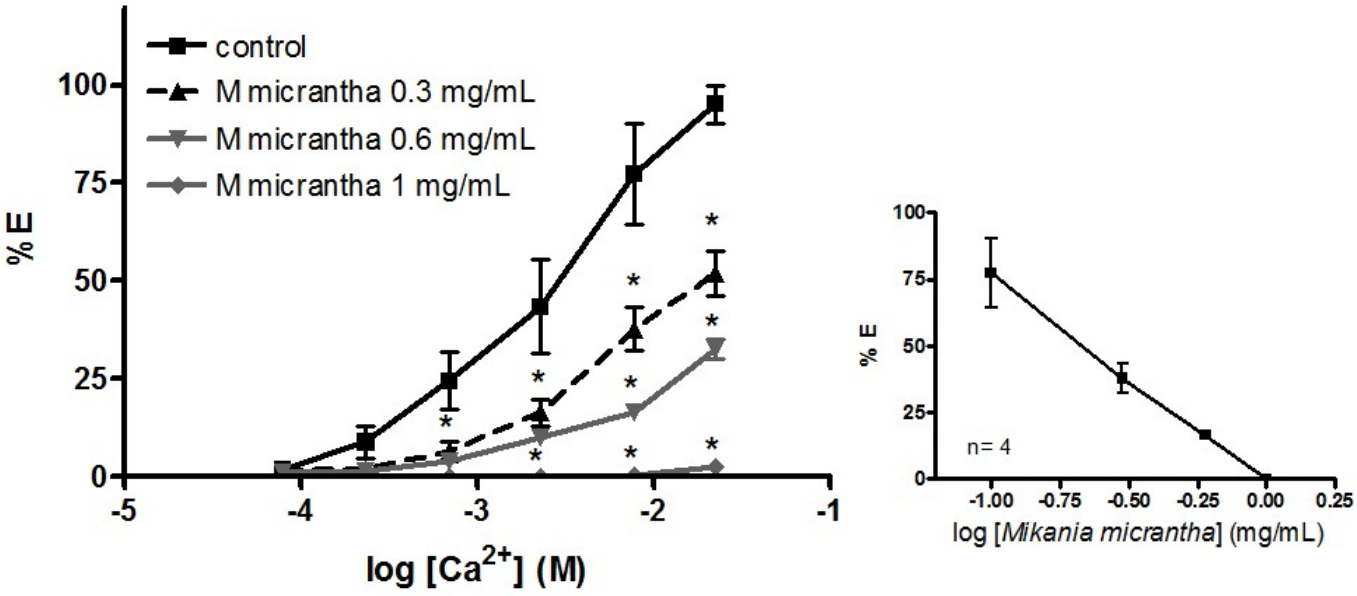

Figure 2: Effects of the aqueous extract of *Mikania micrantha* on the dose-response curves of calcium in high-K Tyrode medium, and inhibition curve (inset) to extrapolate the IC50 of EA-M.m (0.51±0.06 mg/mL). Results as mean±SEM. Concentrations of AE (mg lyophilized/mL) in labels. Two-way ANOVA for (a): by treatment: F=89.12, df 3, p<0.0001, by concentration: F: 60.01, df 5, p<0.0001. Post-tests: $^*p$<0.05 vs control.

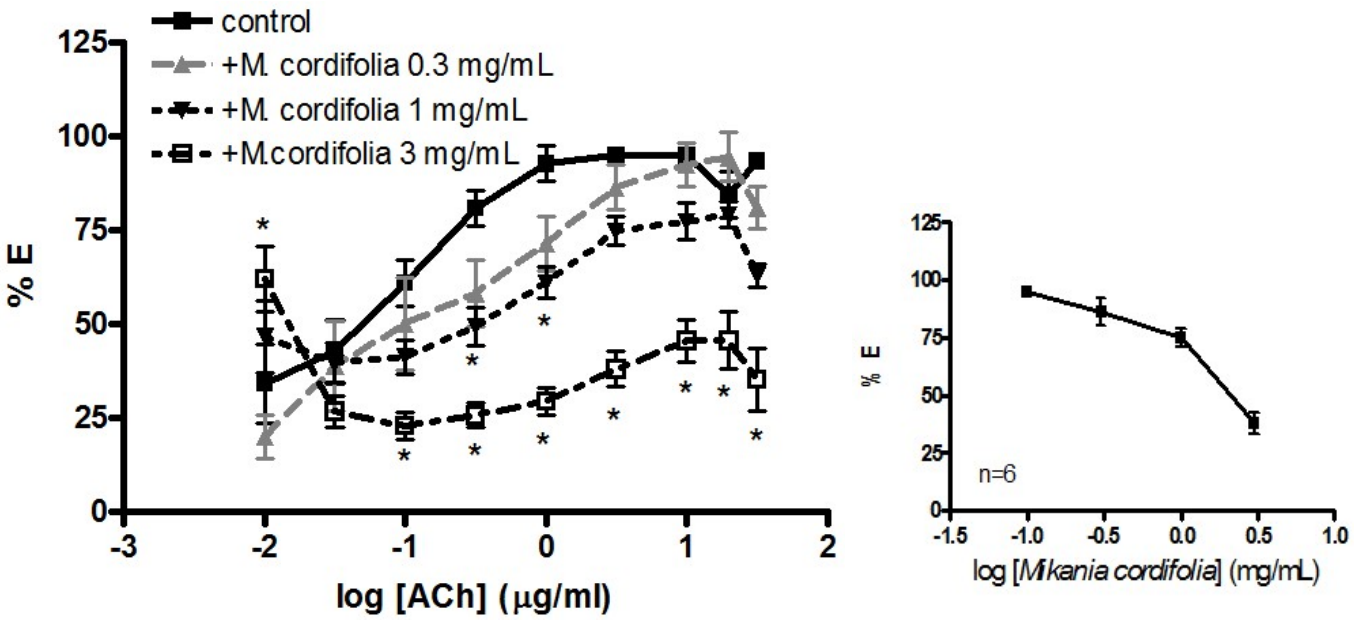

Figure 3: Effects of the aqueous extract of *Mikania cordifolia* on the dose-response curves of acetylcholine (pD_2: 6.34±0.16), and inhibition curve (inset) to extrapolate the IC50 of EA-M.m (2.04±0.06 mg/mL). Results as mean±SEM. Concentrations of AE (mg lyophilized/mL) in labels. Two-way ANOVA for (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.

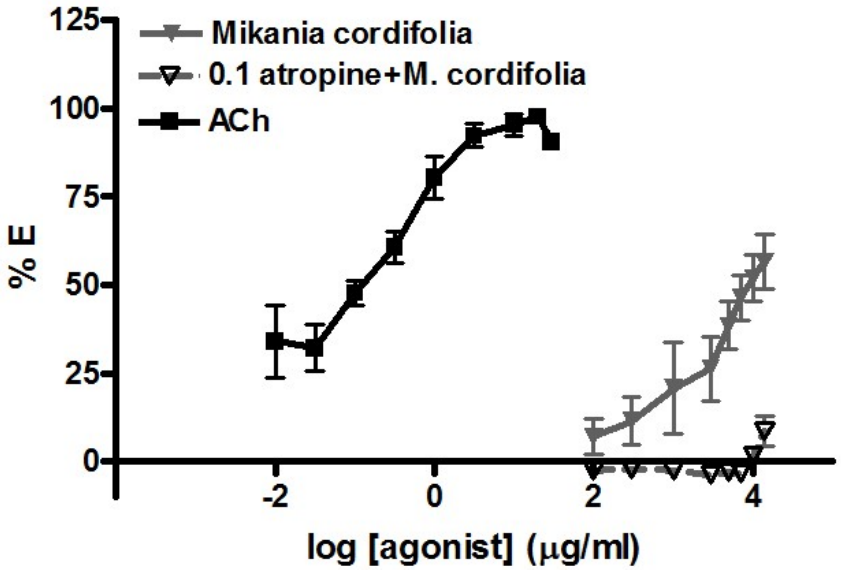

Figure 4: Dose-response curve of *Mikania cordifolia* (ED50: 4168±1.94 µg/mL) in comparison with that of acetylcholine (EC50: 0.15±0.016 µg/mL). Note that 0.1 µg/mL atropine inhibited the DRC of *Mikania cordifolia* running it to the right in the concentration range in which the AE could be evaluated. Results as mean±SEM and concentrations were expressed in µg/mL for Ach and µg lyophilized/mL for the AE-M.c.

Test	*M. micrantha**	M. cordifolia
Shinoda test	+	**++**
Cl_3Fe	+ green	**+ green**
Cl_3Al	+ yellow (UV 366)	**+ yellow (UV366)**
H_3BO_3	++ Light blue (UV 366)	**+ Light blue (UV366)**
KOH	+ yellow (vis)	**+ yellow (vis and UV366)**
Dragendorff Reactive	-	**+ precipitate**
Mayer Reactive	-	**-**
Marme Reactive	-	**-**
Picric acid 1%	-	**+**
Bouchardat Reactive	-	**-**
Tanic acid 1%	-	**+**

Table 1: Preliminary chemicals tests of aqueous extracts of *Mikania* spp

(*extracts with blue fluorescence at UV366 before adding the reactives)

Mobile phase 1		Mobile phase 2	
M. cordifolia	*M. micrantha*	*M. cordifolia*	*M. micrantha*
0 Orange	0 Yellow	0 Yellowish brown	0 Yellowish brown
0.11 yellow	0.11 Yellow	0.10 Orange	0.10 Yellow-green
0.20 Orange	---	---	0.15 Yellow-green
0.28 Light blue	0.28 Light blue	0.20 Light orange	0.20 Yellow-green
0.35 Light blue	---	0.25 Light orange	0.25 Yellow-green
0.44 Light blue	0.44 Light blue	---	0.28 Yellowish
0.51 Light orange	---	---	0.30 Yellowish
---	0.60 Light blue	0.40 Yellowish	0.40 Yellowish
---	0.77 Light blue	---	0.51 Light blue
0.91 Blue	0.91 Yellow	0.55 Yellowish	---
0.96 Light blue	0.96 Light blue	0.60 Yellowish	---
		0.74 Light blue	---
		0.76 Yellowish	---
		---	0.90 Yellowish

Table 2: TLC profiles of aqueous extracts of *Mikania* spp in two mobile phases

The Rf values and fluorescent aspect at UV 366 correspond to the condition after treatment with natural product reactives.