Phytomedicine Plus 1 (2021) 100060

Contents lists available at ScienceDirect

Phytomedicine Plus

journal homepage: www.elsevier.com/locate/phyplu

Intestinal/uterine antispasmodics, sedative effects of *Fuchsia magellanica* Lam. leaves' and flowers' extracts and their flavonolic components

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ARTICLE INFO

Keywords:
Fuchsia magellanica
Antispasmodic
Intestine
uterus
Open-field test
Flavonoids

ABSTRACT

Background: *Fuchsia magellanica* Lam. (*Onagraceae*) is a native plant of the Andean-Patagonian region, and it is also naturalized in Argentina and other parts of the world. Leaves are used for treating indigestion, stomachache, as sedative, for difficult delivery and as antiemmenagogue. However, up to now the pharmacological basis of such uses were not studied.

Purpose: This work evaluates the gastrointestinal, uterine and sedative effects of leaves (L) and flowers (F) tinctures of *F. magellanica* from wild patagonian (T-Fm-P) and naturalized (T-Fm-BA) plants, as well as the mechanism of action and the flavonoids profile of both plants.

Methods: Phytochemical studies were evaluated by using TLC and HPLC methodologies. The *ex-vivo* effects of T-Fm-BA and T-Fm-P were evaluated on contractile concentration-response curves (CRC) of carbachol (CCh) and calcium (Ca^{2+}) in rat isolated intestinal and uterine tissues. *In vivo* tests for intestinal transit, elevated cross plus-maze and open-field tests were performed in mice.

Results: Wild and cultivated leaves and flowers ethanolic extracts induced antispasmodic effect, as a non-competitive inhibitor of the CCh-CRC in intestine, with IC_{50} of 272.8 ± 64.3 μg/ml for T-L-Fm-P and 257.4 ± 36 μg/ml for T-L-Fm-BA. A similar effect was obtained in Ca^{2+}-CRC with IC_{50} of 152.9 ± 29.1 μg/ml and 138.4 ± 48.6 μg/ml respectively. Mice intestinal transit was reduced at oral doses of 73.5 mg/kg of T-L-Fm-P. In uterine tissues, both T-L-Fm reduced the contractions of CCh CRC´s as non-competitive antagonists, with IC_{50} near those in intestine. The T-L-Fm-BA also inhibited the serotonin CRC´s and contribution of other relaxing mechanisms was investigated. Although extracts didn't show anxiolytic effect in the elevated cross plus-maze test, they reduced the spontaneous activity of mice in the open-field at 32.7 mg/kg T-L-Fm-P and 41.7 mg/kg T-L-Fm-BA.

Conclusions: Leaves and flowers ethanolic extracts from wild and cultivated plant *F. magellanica* showed intestinal and uterine antispasmodic effects, mainly by interfering with Ca^{2+} influx. In mice, the ethanolic extracts reduced the intestinal transit and showed sedative effect. The effects agree with the presence of flavonoids, such as quercetin and kaempferol. This is the first study which gives experimental support for some of the traditional uses of *F. magellanica*.

Abbreviations: Ca^{2+}, ionic calcium; CCh, carbachol; CRC, concentration response curve; DF, freedom degrees of variance; EC_{50}, 50% effective concentration; F, Fisher coefficient for variance; HPLC, high performance liquid chromatography; 5-HT, serotonin; IC_{50}, 50% inhibitory concentration; SEM, standard error of media; T-F-Fm-BA, tincture of *F. magellanica* flowers from Buenos Aires; T-F-Fm-P, tincture of *F. magellanica* flowers from Patagonia; TLC, thin layer chromatography; T-L-Fm-BA, tincture of *F. magellanica* leaves from Buenos Aires; T-L-Fm-P, tincture of *F. magellanica* leaves from Patagonia.

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https://doi.org/10.1016/j.phyplu.2021.100060
Received 25 January 2021; Received in revised form 24 March 2021; Accepted 29 March 2021

Introduction

Fuchsia magellanica Lam. (Onagraceae) is known as “fucsia”, “chilco” or “aljaba”. It grows in the Andean-Patagonian forests between Argentina and Chile (Dimitri, 1974). This plant is used for medicinal purposes by the Mapuche native people who inhabit that region and know it as “chilko” (Ladio et al., 2007). The wild plant grows in rivers and lakes shores or in wet places of the phytogeographic region of Valdivian cold forest of Patagonia (Aukanaw, 2010). It is a shrub of 3-5 meters high, with elliptic and opposite composed leaves, characteristic single purple dropping flowers (Berry, 1989). The plant was also naturalized in other parts of South America, east of Africa, New Zealand, Ireland and Hawaii (Berry, 1989). Aqueous and hydroalcoholic preparations are the most popular forms utilized. Mapuche´s people use to macerate leaves in hot wine to drink before and after difficulties in delivery (Estomba et al., 2006; Aukanaw, 2010). Leaves and flowers are used in infusion by the attributed antiemmenagogue properties, that is to interrupt menstruation (Dominguez Díaz, 2010). These traditional uses suggest the hypothesis that the plant would have effects on uterine muscle and could also be anxiolytic. In the north of Argentina, the bark infusions are used against indigestion (locally called “empacho”) (Campos-Navarro and Scarpa, 2013). In the South of Ecuador, *F. magellanica* is considered sedative and effective against stomachache, as a part of the traditional “horchata” infusion mixed with other plants (Ríos et al., 2017). However, there are not scientific reports about the pharmacological basis of these traditional uses.

Some previous phytochemical screenings from flowers and berries of *Fuchsia* sp., found the presence of anthocyanins as pelargonidin-3,5-diglucoside, peonidin, malvidin, and acylated anthocyanins (Crowden et al., 1977). Flavonols and flavones were also reported in flowers and leaves (Williams and Garnock-Jones, 1986). More recently, Csepregi et al. (2020) reported from leaves of *F. magellanica* the presence of anthocyanins (cyanidin and peonidin), flavonoids (quercetin, kaempferol and its galloyl-glycosides) as well as caffeic and gallic acid derivatives, and demonstrated effects as antioxidant, antimicrobial and facilitation of cell chemotaxis.

The aim of this work was to evaluate the pharmacological basis of some of the traditional uses of *F. magellanica*, that is the gastrointestinal and uterine effects, and the possibility of anxiolytic or sedative actions. Moreover, it was of interest to know whether the wild plant from Patagonia (Fm-P) and the naturalized plant from Buenos Aires (Fm-BA) have the same properties. For these purposes, *ex vivo* effects on rat intestinal and uterine smooth muscle preparations and *in vivo* effects on intestinal transit and spontaneous behavior of mice were evaluated after treatment with hydroalcoholic extracts of the wild and the naturalized plants of *F. magellanica*. Their pharmacological and flavonoids patterns were compared and their mechanisms of action were studied.

Materials and methods

Chemicals and reagents

Drugs employed in biological tests included: Carbamylcholine chloride (carbachol, CCh, Sigma-Aldrich, St Louis, MO, USA), Nω-Nitro-L-arginine methyl ester (L-NAME) hydrochloride (Sigma-Aldrich), serotonin hydrochloride (5-HT, Sigma-Aldrich), indomethacin (Indo, Sigma-Aldrich), polyethylene glycol (PEG, Sigma-Aldrich), carboxymethylcellulose (CMC, Sigma-Aldrich), atropine (Sigma-Aldrich), diazepam (Roche, Argentina). Standard flavonoids of HPLC quality were from Carl Roth (Denmark). Tyrode and De Jalon solutions were prepared as previously described (Matera et al., 2016; Jimenez-Hernández et al., 2018; Gavilánez-Buñay et al., 2018). Further details can be found in the supplemental material.

Plant material and extracts

Leaves and flowers of F. magellanica were collected in the field of the Jardín Botánico C. Spegazzini, Facultad de Agricultura y Ciencias Forestales de la Universidad Nacional de La Plata (34° 52′ S, 57° 54′ W) in October 2013 (naturalized plants of Buenos Aires, Fm-BA), and in Villa La Angostura, Provincia de Neuquén (40° 47´ S, 71° 40´ W) during January 2014 (native wild plants from Patagonia, Fm-P).

Aerial parts were allowed to dry at room temperature for 4 weeks and identified as NDBAYON 1644 by M.Sc. Agr. Eng. Marta Colares in the Herbarium (LPAG) from Universidad Nacional de La Plata, accordingly to Holmgren et al. (1990) methodology. The tinctures (T) were obtained by maceration at 10% w/v of dried herbal drug in ethanol 70° for at least 48 h, accordingly to the Argentinian National Pharmacopoeia. Dried leaves (L) and flowers (F) of Fm-BA and Fm-P were used to obtain tinctures. Their residue yields (%w/w) were 37.5% for T-L-Fm-BA, 29.4% for T-L-Fm-P, 19.6% for T-F-Fm-BA and 16.6% for T-F-Fm-P. These yields were used to convert the IC_{50} of the extracts (% w/v) in μg residue/ml or doses in mg residue/kg.

For the biological *ex vivo* studies, the tinctures were diluted either in Tyrode´s or De Jalon´s solution on the day of the experiment (to 0.01, 0.03, 0.1 and 0.3 % w/v), and their concentrations were expressed as μg residue/ml. Negative controls with vehicle (70° ethanol 0.1 and 0.3% v/v) were evaluated. For *in vivo* experiments the tinctures were diluted in saline solution (1/3 and 1/10) and doses were expressed in mg residue/kg, negative controls with saline and saline + ethanolic dilution were assessed.

Analysis of the tinctures

Thin layer chromatography (TLC)

TLC was performed to determine the presence of flavonoids on leaves of both plants, silica gel F254 Merck 0.25 mm and F254 cellulose 0.2 mm plates were used with the following mobile phases: (1) EtOAc/HCOOH/HOAC/H_2O (100:11:11:26), (2) HOAc 15%, (3) HOAc 40%, (4) EtOAc/MEK/HOAc/H_2O (5:3:1:1), (5) EtOAc/HCOOH/MEK/HOAc/H_2O (5:7:3:30:10), (6) CHCl3/AK/HCOOH (7.5:1.65:0.85) and (7) EtOAc/MeOH/H_2O (100:13.5:10), using 1% methanol solutions of apigenin (A), isoquercetin (I), vitexin (V), cynarine (C), quercetin (Q), quercitrin (q), rutin (R), kaempferol (K), chlorogenic acid (CA), hyperoside (H), and caffeic acid (CfA) as standards for flavonoids. Detection of flavonoids bands was developed under visible and UV light (366 nm) with 1% methanolic natural product (AEDB) spray.

High performance liquid chromatography (HPLC)

The separation column used was IB-SIL RP 18 (5 mm, 250 mm, 4.6 mm, ID) Phenomenex. Detection was carried out by a UV (285 nm) Varian 9050 detector and a Varian 9065 photodiode-array detector using a Rheodyne injection valve (20 μl). The separation was achieved by applying a gradient using solvent A (water: acetic acid 98:2) and solvent B (methanol: acetic acid 98:2). The gradient was 85% A from 0 to 30 min; 60% A from 30 to 40 min, 25% A from 40 to 45 min and 15% A from 45 to 60 min. The flow rate was 1.2 ml/min. Quantification was achieved by the external standard method using rutin, quercetin, kaempferol, kaempferol-3-glucoside, quercitrin, hyperoside, isoquercitrin, cinerin, chlorogenic acid, caffeic acid, vitexin, apigenin, apigenin-7-O-glucoside (Filip et al., 2001).

Pharmacological studies

Animals

The research was conducted on both adult male and female Sprague-Dawley rats (200-250 g) as well as 2-3 month-old Swiss albino female mice (25-30 g), following internationally accepted principles of laboratory animal use and care as established by US guidelines (NIH publica-

tion 85-23 revised in 1996) and principles in the Declaration of Helsinki, according to the Resolution 1047 anexo II-2005, of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) de la República Argentina. The protocols and procedures were approved by an ethical local committee of Facultad de Ciencias Exactas de la Universidad Nacional de La Plata (CICUAL) by number D-01-15-16.

Smooth muscles preparations and contractile measurements

Sprague-Dawley rats (200-250 g) were subjected to a 12 h fasting with free access to water before experimentation. After sacrificed by using pentobarbital (60 mg/kg via i.p.), duodenum, ileum (about 2 cm long) and/or uterus were excised. Intestinal tissues were individually mounted in organ chambers containing air-bubbled Tyrode´s solution (20 ml) at 37°C (Ragone et al., 2007; Gavilánez-Buñay et al., 2018). Both uterine horns were divided and immersed in De Jalon´s solution in chambers at 32 °C (Kitchen, 1984; Gavilánez-Buñay et al., 2018). All preparations were equilibrated for at least 30 min at 1g of pre-load. Intestinal tissues were connected to isometric force transducers FORT100 (World Precision Instruments (WPI), Sarasota, FL, USA) and the signals simultaneously amplified by a 4-channel preamplifier (WPI) and recorded by Eagle software (USA). Contractility of both uterine horns was simultaneously detected by PanLab MLT0210/A isometric transducers and recorded by a PowerLab 2/26 system with LabChart A/D program (AD Instruments, New South Wales, Australia).

Concentration-response curves in ex vivo *preparations*

Concentration-response curves (CRC) of contraction to carbachol (CCh-CRC) were simultaneously performed in 4 small bowel portions, as previously described, by using a negative control of vehicle and a positive control of verapamil (Blanco et al., 2013). The tinctures of leaves and flowers of T-Fm were assessed by adding to each preparation before the CRC in sequentially increasing concentrations. The contractile effect of each CCh concentration was expressed as percentage of the maximal contraction of tissue during the second control CCh-CRC (% Emax, percentage of maximal effect).

To evaluate the mechanism of the T-Fm, another protocol was followed with CCh-CRC in the presence of L-NAME (30 μM), an inhibitor of NO-synthases. Moreover, the effects of T-Fm were assessed on Ca^{2+}-CRC´s developed in a depolarizing solution of Tyrode-0 Ca^{2+}-40 mM K^+, after the negative control of vehicle, and using as positive control verapamil, as described (Blanco et al. 2013).

Uterine tissue had spontaneous high phasic contractions, which were minimized with 0.5 mM Ca^{2+} (Kitchen, 1984; Aaronson et al., 2006; Darios et al., 2012; Gavilánez-Buñay et al., 2018). CRC´s with carbachol were developed in the absence (control) and the presence of vehicle (control-vehicle) and growing concentrations of T-Fm. Contractile effects were measured as the increase in the height of the periodical phasic contractions over the basal condition, and expressed as percentage of the maximal increase in the control CRC, after considering the effect of vehicle at the higher concentration. To evaluate the uterine mechanism of T-Fm, other protocols were followed. One group received indomethacin (0.1 μM) before the CRC´s, to inhibit cyclooxigenases. In other protocol, CCh-CRC were developed in the absence (control) and the presence of 10 mM tetraethylammonium (TEA) to block K^+ channels. For another protocol, rats were treated with 5 mg/kg β-estradiol i.p. 24 h before, to induce estro condition, and CRC´s of serotonin (5-HT-CRC) were followed in the absence and the presence of vehicle and T-Fm.

From the CRC performed with CCh, 5-HT and Ca^{2+}, the pEC_{50} (as –log EC_{50}, in M) of the agonist was calculated. For the T-Fm, the 50% inhibitory concentration (IC_{50}) was calculated as previously described (Matera 2016; Gavilanez-Buñay et al., 2018). IC_{50} were expressed as µg residue/ml.

In vivo *evaluation of intestinal transit*

The effect of T-Fm on mice intestinal transit was investigated as previously described (Salako et al., 2015). Briefly, 4 groups of mice fastened by 24 h were respectively treated with: T-L-Fm-BA diluted at 50% in saline per oral (62.5 mg/kg), T-L-Fm-P diluted at 75% in saline per oral (73.5 mg/kg), ethanolic vehicle in saline per oral, and atropine 5 mg/kg via intraperitoneal (i.p.) as positive control. All doses were administered at 0.1 ml/30 g body weight. After 30 min, mice received 0.2 ml polyethylene glycol (PEG) 6% w/v per oral (400 mg/kg) as a volume laxative. After another 30 min, mice received 0.1 ml of aqueous 10% active carbon with 1% carboxymethylcellulose suspension. Following 30 min after, mice were sacrificed by cervical dislocation and the length of intestines was measured, evaluating the distance covered by the carbon content expressed as percentage of the total length from piloro to ileo-cecal limit.

Elevated cross plus-maze test

The test was performed in mice according to previous reports (Mora et al., 2005; Wasowski and Marder, 2011). Briefly, the black wood maze in cross had two open arms (30 × 5 cm each) and two enclosed arms (30 × 5 × 15 cm each) opposite to each other. Mice were respectively treated via i.p. with vehicle, 0.5 mg/kg diazepam as positive control, and the tinctures of *F. magellanica* diluted 1/10 and 1/3 in saline (at doses of 12.5 and 41.7 mg/kg for T-L-Fm-BA; 9.8 and 32.7 mg/kg for T-L-Fm-P). The number of entries to the open and to the closed arms, and the time elapsed there, were counted during 5 min for each mouse, each 30 min from 0 to 150 min, after treatment. Results were expressed as ratios in open arms/closed arms.

Open field test

This test was used to evaluate the spontaneous locomotive and exploratory activity of mice. The open-field apparatus was previously described (Molina-Hernandez et al., 2004; Consolini et al., 2006). Mice (normally feeded) were divided in 5 groups which received respectively one of the following treatments: tinctures (diluted 1/10) as T-L-Fm-BA (41.7 mg/kg), T-L-Fm-P (32.7 mg/kg), vehicle saline, vehicle saline + ethanol 70° (negative control) and 0.5 mg/kg diazepam (positive control), by i.p. injections (0.1 ml by 30 g weight). Each mouse was placed in the open field and counted the crossed lines number and the rearings number during 5 min, at 30, 60, 90, 120 and 150 min after administration.

Statistical analysis

All results are expressed as mean ± SEM (n). Statistical multiple comparisons were performed in each CRC series by two-way ANOVA, as considering two variables influencing the effect, namely the treatment and the x-axis variable, that is, agonist concentration (as pCCh, pCa or p5-HT, in CRC) or time (in the *in vivo* experiments). After ANOVA, Tukey's *post hoc* tests were applied for paired comparisons in CRC´s. For multiple comparisons of IC50 in the CRC groups of T-L-Fm-P and T-L-Fm-BA respectively, the one-way Brown-Forsythe and Welch ANOVA test was applied followed by *a posteriori* unpaired t with Welch's correction *post hoc* tests (significances are indicated in text and figures). Statistical analyses were performed with Graph Pad Prism 8.0 software. The significance was considered at $P < 0.05$ in all tests performed.

Results

Phytochemical profiles of the extracts

Chromatographic profile of polyphenols (caffeoylquinic acids and flavonoids) from both tinctures were compared respectively against

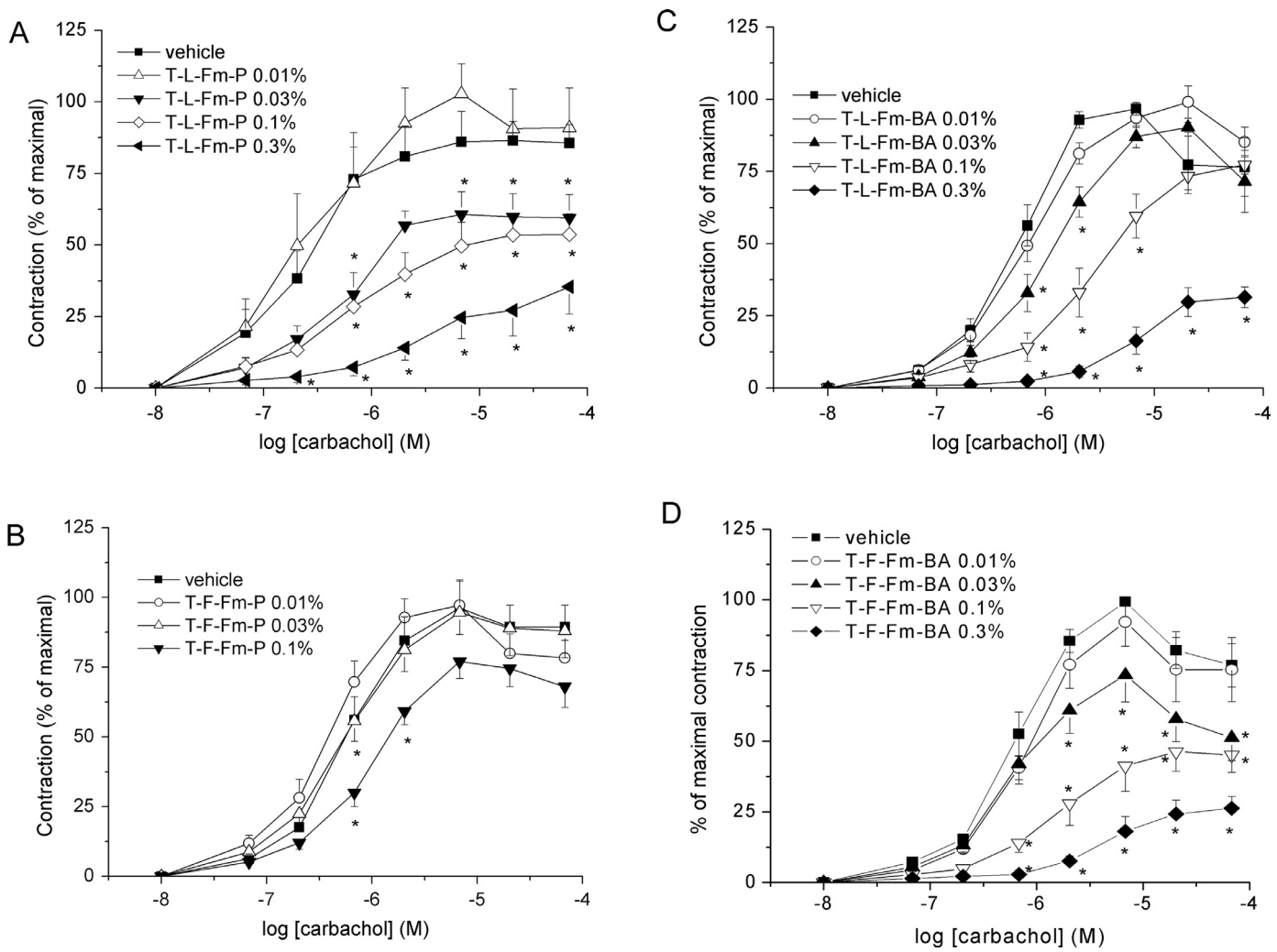


Fig. 1. Effects of *F. magellanica* leaves (L) and flowers (F) tinctures (T) from the wild Patagonian (Fm-P) (A and B) and the naturalized Buenos Aires (Fm-BA) (C and D) plants, on the CRC´s of carbachol in small intestine. Results are shown as media and SEM (n = 6-9). Two-way ANOVA´s: by treatment: $P < 0.0001$; and by log [CCh]: $P < 0.0001$ in each one. Post-tests: * $P < 0.05$ vs. control.

standard compounds in TLC. Systems 1, 2, 4 and 5 showed the same chromatographic profile in both tinctures but a higher content of the analyzed compounds was present in T-Fm-P. A greater intensity of the bands in T-Fm-P in relation to T-Fm-BA can be observed. The presence of quercitrin, isoquercitrin, hyperoside and chlorogenic acid was suggested. The flavonoids: apigenin, vitexin, kaempferol-3-triglycoside and the caffeoylquinic acids (cynarine and caffeic acid) were not detected. Systems 3 and 6 suggested the presence of quercetin and kaempferol in T-Fm-P. Appendix Fig. A.1.

HPLC analysis confirmed the presence of quercitrin and hyperoside (in a lower amount) in both the wild and naturalized plants, expressed as mg flavonoid per 100 g leaves (mg%) (Table 1). For both flavonoids, the T-Fm-P showed greater amounts than T-L-Fm-BA. Quercetin and kaempferol were found present only in T-Fm-P (Fig. A2), since they were undetectable in T-Fm-BA (Table 1). Rutin, isoquercitrin and chlorogenic acid were not detected.

Table 1
Yields of flavonoids present in T-L-Fm-BA and T-L-Fm-P. Mean ± ESM (expressed in mg%, that is mg flavonoid per 100 g dried leaves).

Flavonoid Rt (min)	T-L-Fm-BA (mg%)	T-L-Fm-P (mg%)
QuercitrinRt 36.021	473.1 ± 23.6	597.4 ± 19.1
HyperosideRt 36.728	87.9 ± 5.0	201.8 ± 0.2
QuercetinRt 38.710	ND	18.8 ± 0.6
KaempferolRt 40.302	ND	NQA

ND (not detectable): < 0.2 ppm; NQA (not quantified amount): < 1 ppm.

Effects of F. magellanica *tinctures on the small bowel*

The 4 tinctures of *F. magellanica*, T-L-Fm-BA, T-L-Fm-P, T-F-Fm-BA and T-F-Fm-P inhibited the intestinal contractions of CCh-CRC as non-competitive antagonists (Fig. 1). The CRC´s patterns were similar, although the T-F-Fm-P inhibited the CCh-CRC contractions less than T-L-Fm-P without reaching 50% (IC_{25} 90.1 ± 25.0 μg/ml, n = 9). Table 2 shows the IC_{50} values of T-L-Fm-P, T-L-Fm-BA and T-F-Fm-BA, which were similar among them. Fig. A.3 shows the inhibition curves.

When the tinctures were assessed on Ca^{2+}-CRC in depolarizing solution in order to evaluate whether they affect Ca^{2+} channels influx, both T-L-Fm-P and T-L-Fm-BA inhibited the Ca^{2+}-CRC´s in a non-competitive way (Fig. 2A-B). Table 2 shows that IC_{50} values were not significantly different to those respectively obtained in the CCh CRC´s despite a tendency to be lower ($P = 0.065$ and $P = 0.167$ for T-L-P and T-L-BA, respectively) (inhibition curves in Fig. A.3 C vs A). The non-competitive effects of tinctures had the same pattern that verapamil, which was assessed as a positive control (Fig. 2 C-D).

Table 2
Comparison of the IC_{50} values (in μg/ml) of tinctures (T) obtained from *Fuchsia magellanica* (Fm) leaves (L) and flowers (F), either from Patagonia (P) and Buenos Aires (BA), in the several CRC´s (n: number of experiments), and multiple comparisons results.

Tincture	Intestinal CRC of carbachol	Intestinal CRC of calcium	Uterine CRC of carbachol	Uterine CRC of 5-HT
T-L-Fm-P	272.8 ± 64.3 (8)	152.9 ± 29.1 & (6)	393 ± 64 (10)	
T-F-Fm-BA	266.7 ± 45.5 (6)			
T-L-Fm-BA	257.4 ± 36.0 (6)	138.4 ± 48.6 # (13)	209.3 ± 37.8 # (8)	234.1 ± 25.8 # (8)
T-L-Fm-P + L-NAME	212.3 ± 44.3 & (6)			
T-L-Fm-BA + L-NAME	185.4 ± 32.2 # (6)			
T-L-Fm-BA + Indomethacin			341 ± 31.8 (7)	
T-L-Fm-BA + TEA			90.5 ± 13.4 *# (8)	
Brown-Forsythe ANOVA test for T-L-Fm-BA	F = 6.575	P < 0.0001	DFn = 7	DFd = 56
Brown-Forsythe ANOVA test for T-L-Fm-P	F = 3.883	P < 0.0224	DFn = 4	DFd = 30

Post-tests for comparing groups in the series of T-L-Fm-BA: * p<0.05 vs intestinal CCh-CRC, # p<0.05 vs Indomethacin; and for the series of T-L-Fm-P: & p<0.05 vs uterine CCh-CRC.

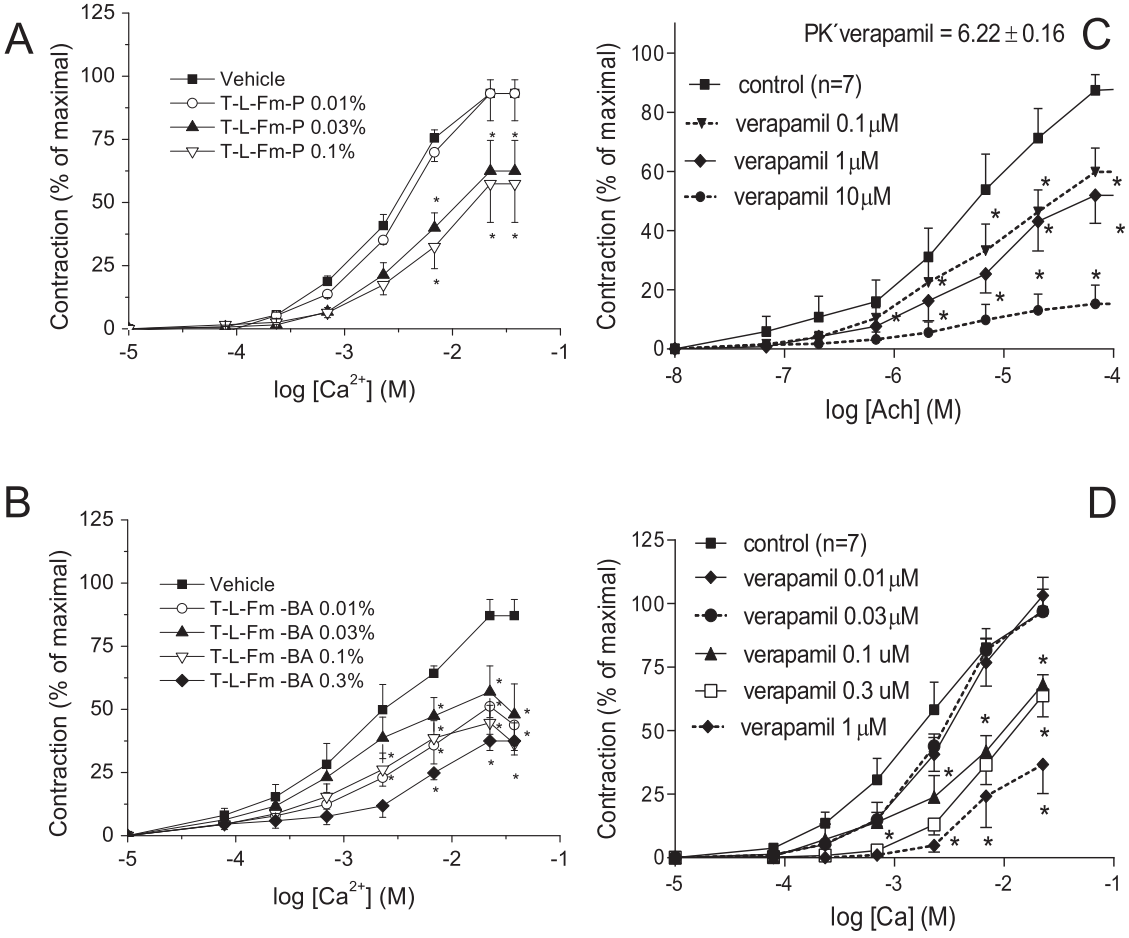


Fig. 2. Effects of *F. magellanica* leaves tinctures (T-L), from the wild Patagonian (Fm-P) (A, n = 6) and the naturalized Buenos Aires (Fm-BA) (B, n = 13) plants, on the Ca^{2+}-CRC´s obtained in isolated rat intestine under depolarizing media. Results are shown as media and SEM. Two-way ANOVA´s: by treatment: $P < 0.0001$ and by log $[Ca^{2+}]$: $P < 0.0001$ in both, (A) and (B). Post-tests: $^{*}P < 0.05$ vs. control. In C-D: Effects of verapamil (as positive control) on the CRC´s of acethylcholine (C) and calcium (D) in rat isolated intestinal muscles. Observe the non-competitive inhibition, as well as it was obtained with the *F. magellanica* tinctures.

When the NOS was blocked by the presence of L-NAME on the CCh-CRC´s (Fig. 3), the IC_{50} values of *F. magellanica* tinctures T-L-Fm-BA and T-L-Fm-P were not significantly different to the respective values obtained in the absence of L-NAME (Table 2, $P = 0.16$ and $P = 0.45$, respectively, by *post-hoc* tests) (Fig. A.3, B vs A).

Effects of F. magellanica *tinctures on the isolated uterus*

In uterine tissue, the consecutive CRC´s of carbachol increased the amplitude of phasic contractions (Fig. 4A). T-L-Fm-P and T-L-Fm-BA reduced the contractions of the CCh-CRC´s as non-competitive antagonists (Fig. 4, B-C). Table 2 shows the IC_{50} of T-L-Fm-P and T-L-Fm-BA, being the first higher than the respective value in the intestinal Ca-CRC´s ($P = 0.005$) (Fig. A.3 D shows the inhibition curves). When cyclooxygenases were inhibited with indomethacin in the CCh-CRC´s, the T-L-Fm-BA maintained the non-competitive antagonism (Fig. 5A) but with an IC_{50} higher than in the absence of indomethacin ($P = 0.018$, Table 2, Fig. A.3 E). On the other hand, in the presence of TEA, a blocker of K^+ channels, the uterine tissues increased the amplitude of phasic contractions, but it maintained the non-competitive inhibition of T-L-Fm-BA on the CCh-CRC (Fig. 5B), with an IC_{50} significantly lower than that obtained in the absence of TEA ($P = 0.0047$, Table 2, Fig. A.3 E). Moreover,

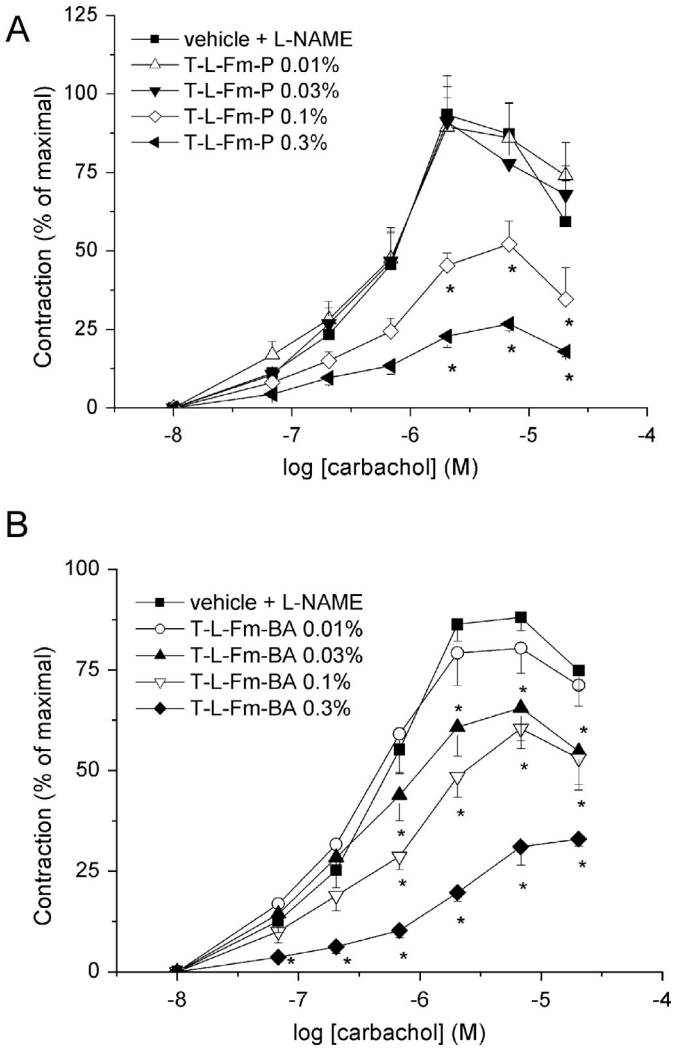


Fig. 3. Effects of *F. magellanica* leaves tinctures (T-L) from the wild Patagonian (Fm-P) (A, n = 6) and naturalized Buenos Aires (Fm-BA) (B, n = 6) plants, in the presence of L-NAME to inhibit NO-synthases, on the carbachol CRC´s in rat intestine. Results are shown as media and SEM. Two-way ANOVA´s: by treatment: $P < 0.0001$ and by log [CCh]: $P < 0.0001$ in both, (A) and (B). Post-tests: $^{*}P < 0.05$ vs. control.

Fig. 5C shows that the T-L-Fm-BA was also a non-competitive antagonist in the 5-HT-CRC obtained in uterus, with an IC_{50} value not significantly different from those obtained in the intestinal and uterine CCh-CRC´s ($P = 0.61$, and 0.59, respectively) (Table 2, Fig. A.3 F).

Effects of F. magellanica *tinctures on the mice intestinal transit*

The oral administration of T-L-Fm-P at doses of 73.5 mg/kg (n = 8) significantly reduced the intestinal transit in mice treated by PEG 3350 from 55.1 ± 6.4% (saline + ethanolic vehicle, n = 6) to 28.6 ± 6.8 % ($P = 0.024$ in post-test to ANOVA), almost as did atropine 5 mg/kg (n = 8) injected via i.p. as positive control (15.3 ± 6%, $P = 0.0005$ vs vehicle) (Fig. 6). T-L-Fm-BA at doses of 62.5 mg/kg (n = 7) did not significantly reduce the intestinal transit (48.6 ± 3.5%, $P = 0.88$ vs vehicle), although it had a tendency to decrease.

Effects of F. magellanica *tinctures on spontaneous mice behavior*

The tinctures of leaves and flowers of the wild and naturalized *F. magellanica* plants, diluted at 1/10 and 1/3 in saline, did not show anxiolytic effects in the elevated cross plus-maze test, as it was shown for 0.5 mg/kg diazepam, which increased the entries to open arms. Contrarily, the elapsed time (Fig. 7A) and entries number (Fig. 7B) shown as ratios in open/closed arms were in general significantly reduced with T-L-Fm-BA (at 12.5 and 41.7 mg/kg, both n = 9), T-L-Fm-P (at 9.8 and 32.7 mg/kg, both n = 9), T-F-Fm-BA (at 6.5 and 21.8 mg/kg, both n = 6) and T-F-Fm-P (at 5.5 and 18.4 mg/kg, both n = 6), in comparison to the effects of the positive control of anxiolytic (0.5 mg/kg diazepam, n = 9) and the negative control (saline with ethanol) (ANOVA: $F = 11.57$, $P < 0.0001$ in A, $F = 3.882$, $P < 0.0001$ in B, DFn= 10, DFd = 332). Ratios obtained at 30 to 150 minutes are shown in Appendix Fig. A.4. Since they did not correlate with time, results were averaged as shown in Fig. 7.

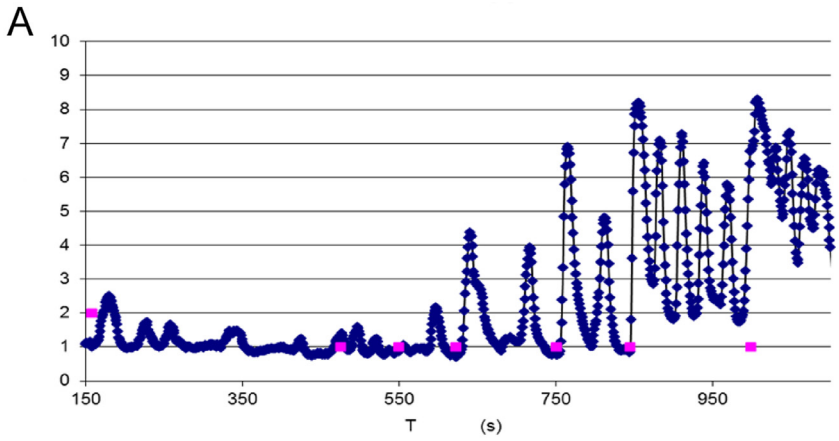
In order to distinguish whether these results were due to anxiogenic or sedative effects, the tinctures were assessed on mice in the open-field test. Fig. 7C-D shows that the ethanolic vehicle increased spontaneous locomotion and exploration versus saline at 30 min of administration, that is in the first exposition to field (2-way ANOVA: In A: by treatment: $F = 12.08$, $P < 0.0001$, by time: $F = 32.31$, $P < 0.0001$; in B: by treatment: $F = 19.93$, $P < 0.0001$, by time: $F = 11.37$, $P < 0.0001$, DFn = 4, DFd = 275). Both 1/3 dilutions of T-L-Fm-P (32.7 mg/kg, n = 12) and T-L-Fm-BA (41.7 mg/kg, n = 12) reduced the activities during the first 30-60 min in the open-field with respect to the ethanolic vehicle, suggesting a sedative effect.

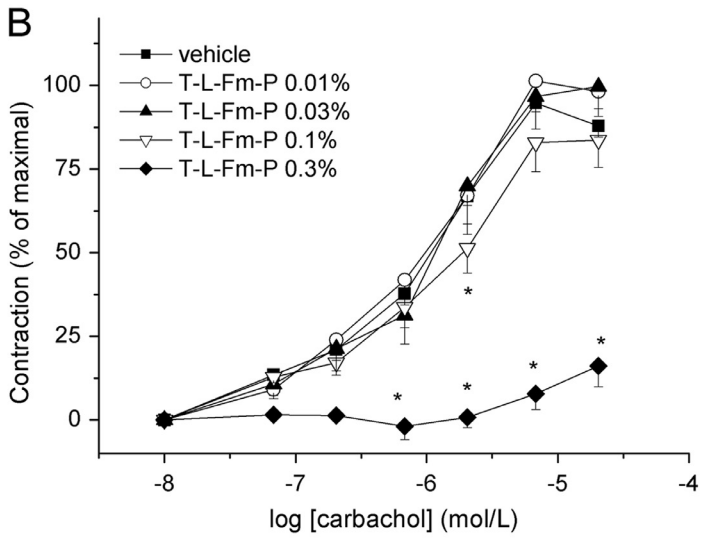
Discussion

This is the first pharmacological study demonstrating the antispasmodic effect that supports the traditional use of leaves and flowers from *F. magellanica* for treating gastrointestinal and uterine spasms. We showed that both, the wild plant used by Mapuche communities in Patagonia and the naturalized plant from the temperate Pampean region of Buenos Aires have the same properties.

By oral administration, the tincture of the Patagonian plant leaves, T-L-Fm-P at 73.5 mg/kg, significantly reduced the stimulated intestinal transit almost as well as 5 mg/kg atropine. A doses of 62.6 mg/kg of T-L-Fm-BA showed only a tendency to reduce transit. It could be due to the slightly lower doses, because both plants demonstrated to reduce intestinal contractility in the *ex vivo* carbachol CRC´s. Either leaves or flowers tinctures inhibited the contraction induced by carbachol in a non-competitive way, with similar IC_{50} values, except for the less effective flowers tincture of the Patagonian plant (Table 2). The mechanism involves the inhibition of Ca^{2+} influx to the smooth muscle, with a non-competitive blockade, in a similar pattern to that of the known Ca^{2+}-channels blocker verapamil (Blanco et al., 2013; Gavilánez-Buñay et al. 2018). The effect of the tinctures on Ca^{2+} influx is highly determinant of the antispasmodic properties. Moreover, the inhibition of Ca^{2+} influx could explain the described hypotensive effect of this plant (Rodríguez et al. 1994). On the other hand, the effect and IC_{50} of T-L-Fm-BA on CCh-CRC´s were not significantly changed by L-NAME, suggesting that the tinctures effect was not due to NO release.

The uterine tissue basal tone, with spontaneous wide phasic contractions, depends on K^{+}-channels inactivation and Ca^{2+}-channels activation, while cholinergic or serotoninergic stimulation develop tonic contractions (Darios et al., 2012). The wild and naturalized *F. magellanica* leaves tinctures inhibited the CRC´s of carbachol in a non-competitive way. The T-L-Fm-BA also inhibited the CRC´s of serotonin in a non-competitive way, with similar IC_{50} to that found in CCh-CRC´s, suggesting that the mechanism of inhibition is independent on the agonist receptor. So, this result agrees with the inhibition of L-type Ca^{2+} channels found in intestinal muscle. Moreover, the possibility that other relaxing mechanisms contribute to the effect in uterus was investigated. The non-competitive inhibition of T-L-Fm-BA on uterine CCh-CRC´s was not changed by indomethacin, but the IC_{50} was significantly increased, suggesting that the tincture could also induce the local production of a relaxant prostaglandin (PGI_2). Contrarily, the IC_{50} of T-L-F.m-BA was reduced by the presence of TEA, condition in which basal uterine tone was increased by the K^{+}-channels blockade and consequent activation





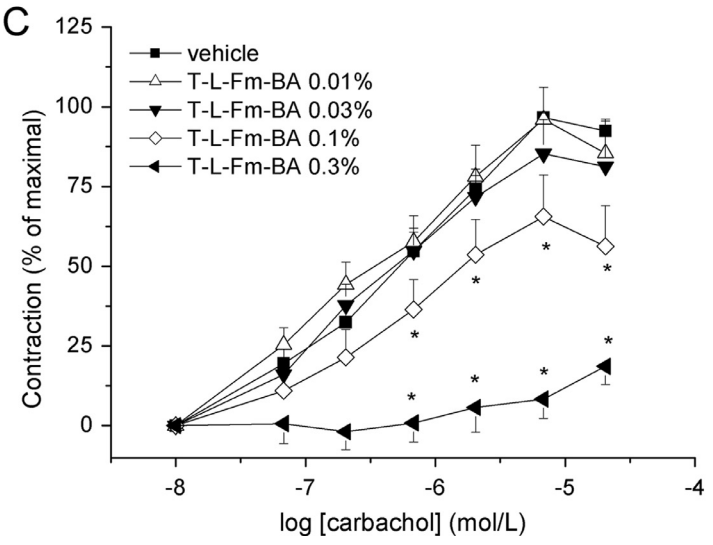


Fig. 4. Effects of *F. magellanica* leaves tinctures (T-L) from the wild Patagonian (Fm-P) and the naturalized Buenos Aires (Fm-BA) plants, on the uterine contractions and carbachol CRC´s. In A, a typical recording. The CRC´s in the presence of T-L-Fm-P (B, n = 13) and T-L-Fm-BA (C, n = 8) are shown as media and SEM. Two-way ANOVA´s: by treatment: $P < 0.0001$ and by log [CCh]: $P < 0.0001$ in both, (A) and (B). Post-tests: $^{*}P < 0.05$ vs. control.

of voltage-dependent L-type Ca^{2+}-channels. Under this condition, the Ca^{2+}-channels blockade induced by T-L-Fm-BA was facilitated.

The presence of flavonoids in *F. magellanica* was found by TLC, including quercetin, isoquercetin, hyperoside and kaempferol. Some of them, quercetin, hyperoside and other glycosylated phenolic compounds were identified by HPLC. Despite quercetin was present only in the Patagonian plant, and the content of quercitrin and hyperoside was higher in T-L-Fm-P than in T-L-Fm-BA, the effect was not influenced by these differences. Some flavonoids were reported as responsible of antispasmodic effects in intestinal and uterine smooth muscles. In particular, some of them interfere with the Ca^{2+} influx to rat intestine, such as vitexin (Ragone et al., 2007), quercetine, kaempferol and oth-

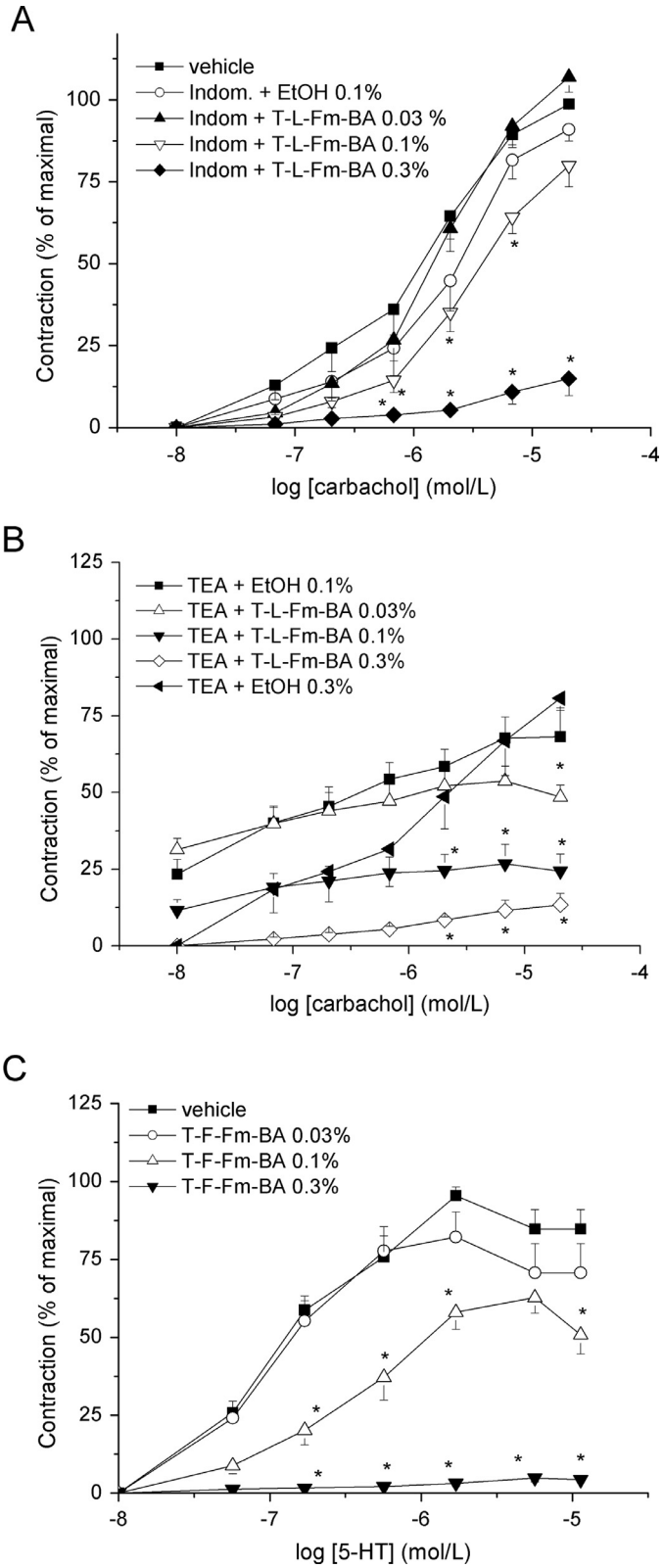


Fig. 5. Effects of *F. magellanica* leaves tinctures from the naturalized Buenos Aires plant (T-L-Fm-BA), in the presence of indomethacin to block COX (A, n = 6) and TEA to block K^+ channels (B, n = 6) on the carbachol CRC´s, as well as on the CRC´s of serotonin (C) in rat intestine. Results are shown as media and SEM. Two-way ANOVA´s: by treatment: $P < 0.0001$ and by log [CCh]: $P < 0.0001$ in (A), (B) and (C). Post-tests: $^{*}P < 0.05$ vs. control.

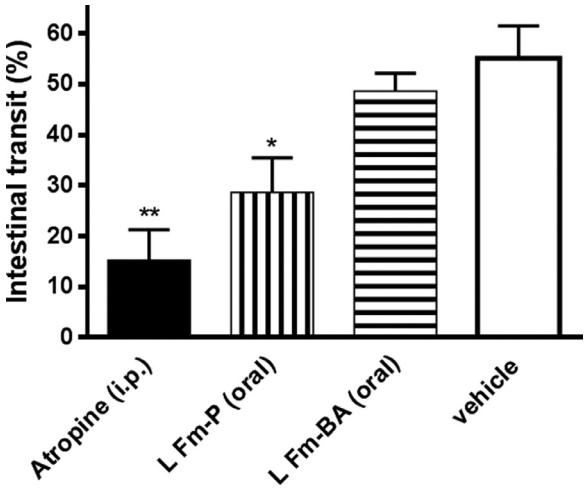


Fig. 6. Effects of oral *F. magellanica* leaves tinctures from the wild Patagonian (73.5 mg/kg T-L-Fm-P) and the naturalized Buenos Aires (62.5 mg/kg T-L-Fm-BA) plants on the intestinal transit test in mice treated with PEG 3350, distance run as % of intestinal length. Comparison with 5 mg/kg i.p. atropine. ANOVA: F = 9.39, $P = 0.0002$, post-test * $P < 0.05$ vs vehicle, ** $P < 0.01$ vs vehicle.

ers (Hammad and Abdalla, 1997; Revuelta et al., 1999; Nigusse et al., 2019). More recently, quercetine and related polyphenols were described as antioxidant and preventive of cardiovascular and intestinal diseases (Kawabata et al., 2015). Moreover, quercetine and kaempferol are present in *Tilia* genus, in which demonstrated to have sedative properties (Aguirre-Hernández et al., 2010). In agreement with the presence of these flavonoids, *F. magellanica* leaves and flowers tinctures demonstrated to reduce mice explorative and locomotive spontaneous behavior respect to ethanolic vehicle in the open field test and the plus-maze, although they did not show anxiolytic effect. Indeed, leaves tinctures of wild and naturalized *F. magellanica* plants demonstrated to induce short sedative effect, at i.p. doses of 32.7 mg/kg T-L-Fm-P and 41.7 mg/kg T-L-Fm-BA. These doses are nearer to those that under oral administration reduce the intestinal transit, and so it could be expected that this central inhibition could contribute to the therapeutic antispasmodic effect.

Conclusion

This is the first study showing that both, wild and naturalized plants of *F. magellanica* exhibited antispasmodic and sedative effects with little differences in flavonoids composition. The intestinal and uterine antispasmodic effects of leaves and flowers tinctures were a consequence of non-competitive Ca^{2+}-channels blockade. These results and the effects of reducing the stimulated intestinal transit and spontaneous behavior in mice, give a basis to some of the traditional uses of this plant. The presence of flavonoids as kaempferol, quercetin and hyperoside could be at least partially responsible for the antispasmodic and sedative properties.

Declaration of Competing Interest

We have not conflicts of interest in the publication of this article.

Acknowledgments

We are thankful to Bot. Nora Duzevich for the generous collection of the plants in Villa La Angostura, Neuquén province, Argentina.

This work is part of the Magister Thesis in Medicinal Plants of MSc. Biol. Eng. Ana María Bernal Ochoa (Facultad de Ciencias Exactas, Universidad Nacional de La Plata). Funding for this work were supported by Grants 11-X-642, 11-X-795 and PPID-X046 from Universidad Nacional de La Plata (UNLP), by funds from the postgraduate courses of the MSc

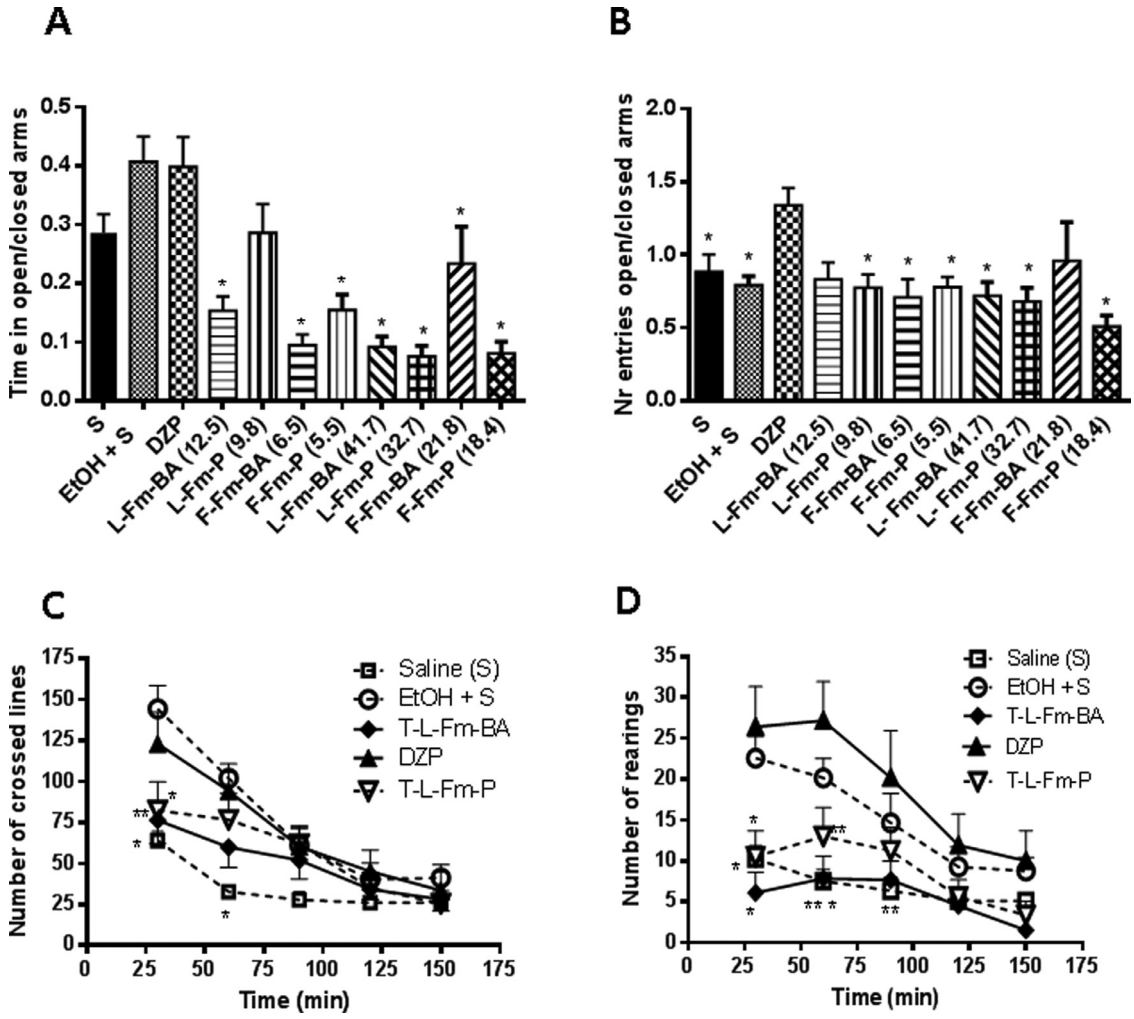


Fig. 7. Effects of *F. magellanica* leaves (L) and flowers (F) tinctures (T) from the wild Patagonian (P) and the naturalized Buenos Aires (BA) plants on mice spontaneous behavior. Elapsed time (A) and number of entries (B) as ratios in open/closed arms of the elevated cross plus-maze test with the indicated doses (in mg residue/kg) for T-L-Fm-BA (n = 9), T-L-Fm-P (n = 9), T-F-Fm-BA (n = 6) and T-F-Fm-P (n = 6). Number of crossed lines (C) and rearings (D) in the open field over time of 41.7 mg/kg T-L-Fm-BA and 32.7 mg/kg T-L-Fm-P. Two-way ANOVA, * $P < 0.05$ vs vehicle EtOH+S, and ** $P < 0.05$ vs. DZP (diazepam 0.5 mg/kg).

in Medicinal Plants, and by a grant from Universidad de Buenos Aires (UBACYT 20020130100686BA).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phyplu.2021.100060.

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