Pathophysiology 26 (2019) 349–359

Contents lists available at ScienceDirect

Pathophysiology

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

Gastrointestinal effects of standardized Brazilian phytomedicine (Arthur de Carvalho Drops®) containing *Matricaria recutita*, *Gentiana lutea* and *Foeniculum vulgare*

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ARTICLE INFO

Article history:
Received 27 May 2019
Received in revised form 22 August 2019
Accepted 6 September 2019

Keywords:
Matricaria recutita
Gentiana lutea
Foeniculum vulgare
Gastroprotective effect
Myorelaxant effect
Phytomedicine

ABSTRACT

Arthur de Carvalho Drops® (ACD) is a traditional Brazilian herbal medicine used to treat functional gastrointestinal disorders (FGIDs). ACD is a formulation of herbal extracts from *Matricaria recutita* (chamomile), *Foeniculum vulgare* (fennel) and *Gentiana lutea* L. (gentian). Considering the popular use for FGIDs, the aim of this work was to investigate the ACD effect on gastric and intestinal parameters with emphasis in a mechanistic approach using isolated duodenal preparations of rodents. Analytical method was developed and validated for quantify three actives principles/markers (Apigenin-7-glucoside, gentiopicroside and anethole) in ACD. The treatment with ACD significantly reduced the emetogenic stimuli induced by cisplatin in rats, showed a laxative effect, reduced the bethanechol-enhanced gastrointestinal transit and completely reversed the contraction induced by carbachol in rat duodenum. However, ACD did not alter the secretory gastric volume or total gastric acidity. The ACD affect the contractions of duodenal smooth muscle mediated by Ca^{2+} channels and it is also able to inhibit the contractile response mediated by the release from its intracellular store. Furthermore, the relaxant effects of ACD appear independent of the nitric oxide pathway in rat duodenum. These results suggest that ACD could be beneficial for the treatment of disorders of the gastrointestinal tract.

1. Introduction

The gastrointestinal tract develops innumerable functions in the human organism, such as digestion, protection and motility through the secretion of acids, enzymes, control in the absorption of water, electrolytes and nutrients [1]. Functional gastrointestinal disorders (FGIDs) represented by functional dyspepsia, irritable bowel syndrome, diarrhea, constipation and other motility gastrointestinal disorders are typical examples of how the digestive system plays a crucial role in the life of humans, so that when triggered, they have a considerable impact on patients' quality of life [2,3].

The high prevalence of FGIDs, disadvantages and healthcare costs associated of the actual pharmacotherapy have justify the researches of new medicines. In this context, medicinal plants have been shown a promise source, including molecule, extracts or phytomedicines, such as Colimil [3], peppermint oil (*Mentha piperita*) [4], anethole [5], red pepper powder (*Capsicum annuum*) [6], senna (*Cassia acutifolia*), and cascara (*Rhamnus purshiana*) [7].

Arthur de Carvalho Drops (ACD) is an herbal medicine extensively used by the population of the Northeast of Brazil for the treatment of gastrointestinal disorders. ACD is produced by

Abbreviations: ACh, acetylcholine; ACN, acetonitrile; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; Eq, equivalent; HPLC-PDA, high performance liquid chromatography - photo diode array; L-NAME, N omega-Nitro-L-arginine methyl ester hydrochloride; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

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https://doi.org/10.1016/j.pathophys.2019.09.002
0928-4680/

the combination of three tinctures; 50% of *Matricaria recutita* (chamomile) flowers, 25% of *Gentiana lutea* (gentian) roots, and 25% of *Foeniculum vulgare* (fennel) fruits. The ACD is commercialized in the Brazilian pharmacies since the early years of the 20th century. There are not reports available in the literature justifying the widespread popularity of the ACD for treatment of gastrointestinal disorders. For instance, *M. chamomilla* is one of the plants that compose the herbal preparation STW5 (Iberogast®), which is useful for the treatment of patients with functional dyspepsia [8]. In combination with *F. vulgare*, *M. chamomilla* is also a component of ColiMil®, another herbal formulation effective in the treatment of breastfed colic in infants. To these herbal formulations, the ability to interfere directly with the motor behavior of the gastrointestinal tract was already demonstrated experimentally [9]. Fixed and volatile bioactive principles, such as apigenin-7-glucoside and anethole (1-methoxy-4-((E)-propenyl)-benzene) from *M. chamomilla* and *F. vulgare*, respectively are responsible at least in part by the beneficial effects of these species for the treatment of gastrointestinal disorders [10].

Like *F. vulgare* and *M. chamomilla, Gentiana lutea* L. (Gentianaceae), known as gentian, is a medicinal plant present in several pharmacopeias. Its widely used in traditional Chinese medicine for their activities of anti-inflammatory, analgesia and strengthening gastric motility [11,12]. Its properties can be attributed mainly to the presence of some bitter compounds, such as secoiridoides glycosides including gentiopicroside [13]. Spasmolytic actions were described on isolated ileum preparations as for extracts as for some individual constituents of these plants [14,15], although the mechanism of action of these botanicals has not been completely elucidated. In the present report, the effects of ACD on rodent's gastrointestinal system were studied, with emphasis in a mechanistic approach using isolated duodenal preparations regarding the direct antispasmodic effects of this phytomedicine on intestinal smooth muscle cells.

2. Materials and methods

2.1. Solutions and chemicals

Modified Tyrode's solution at 37 °C was used with the following composition (in mM): 136.0 NaCl, 5.0 KCl, 0.98 $MgCl_2.6H_2O$, 0.36 NaH_2PO_4, 11.9 $NaHCO_3$, 2.0 $CaCl_2.6H_2O$ and 5.5 glucose. Salts and reagents were purchased from Sigma Chemical Co. or Merck (Germany). Acetylcholine, carbachol, scopolamine, verapamil, L-NAME were purchased from Sigma Chemical (St. Louis, MO, USA) and were dissolved directly in distilled water. Apigenin-7-glucoside – APG (Sigma-Aldrich; purity > 97.0%), Gentiopicroside - GTP (USP –USA; purity 98%) and Anethole – ANT (Sigma-Aldrich; purity 99%).

2.2. Herbal preparation

The Arthur de Carvalho Drops (ACD) was obtained from Ravick Chemical Products and Cosmetics Laboratory (Fortaleza, CE, Brazil). The ACD was made from a fixed combination of three plant tinctures (*Matricaria recutita, Gentiana lutea* and *Foeniculum vulgare*) with 88 mg/mL of solid residue.

2.3. Chemical characterization of Arthur de Carvalho Drops (ACD) by HPLC-PDA: validation method

The method was validated according to the International Conference of Harmonization (ICH), considering the following parameters: specificity, linearity, precision, accuracy, recovery and robustness. To assess specificity, comparative analyses of markers and ACD were performed in order to evaluate if any formulation components interfered with markers quantification. The Linearity determination was conducted by quantifying three different markers standard curves (ranging from 0.14 to 14.0 $\mu g.mL^{-1}$). The results were processed in Microsoft Excel. The curve equation was obtained by plotting substances peak mean areas against the respective nominal concentration on a Cartesian axis. By using linear regression analysis (least square regression method) the slopes, intercepts and determination coefficients were calculated. The validity of the assay was verified by means of the one-way ANOVA ($\alpha = 0.05$). The precision assay was investigated with respect to repeatability Intra-day precision (repeatability) and Inter-day precision (intermediate precision).

The accuracy was determined by recovery test by adding a known amount of each marker in three concentrations (low, medium and high), following the linear range of the method and comparing the results obtained with the same concentrations of standard reference. The robustness of the method was assessed by varying the parameters of the column temperature, flow rate of mobile phase and column manufacturer. Statistical analysis was by the ANOVA analysis of variance followed by Tukey's test, the results were considered significant when $p < 0.05$.

The assays were performed on an Alliance - Waters 2695 (Milford, MA) chromatograph with a binary pump, auto-sampler, and photodiode-array detector (Waters-2996 PDA) at 310 nm. The separations were performed with a reverse-phase column (Phenomenex®RP-8, 250 x 4.6 mm x5 μm) at 40 °C in a thermostatic oven. The mobile phase was made from 0.1% TFA/H2O (A), ACN (B) and THF (C) (gradient elution at 40 °C) and 10 μL of sample was injected. The wavelengths selected were 273 nm, 267 nm and 259 nm, varying according to the retention time of each marker, such as (APG/*Matricaria recutita*, GTP/*Gentiana lutea* and ANT/*Foeniculum vulgare*). The identification of chemical markers/plant tincture in ACD by HPLC were based on the retention time (rt) of external standards. The data was processed by the Empower® (Waters, USA) software. The majority volatile constituents of *F. vulgare*, anethole (isoestragole) and estragole [16,17] were not distinguished in this study being denominated as anethole.

2.4. Animals

The experiments were conducted with adult male Swiss mice (25–30 g) or Wistar rats (200–300 g) obtained from our local colonies maintained at the Federal University of Ceará. They were divided into groups of 6–8 animals per cage and were subjected to periods of light/dark of 12 h with access to water and food ad libitum. All experiments were performed in accordance with the Guide for Care and Use of Laboratory Animals (National Institute of Health, Bethesda, MD, USA) and were approved by the Ethics Committee in Research of the Federal University of Ceará (protocol nº.19/2008).

2.5. Acute Toxicity of Arthur de Carvalho Drops® in mice

Mice of both sexes (n = 6) were treated with a single oral administration of ACD at 250, 500, 1000 and 2000 mg/kg, p.o. (10 mL/kg), and the control group received only sterile water (vehicle). After the treatment, all groups were observed during the first 12 h for any alteration including the symptoms of mobility, posture, piloerection, respiratory pattern and for mortality. The mice were observed for fourteen days. Following treatment, their weights, feed and water intakes were registered. After 14 days of observation, blood was collected from the retro orbital plexus of the animals for biochemical parameters.

2.6. Evaluation of the effect of ACD on nausea and emesis induced by cisplatin in rats

Male rats (n = 6) were divided into groups and one week before starting the experiment, the animals were habituated to kaolin and feed. After this adaptation period, the animals received vehicle (water, 10 mL/kg, p.o), ACD (100, 200 and 400 mg/kg, p.o) or ondansetron (1 mg/kg, i.p.), and immediately after cisplatin (1 mg/kg, i.p.). The animals were offered standard feed and kaolin previously weighed and placed separately in the cages. After the 7-days period, the animals were again given drug and vehicle administration (cisplatin, ACD and ondansetron) and repeated for 5 weeks. Daily consumption of standard feed and kaolin was recorded and expressed in weekly values (g) [18].

2.7. Evaluation of the effect of ACD on castor oil induced diarrhea

Male mice (n = 8) maintained in 12 h solids fast were treated orally with vehicle (water), ACD (100, 200 and 400 mg/kg) or loperamide (20 mg/kg, positive control). After 60 min, the animals received castor oil (0.2 ml/animal, p.o). The animals were placed in individual boxes and 2 h after the administration of castor oil the amounts of feces, in mg, eliminated from each animal was recorded and expressed in terms of the amount of feces per kg of body weight of the animal [19].

2.8. Evaluation of the effect of ACD on secretory volume and total gastric acidity in rats with pyloric ligation

Male rats (n = 6) in 12 h solids fast were anesthetized with xylazine (10 mg/kg, i.p.) and ketamine (90 mg/kg, i.p.). After anesthesia, the abdominal cavity was surgically opened, and the pyloric ligation was performed with cotton thread. After the pyloric ligation, vehicle (water), ACD (100, 200 and 400 mg/kg) or cimetidine (2 mg/kg) were administered intraduodenally. After suturing to abdominal cavity closure, animals were housed in cages and 4 h after pyloric ligation, they were euthanized. The abdominal cavity was opened, the cardia was tied with the aid of cotton thread to avoid the loss of the gastric contents, and the stomach was removed, washed externally with saline, and opened by the great curvature and the gastric contents collected in the test tube. The volume obtained was centrifuged at 1500 rpm for 15 min. The gastric juice was collected and transferred to the beaker to determine the volume of gastric juice (mL). Total gastric acidity was determined by titrating the entire gastric juice content with 0.1 N NaOH using phenolphthalein [20].

2.9. Evaluation of the effect of ACD on normal gastrointestinal motility and on bethanechol-induced gastrointestinal transit in mice

Male mice (25–30 g) were used for the measurement of gastrointestinal transit index, which was assessed by means of the classical technique that consider the passage of a charcoal meal through the gastrointestinal tract in mice. Briefly, the animals were fasted for 24 h before the experiment, but were allowed unrestricted access to drinking water. Each animal received a quantity of charcoal (10% suspension with 5% arabian gum in distilled water) corresponding to 1% body weight (10 mL/kg intragastrical) 60 min after receiving ACD (100, 200 or 400 mg/kg) or vehicle (water) administered intragastrical. After 30 min, the animals were euthanized for removal of the gastrointestinal organs, from stomach to colon. The gut was carefully stretched along a meter stick on a plain tabletop and the distance travelled by the marker was measured and expressed as a percentage of the total intestinal length (from the pylorus to the caecum) according to the following equation:

$$Distance\ (\%\ of\ total\ length) = \frac{distance\ traveled\ by\ the\ marker}{total\ length\ of\ the\ small\ intestine}\ x\ 100$$

In addition, some animals were used to determine the effect of ACD on bethanechol-induced alteration of intestinal transit. The protocol adopted involved administration of castor oil (1% body weight) 60 min after dosing with either ACD (100–400 mg/kg, p.o.) or vehicle (water). In a manner like previous experiments, the charcoal meal was administered 30 min later and the experimental protocol for determination of intestinal transit was identical [21].

2.10. Isolated tissues

After animal euthanasia, longitudinal strips of duodenum and ileum were rapidly removed and immersed in a Petri-dish containing physiological salt solution (pH 7.4, at room temperature) to remove adhering fat and connective tissue. Thereafter, smooth muscle preparations were suspended in conventional isolated bath chambers (5 mL glass organ bath containing physiological salt solution; pH 7.4; 37 °C; continuous bubbling with 5% CO2 in O2). The strips were tied at both ends by thread and then suspended longitudinally in organ bath for tension measurement. Changes in muscle tension were measured isometrically by attaching the upper end of the ring or strip to a force transducer (Grass, model FT03, USA) connected to a digital data acquisition system (Dataq, model PM-1000, USA) and were expressed in g. Resting tension was 1 g.

2.11. Experimental protocols

After the equilibration period (1 h) all tissues were repeatedly stimulated with 60 mM K+ to evaluate the tissue viability and preparations without reproducible contractions were discarded. This procedure was executed until observation of two successive contractions with similar amplitude to begin the experiment. These contractions also served as reference for expression the results showed in this study.

Experiments were performed with smooth muscle preparations maintained under resting tonus or on the steady state of a sustained contractile stimulus induced by a pharmacological agent (carbachol, 1 μM). All concentration-effect curves were obtained by exposing the preparation to increasing concentrations of the ACD (1–3000 μg/mL), carbachol (0.01–10 μM) or scopolamine (1–3000 ng/mL) which were added to the bath and maintained at a given concentration during 4–5 min. Scopolamine was used as positive control for comparison with ACD.

To measure the relaxation of muscle tonus, a piece of tissue was set up and exposed to ACD and the relaxation was taken as the difference between baseline observed at a given concentration and the baseline immediately before the ACD addition. Similar experiments were performed using only the vehicle (water) at equivalent concentrations that dissolved ACD. Contractions were measured at the peak deflections. In other experiments, preparations were maintained in Ca2+-free medium containing EGTA (1 mM). Under such conditions, the preparations were then stimulated with high K+ (60 mM), acetylcholine or carbachol in absence or in presence of ACD to study its effects on the cellular pathways involved in the cytosolic Ca2+ dynamics.

2.12. Statistical analysis

Data are expressed as mean ± standard error of the mean. Values of EC50 or IC50, i.e. the concentration that can be expected to cause a defined effect on 50%, were shown as geometric mean [95% confidence interval]. Significance of the results was determined using

one- or two-way analysis of variance (ANOVA) and when significant, it was followed by a multiple comparison. Comparison of IC50 values was performed by the Mann-Whitney test. Statistical significance was accepted when $p < 0.05$.

3. Results

3.1. Validation of a HPLC method to quality control of the phytomedicine Arthur de Carvalho Drops (ACD) based on M. recutita, G. lutea and F. vulgare

The analysis of secondary metabolites (chemical markers) in phytomedicines are essential for the quality control of these. In this context, it was investigated the chromatography profile and content of secondary metabolites/bioactive markers in ACD by HPLC through a validated analytical method.

The chromatography method was linear in the range studied with a good correlation coefficient (apigenin-7-glucoside-APG / r = 0.9994; gentipicroside-GTP/ r = 0.9975 and anethole-ANT/ r = 0.9969) and the following straight-line equation: APG: y = 2E+07x – 34707; GTP: y = 7E+06x – 605.8 and ANT: y = 1E+08x + 1202.5. The procedure proved to be specific, and precision and accuracy analysis showed low relative standard deviation (maximal 4.7%) and a good recovery percentage (97.2–98.2%).

The HPLC analysis permitted to determine the chromatography profile of ACD and quantify simultaneously three bioactive markers in it. The retention time for GTP, APG and ANT were 14.1; 26.2 and 43.0 min, respectively (Fig. 1). The contents of GTP, APG and ANT in ACD were of 1.51 (0.4%), 0.22 (0.9%) and 0.05 (2.3%) mg/mL, respectively.

3.2. Acute toxicity study

The acute treatment of the mice (both sexes) with ACD (250–2000 mg/kg, p.o) did not cause the death of the animals during the period of observation (14 days). However, 17% of the animals (male) treated with ACD (500 mg/Kg) presented sleepiness for up to 90 min after the treatment with phytomedicine, while the females (ACD 500 and 1000 mg/Kg) presented diarrhea and at times, piloerection. The treatment with ACD, at a greater dose (2000 mg/Kg), induced piloerection, lethargy and sedation in all male mice, while the females presented wheezing and lethargic behavior. Therefore, based on these results, the DL_{50} of ACD when administrated orally seems to be above 2 g/Kg.

Until the 14th day after the acute treatment of the animals with ACD, it was not observed a significant interference in the corporal weight gain of the groups treated when related to control group. The biochemical analysis (urea, creatinine, glucose, AST and ALT) of the animals treated with ACD group (2 g/Kg) realized on the 14th day after the treatment did not interfere significantly in the majority of the parameters. Only the glucose levels of the male mice treated was significantly elevated when compared to the control group (data not shown). Given the results obtained, it was then investigated the possible pharmacological effect of ACD (non-toxic doses) in gastrointestinal system including experimental models of emesis and diarrhea.

3.3. Effect of ACD on nausea and emesis induced by cisplatin in rats

For the evaluation of the effect of the ACD on dyspepsia, animals were tested on the parameter p̈ica(̈the behavior of ingestion of non-nutritive substances), a parameter observed in rodents with induction of emesis. In this stage a special type of feed was prepared, kaolin (pellets of 1% of arabica gum/water and kaolin) for the measurement of this behavior [22]. The effects of cisplatin 1 mg/kg/week on food ingestion are shown in Table 1. Cisplatin 1 mg/kg induced a significant reduction on food ingestion in rats without kaolin when related to not treated group for ACD (100–400 mg/kg, p.o.). On the other hand, after exposure of the animals to cisplatin, a significant increase on kaolin ingestion (vehicle/control group: 2.5 ± 0.48 g) was observed when compared to not treated group (1.5 ± 0.51 g) corresponding an increase of 66%. The treatment of the animals with ACD since from the lowest doses (100 and 200 mg/kg) abolished the increase on kaolin intake (0.7 ± 0.14 and 0.7 ± 0.10 g, respectively) reaching values lower than not treated group.

Table 1
Effect of ACD on altered feeding behavior induced by long-term cisplatin in rats: an experimental model of nausea and emesis.

		Feed Consumption	
Group	Dose (mg/kg)	No Kaolim (g)	Kaolin (g)
Not treated	–	167 ± 10.7	1.5 ± 0,51
Control	–	124.0 ± 2.8[a]	2.5 ± 0.48[a]
ACD + Cisplatin	100	112.4 ± 4.5[a]	0.7 ± 0,14[a,b]
	200	109.0 ± 4.2[a]	0.7 ± 0.10[a,b]
	400	110 ± 5.2[a]	1.3 ± 0.29[a,b]
Ondansetron + Cisplatin	1	108.5 ± 4.6[a]	1.64 ± 0,46[a,b]

Rats (n = 6) received vehicle (10 ml/kg, p.o, control group), ACD (100, 200 and 400 mg/kg, p.o) or ondansetron (1 mg/kg, i.p.) and immediately after received cisplatin (1 mg/kg, i.p.) once weekly for 5 weeks. Values represent the mean ± S.E.M. of the standard feed intake with or not kaolin for 5 weeks. [a] vs not treated group; [b] vs control group (ANOVA, Tukey test).

Table 2
Effect of ACD on the model of induced diarrhea by castor oil in mice.

GroupDose (mg/kg)Feces removed (g)		
Not treatedControl ACD	–	77.89 ± 6.19
	100	191 ± 14,4[a]
		346,3 ± 56,1[a,b]
	200	295,9 ± 57,8[a,b]
	400	379,3 ± 63,0[a,b]
Loperamide	20	12,5 ± 2,32

The animals (mice, n = 6) were orally treated with vehicle (control), ACD (100, 200 and 400 mg/kg) or loperamide (20 mg/kg) and after 60 min 0.2 ml castor oil. Results are expressed as mean ± S.E.M. of the amount in grams of feces eliminated by the animals after the administration of the phytotherapic. [a] $p < 0,05$ vs loperamide; [b] $p < 0,05$ vs not treated (ANOVA, Tukey test).

3.4. Effect of ACD on castor oil-induced diarrhea

Castor oil is reported to induce diarrhea via increasing the volume of intestinal content by prevention of reabsorption of water [23]. The administration of castor oil in rats produced copious diarrhea increasing in 2.4 times the weight of stools when compared to not treated group. The pre-treatment of the animals with ACD (100, 200 and 400 mg/kg) significantly increased the castor oil-induced diarrhea. On the other hand, loperamide (2 mg/kg), standard drug, showed a potent antidiarrheal activity (Table 2).

3.5. Effect of ACD on secretory volume and gastric acidity in rats

Increased hydrochloric acid secretion, as well as alterations in mucosal integrity and gastric cytoprotection factors may contribute to the pathogenesis of peptic ulcer, one of the symptoms associated with dyspepsia [24]. The administration of the ACD (100–400 mg/kg, p.o.) did not significantly alter the secretory gastric volume (1.30 ± 0.08, 1.70 ± 0.16, 1.52 ± 0.26 mL, respectively) or total gastric acidity (17.92 ± 4.67; 21.25 ± 4.37; 14.58 ± 2.53 μEq [H+]/h, respectively) in rats with pyloric ligation when compared to control group (1.62 ± 0.25 mL and 12.00 ± 1.46μEq [H+]/h, respectively). Cimetidine (standard drug) significantly reduced secretory

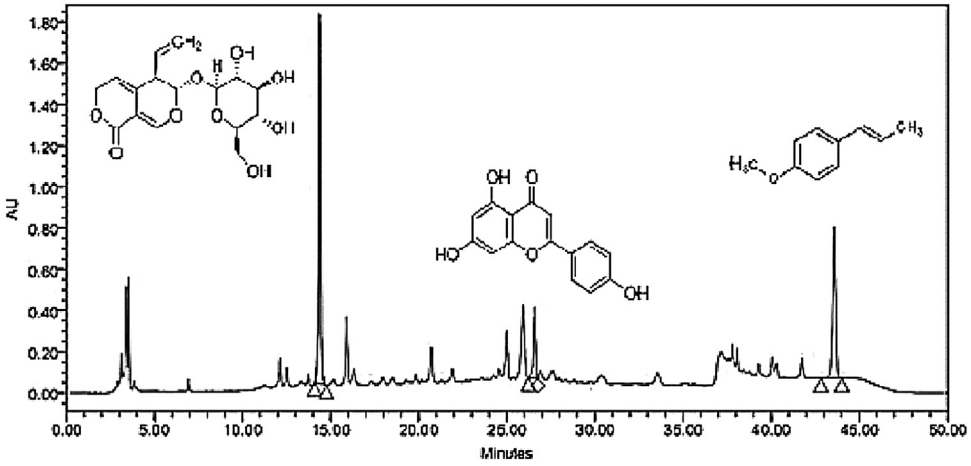

Fig. 1. Chromatogram generated by the HPLC-PDA system for ACD. Gentiopicroside (A); apigenin (B) and anethole (C). Conditions: (Phenomenex ® RP-8, 250 x 4.6 mm x5 μm), mobile phase (0.1% TFA/H2O (A), ACN (B) and THF (C)) (gradient elution at 40 °C), The wavelengths selected were 273 nm, 267 nm and 259 nm, varying according to the retention time of each component.

volume (0.66 ± 0.13 mL) and total gastric acidity (6.07 ± 0.50μEq [H +]/h) when compared to control group.

3.6. Effects of ACD on the intestinal transit of mice

The use of activated charcoal, a very useful tool in the pharmacological evaluation of a new drug, capable of determining its possible inhibitory or stimulatory effect on peristaltic activity. The oral administration of ACD (100–400 mg/kg) did not alter intestinal transit index under normal conditions (Fig. 2a). However, the morphine (2.5 mg/kg, s.c.), standard drug, reduced significantly (36.2 ± 2.4%) the distance travelled by the marker when related to control group (59.6 ± 3.4%). Animals that received bethanechol (10 mg/kg, s.c) increased the distance achieved by the marker to 80.5 ± 2.7%, value significantly higher than 58.8 ± 1.0% in control group (Fig. 2b). In contrast, animals treated conjunctly with bethanechol and ACD (100, 200 and 400 mg/kg) showed decreased values in comparison with bethanechol-treated animals, whose magnitude was comparable to the distance observed in control group. Treatment with atropine (5 mg/kg, i.p.) further reduced the index of intestinal transit in bethanechol-treated mice to a level significantly lower than that observed in control animals (38.9 ± 1.0%). Following the study, it was investigated if ACD affects the intestinal smooth muscle of rats.

3.7. Relaxant effect of ACD on the sustained contraction induced by carbachol on strips of rat duodenum

To determine the mechanism of the reduction of contractility in strips of rat duodenum of ACD, we conducted most of the experiments using carbachol as the contractile agent. Duodenal preparations maintained under basal tonus contracted typically after stimulation with carbachol (1 μM), showing a biphasic response characterized by an initial peak (phasic contraction) that relaxed slightly and was followed by a sustained phase, which remained stable during the time that carbachol was present in the extracellular solution (Fig. 3a, Control). In separate sets of experiments, duodenal preparations relaxed, in a concentration-dependent manner, when they were exposed to increasing concentrations of ACD (1–3000 μg/mL; Fig. 3b) or scopolamine (1–3000 ng/mL; Fig. 3c), standard drug. In both cases, relaxation was complete and significantly exceeded the tonus recorded under basal conditions. Maximal relaxation was similar comparing preparations treated with ACD or scopolamine and

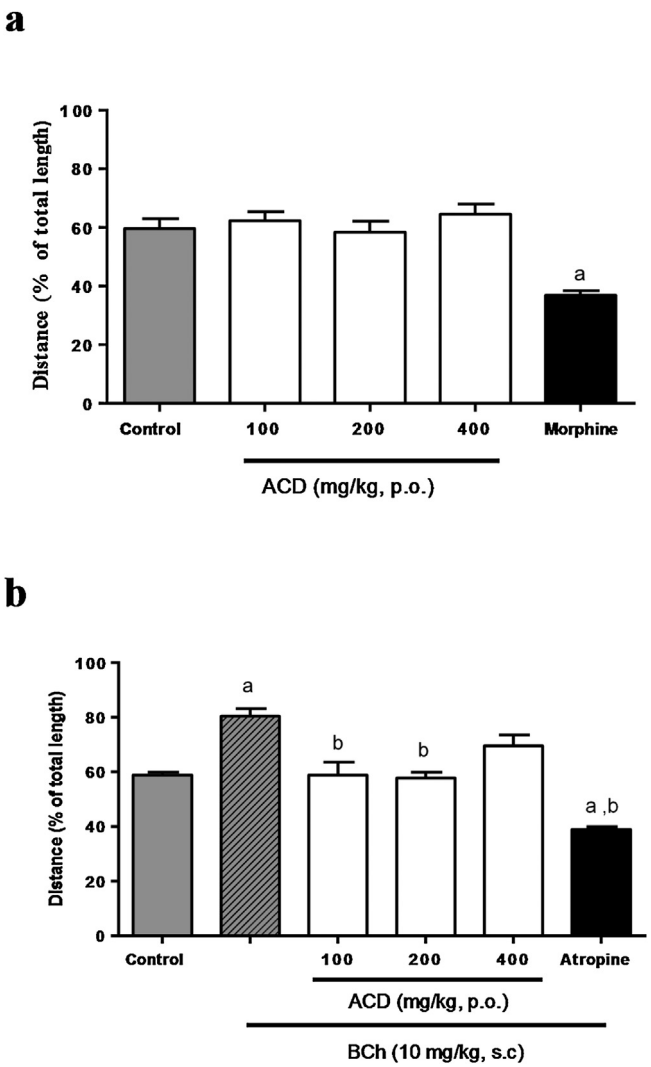

Fig. 2. Effects of ACD on gastrointestinal transit of mice. Gastrointestinal transit was measured by the passage of a charcoal meal through the gastrointestinal tract in mice under normal conditions (panel a) or under bethanechol- increased gut motility (panel b). Bar indicates the distance achieved by the marker in animals treated with vehicle (Control group) or ACD (100, 200 or 400 mg/kg). Morphine (2.5 mg/kg, s.c.) and atropine (5 mg/kg, p.o.) were used as positive controls. [a] vs Control; [b] vs BCh alone (ANOVA, Tukey test, $p < 0.05$).

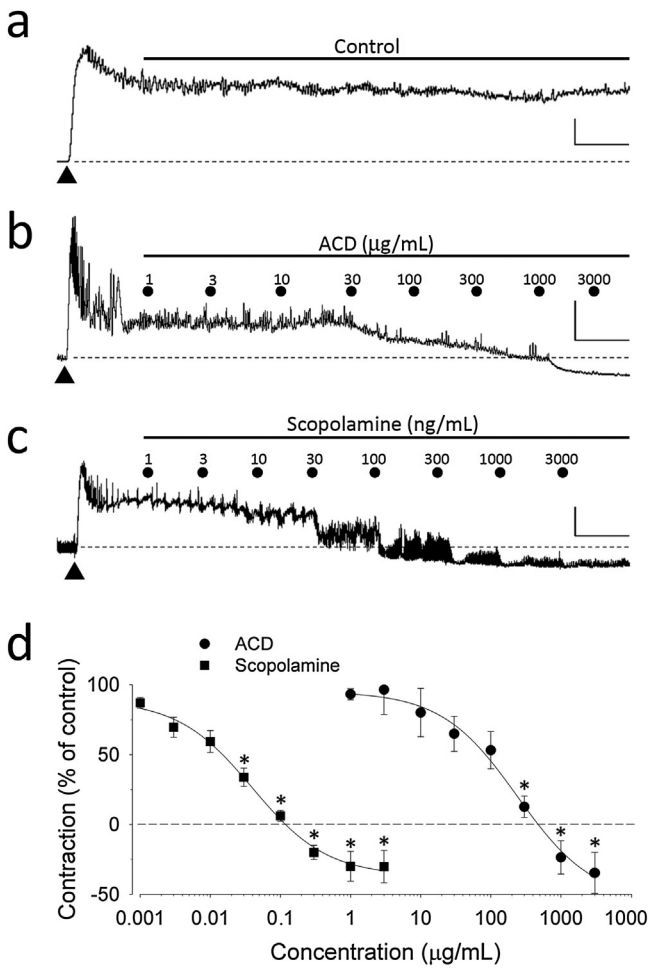

Fig. 3. Relaxant effect of ACD on the sustained contraction induced by carbachol on strips of rat duodenum. Panels a to c show experimental traces of duodenal strips treated with carbachol (1 μM; ▲) for several minutes to produce a contraction that was stable during the time that carbachol was present in the extracellular solution (panel a). When treated with ACD (1–3000 μg/mL; panel b) or scopolamine (1–3000 ng/mL; panel c), duodenal preparations relaxed to a level beyond basal tonus indicated by dotted lines. Calibration: vertical = 0.5 g; horizontal =5 min. In panel d, one can see the mean values of these relaxant effects. *, $p < 0.05$ compared with the value observed in the plateau of the carbachol-induced response in before addition of ACD or scopolamine.

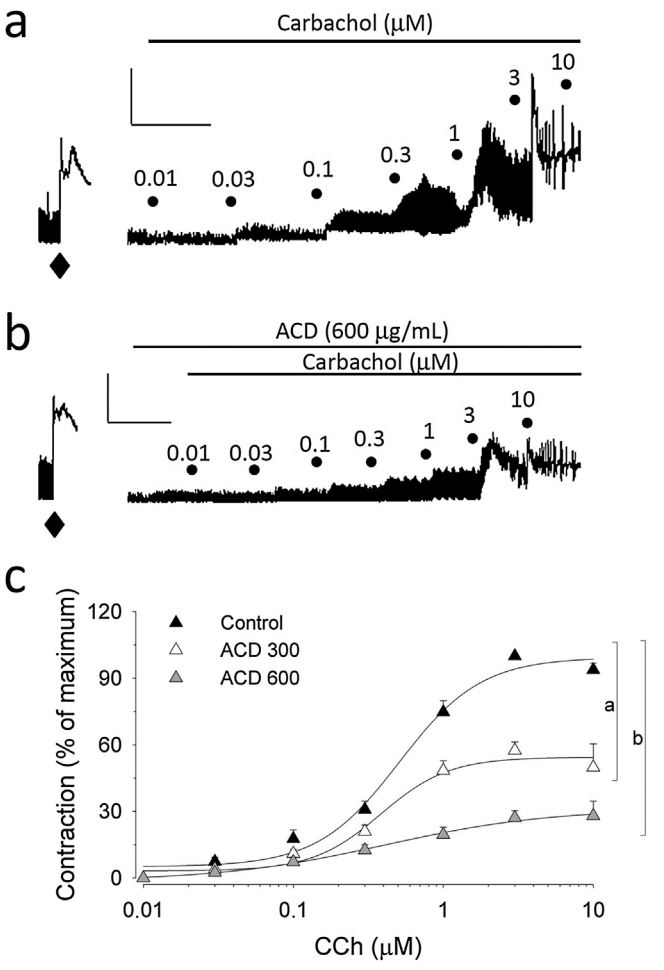

Fig. 4. Inhibitory effect of ACD on the concentration-effect curve induced by carbachol in strips of rat duodenum. Panels a and b show experimental traces of duodenal strips receiving increasing concentrations of carbachol (0.01–10 μM) in absence (panel a) or in presence of ACD (600 μg/mL; panel b). All traces show an initial K^+ (60 mM; ▲)-induced contraction as reference to indicate that maximal effect achieved by carbachol was lower in presence of ACD than in its absence. Calibrations: vertical = 0.5 g; horizontal =5 min. In panel c, graph showing the mean values for the inhibitory influence of ACD applied at 300 (white triangle) or 600 (gray triangle) μg/mL on the concentration-effect curve induced by carbachol. a and b, $p < 0.05$, compared to control, two-way ANOVA and Holm-Sidak test.

reached -34.7 ± 14.7% (n = 6) and -30.2 ± 11.6% (n = 8), respectively, taking as reference the amplitude of the carbachol-induced sustained phase under control conditions (negative values indicate that experimental trace was beyond basal level; Fig. 3d). Notwithstanding, values of IC_{50} were significantly lower for scopolamine (11.0 [4.4–27.4] nM) than for ACD (60.7 [16.4–224.4] μM).

Some duodenal preparations were treated with L-NAME (50μM), but the relaxing properties of ACD (10–3000 μg/mL) did not change because its concentration-effect curve showed values for IC_{50} of 105.6 [33.6–331.7]μg/mL and maximal relaxing effect of -22.3 ± 4.0%, which did not achieve significant difference compared to values observed in preparations in absence of L-NAME.

3.8. Inhibitory effect of ACD on the concentration-effect curve induced by carbachol in strips of rat duodenum

Full concentration-response curves for carbachol (0.01–10 μM) were constructed showing concentration-dependent contractions. In ACD-untreated duodenal strips (Fig. 4a), tissues displayed contractile responses to carbachol with maximal effect observed at 3 μM and EC_{50} of 0.26 [0.21-0.33] μM (n = 9). In tissues treated with ACD (Fig. 4b), maximal effect was significantly reduced, reaching levels correspondent to 57.5 ± 3.7% and 27.1 ± 3.2% of the maximal effect observed in control conditions, when preparations were maintained in presence of 300 and 600 μg/mL of ACD, respectively (Fig. 4c).

3.9. Inhibitory effects of ACD on the contractions induced by stimulation of the transmembrane Ca^{2+} influx through voltage- or receptor-operated pathways

Under Ca^{2+}-free conditions (Tyrode solution without $CaCl_2$), duodenal strips were stimulated with either K^+ (60 mM; Fig. 5a) or carbachol (1μM, in presence of verapamil (1 μM); Fig. 5b) producing both small contractions that rapidly returned to the basal levels observed before addition of K^+ or carbachol. Subsequently and still in the presence of a contractile agent, Ca^{2+} (0.1–15 mM) was cumulatively added and concentration-response curves were constructed (n = 6) either in absence or in presence of ACD (600 μg/mL). ACD changed the profile of the concentration-response curve to Ca^{2+} because it reduced significantly the maximal effect for Ca^{2+} (at 10 mM) to 15.5 ± 8.2% in preparations stimulated with K^+ and

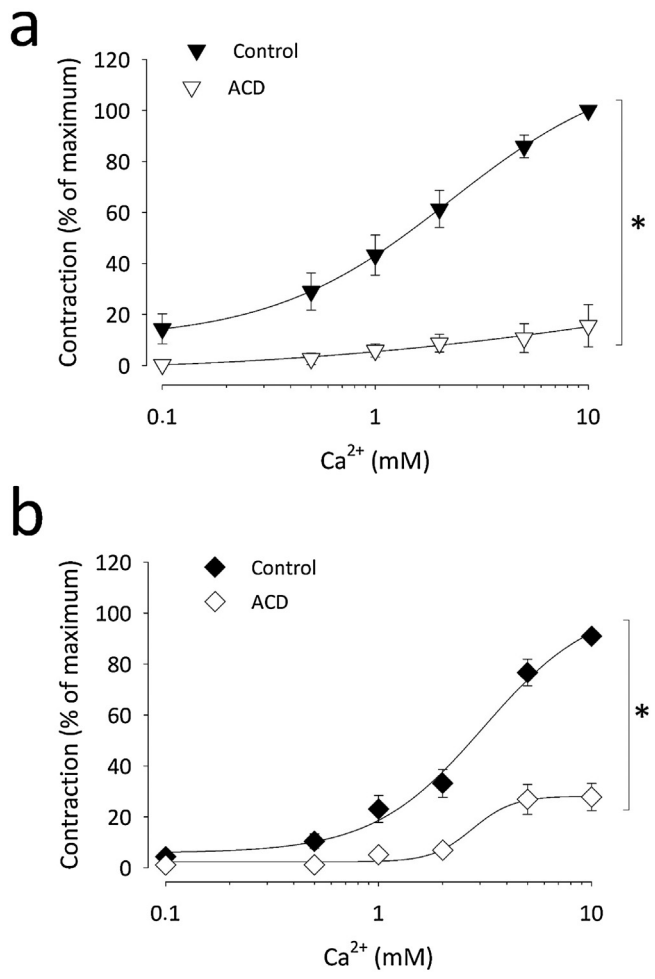

Fig. 5. Inhibitory effects of ACD on the contractions induced by stimulation of the transmembrane Ca^{2+} influx through voltage- or receptor-operated pathways. Graphs showing the inhibitory effects of ACD (600 μg/mL) on the contractions induced by stimulation of duodenal strips under Ca^{2+}-free conditions with either K^+ (60 mM; panel a) or carbachol (1 μM in presence of verapamil also at 1 μM; panel b) to produce opening of Ca^{2+} entry pathways. After a short period, Ca^{2+} was cumulatively added to the extracellular medium and a concentration-dependent contraction was observed in absence (black symbols) or in presence of ACD (open symbols). *, $p < 0.05$, two-way ANOVA and Holm-Sidak test.

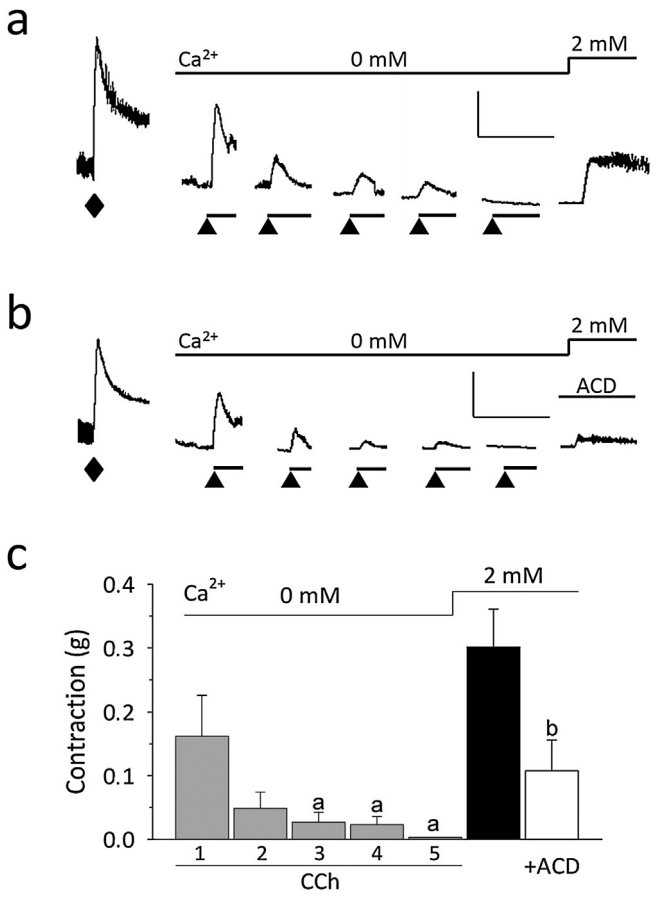

Fig. 6. Inhibitory effects of ACD on the contractions induced by activation of store-operated Ca2+ influx in isolated duodenal preparations. Panels a and b show experimental traces of duodenal strips submitted to Ca^{2+}-free conditions being repeatedly stimulated with a high concentration of carbachol (100 μM; ▲) to deplete intracellular Ca^{2+} stores. Bar above triangle indicates the time of exposition to carbachol in Ca^{2+}-free medium. After abolishment of phasic contractile responses, carbachol was removed by bath washing with Ca^{2+}-free solution. Then, Ca^{2+} (2 mM) was added to the extracellular medium and a sustained contraction was recorder in absence (panel a) or in presence (panel b) of ACD (600 μg/mL). All traces show an initial K^+ (60 mM; ♦)-induced contraction only as reference. Calibrations: vertical = 0.3 g; horizontal =5 min. In panel c, graph with the mean values of the decreasing responses of repeated stimulation (5 subsequent applications) of carbachol in Ca^{2+}-free medium to deplete internal stores (gray bars) followed by the contractions induced by Ca^{2+} restoration in extracellular medium in absence (black bar) or in presence (white bar) of ACD (600 μg/mL). a, $p < 0.05$ compared to value of gray bar (nº 1), Holm-Sidak test; b, $p < 0.05$, compared to black bar, Student's t test.

37.3 ± 5.6% in preparations stimulated by carbachol, in comparison to maximal force developed by each contractile agent in absence of ACD.

3.10. Effects of ACD on the contractions induced by activation of store operated Ca^{2+} influx in isolated duodenal preparations

Under Ca^{2+}-free conditions, duodenal preparations were repeatedly stimulated with carbachol until phasic contractile responses were no longer observable (Fig. 6, panels a and b). Repeated stimuli in Ca^{2+}-free medium showed significant decrease in the amplitude of this carbachol-induced contraction, and such event indicated full Ca^{2+} internal store depletion. Afterwards, carbachol was removed by replacement of the extracellular solution by another without this muscarinic substance, but still maintaining the tissue under Ca^{2+}-free conditions. Then, extracellular Ca^{2+} (2 mM) was restored and a sustained contractile response was observed with amplitude corresponding to 0.30 ± 0.06 g (n = 6). Such contractile response was significantly reduced to 0.11 ± 0.05 g in presence of ACD (600 μg/mL) (Fig. 6c).

3.11. Effects of ACD on the phasic contractions of isolated duodenal preparations maintained under Ca^{2+}-free conditions

Some duodenal strips contracted in response to acetylcholine (3μM) addition in bath chamber with amplitude corresponding to 1.13 ± 0.09 g (n = 24) (Fig. 7). When they were submitted to Ca^{2+}-free conditions (solution without $CaCl_2$ containing 1 mM EGTA) acetylcholine (3μM) produced typical unstained phasic contractions that corresponded to 0.53 ± 0.05 g (n = 24), value significantly lower than that recorded when acetylcholine was added in Ca^{2+}-containing medium ($p < 0,05$, Student's t test). On the other hand, tissues treated with ACD (300, 600 or 1000 μg/mL) showed phasic contractions corresponding to 0.47 ± 0.11 g (n = 8), 0.25 ± 0.05 g (n = 8) and 0.10 ± 0.02 g, respectively, in response to acetylcholine (3μM). Such values reached significant difference at 600 and 1000 μg/mL, compared to acetylcholine-induced response in Ca^{2+}-free medium in absence of ACD.

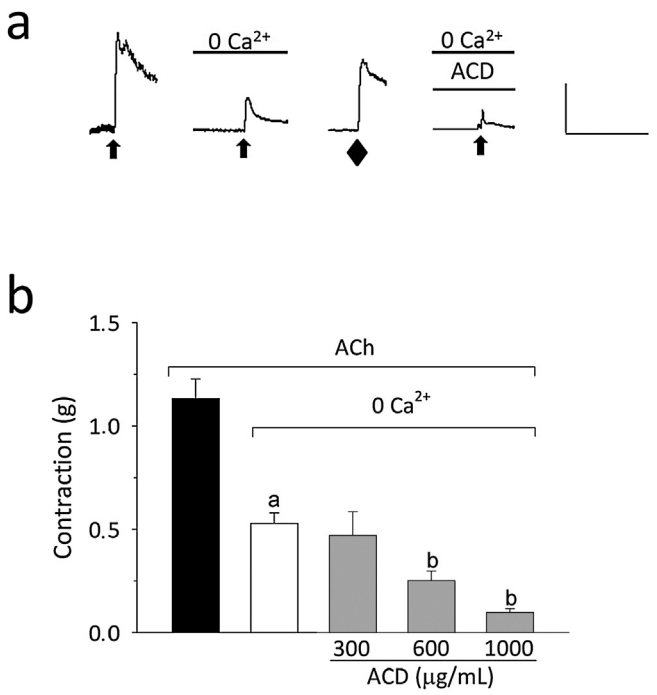

Fig. 7. Effects of ACD on the phasic contractions of isolated duodenal preparations maintained under Ca^{2+}-free conditions. Panel a shows experimental trace of duodenal strip being initially stimulated with acetylcholine (3 μM; ↑) in Ca^{2+}-containing or in Ca^{2+}-free (0 Ca^{2+}) medium. Note the reduction of the contractile response in Ca^{2+}-free medium. An intermediary K^+-induced contraction was performed in Ca^{2+}-containing medium to replenish Ca^{2+} stores. Afterwards, acetylcholine (3 μM) was added again in Ca^{2+}-free medium in presence of ACD (1000 μg/mL), which produced a decreased contraction compared to that observed in absence of ACD. Calibrations: vertical = 0.5 g; horizontal =5 min. In panel b, graph showing the mean values of the acetylcholine (ACh)-induced contractions in Ca^{2+}- containing (black bar) or Ca^{2+}-free (0 Ca^{2+}) medium in absence (white bar) or in presence of ACD (300–1000 μg/mL). a, $p < 0.05$ compared to values of black bar; b, $p < 0.05$, compared to values in white bar; Holm-Sidak test. Ach: acetylcholine.

4. Discussion

To contribute for validation of medicinal use of the standardized phytomedicine Arthur de Carvalho Drops (ACD) on functional gastrointestinal disorders (FGIDs), this study was designed to determine its effect on gastric secretory volume, gastric acidity, emesis, diarrhea, intestinal transit and smooth muscle using the in vivo and in vitro assays.

The acute treatment with ACD on animals of both sexes by the oral route did not induce death. However, it at the highest doses induced changes in clinical signs of the animals, such as diarrhea, sedation, piloerection, lethargy and analgesia. These effects are corroborated by previous works [25–27] which showed that the extracts or chemical constituents from medicinal plants present in ACD (chamomile, fennel or gentian) have effects in gastrointestinal and central nervous systems. According to [28] the chamomile extract was able to significantly reduce intestinal motility and has a sedative effect, while the administration of fennel, also induced sedation and increased spontaneous gastric motility and gastric acid secretions in rodents.

The blocked of the nausea and emesis induced by cisplatin in rats by ACD was greater than that showed with well-characterized anti-emetic ondansetron (5-HT_3 antagonist) [29]. However, ACD did not interfere in the reduction intake of normal feed induced by cisplatin.

Rats and mice which are very convenient laboratory animals for initial trials of new medicines do not have an emetic reflex. However, several emetogenic stimuli like cisplatin can induce an altered feeding behavior in these animals called pica, which consisting of the ingestion of non-nutritive foods, such as kaolin [22]. Therefore, kaolin consumption has been evaluated as an index of cisplatin-induced emesis in rats, while normal feed consumption as an indicator of anorexia nervosa. Similar to nausea and emesis in humans, the pica in rats is associated with multiple mechanisms, including serotonin release from the enterochromaffin cells, increased c-fos labelling in the area postrema and the *nucleus tractus solitarius*, and a delay in gastric emptying. The ACD inhibited feeding behavior against cisplatin-induced pica in rats probably due a synergic effect of its herbal extracts through their active principles, such as gentiopicroside and anethole which have been shown the ability to restore the delayed gastric emptying in rats [30]. Corroborating with our results, a clinical study with the Lomatol® product (preparation composed of extracts of *Carum carvi*, *Foeniculum vulgare*, *Mentha piperita* and *Artemisia absinthium*) showed to be quite effective in the treatment of patients with gastrointestinal disorders when compared to the use of metoclopramide, a dopaminergic antagonist [31].

Among the typical symptoms present in gastrointestinal disorders we may abdominal discomfort, flatulence, nausea, vomiting, and change in bowel habit [32]. In this sense, continuing the study of ACD, its effect on the diarrhea induced by castor oil in mice was investigated. The pre-treatment of the animals with ACD increased the castor oil-induced diarrhea.

Castor oil-induced diarrhea is especially related to the presence of ricinoleic acid, which acts to increase peristaltic activity and alter the permeability of the membrane to water and electrolytes, reducing Na^+ and K^+ uptake and decreasing Na^+ and K^+ ATPase activity [33]. Furthermore, there is a consensus that castor oil induces diarrhea by stimulating prostaglandin synthesis [19]. In the present study, the widening of the diarrheal effect of castor oil by ACD may involve one or more mechanisms of action promoting diarrhea. Previous studies determined the laxative effect of fennel as well as of chamomile on the experimental model of diarrhea induced by castor oil [34]. However, other study with chamomile decoction extract showed the antidiarrheal effect of non-standardized aqueous extract. This contradictory effect is possibly related to nature of the plant extract, which differed among other aspects in the type of extractive solvent (aqueous) when compared to extract of chamomile (hydroethanolic) used in the formulation of ACD. In addition, aqueous extract of chamomile is probably rich in tannins, a secondary metabolite class known by its antidiarrheal potential. Thus, at least part of the effect of the ACD are related to the presence of chamomile and fennel in the herbal formulation.

Since the increased secretion of hydrochloric acid may contribute to the pathogenesis of peptic ulcer, one of the symptoms associated with dyspepsia, a FGID [35], it was investigated the effect of ACD on gastric secretion volume and gastric acidity in rats. However, the oral treatment of the animals with ACD did not significantly interfere in the gastric secretion volume and gastric acidity in rats submitted to the pylorus attachment model. However, there are several studies showing the gastroprotective effect these species. Chamomile extracts has anti-ulcer activity in rats related partially to its antioxidant properties. Methanol extract from *G. lutea* have protective effect toward gastric ulcers induced by aspirin plus pyloric ligation on ethanol-induced gastric in rats, and its active principle (gentiopicroside) contribute to this activity. Furthermore, aqueous extract of fruit fennel also has gastroprotective effect and antioxidant properties [36]. Thus, although the three species that compound ACD formulation have gastroprotective effect, together they did not show a synergic activity on gastric secretion volume and gastric acidity.

We investigated the possible effects of ACD on gastrointestinal motility, and it was observed that the oral administration of this herbal medicine did not induce alteration in the normal intestinal

transit of mice. ACD also reduced the bethanechol-enhanced gastrointestinal transit indicating that such compound may be useful in conditions that involve increased motor input to the gut.

Bethanechol is a selective cholinergic agonist for muscarinic receptors, resistant to hydrolysis by acetylcholinesterase, and considered a prokinetic drug capable of increasing peristaltic activity of the gastrointestinal tract [37]. Also, it was demonstrated that methanol extracts of *M. recutita* has inhibitory activity in model of hyperperistalsis intestinal in rats [38]. It is reasonable to think that its pharmacological actions may be due to direct influence on gut smooth muscle behavior. In vitro, such direct actions were confirmed since ACD inhibited the contractile activity of isolated duodenal preparations stimulated to contract by various stimuli. Taken together, the findings reported herein corroborate the general comprehension that such plant species have pharmacological properties that confer their abilities to treat gut dysfunctions [39]. In addition, apigenin, the bioactive flavonoid of chamomile, has an inhibitory action on intestinal transit, as well as accumulation of intraluminal fluid, besides having antidiarrheal activity in rats [40].

Despite the widespread use of these species to treat gut dysfunctions, information regarding their mode of action is yet scarce. Then, the present findings reveal a few aspects of the underlying mechanism involved in the inhibitory actions of ACD on motor gastrointestinal behavior, as showed in bethanechol-treated mice. Using isolated rat duodenum as experimental model, it was showed that ACD is a myorelaxant agent because it completely reversed the contraction promoted by cholinergic stimulation using carbachol, a cholinergic agent analog of acetylcholine, the main parasympathetic neurotransmitter in gut [41]. The maximum amplitude of relaxation induced by ACD was like that obtained with the well-characterized smooth muscle relaxant scopolamine [42], although with lesser potency. Once added to the bath solution, cholinergic stimulus produces vigorous contractions, which are characterized by a first phasic peak followed by a sustained phase. While the phasic peak is related to Ca^{2+} release from the internal stores such as the sarcoplasmic reticulum, the sustained phase is related to the transmembrane Ca^{2+}influx from the extracellular milieu through Ca^{2+}-permeable ion channels found in plasmalemma [43]. With such concepts in mind, the study was then conducted in order to understand how this compound produces inhibitory actions on motor behavior of intestinal cells.

Initially, a putative anticholinergic action of ACD in inhibiting the contractile behavior of the rat gastrointestinal system is unlikely because it showed a similar efficacy to inhibit smooth muscle contractions induced by electromechanical (with high K^+ concentration) or pharmacomechanical (with carbachol) coupling [44]. Under such conditions, is expected that an anticholinergic agent will inhibit carbachol-induced pharmacomechanical coupling more selectively than those electromechanical events elicited by K^+-enriched depolarizing solutions. In addition, ACD reduced the distance travelled by the marker after stimulation with bethanechol to an intermediary level (like control animals), whereas a typical anticholinergic agent such as atropine promoted a more significant reduction of the gastrointestinal transit index, reaching levels beyond of those obtained in animals treated only with the vehicle [45]. Thus, a selective blockade of muscarinic receptors on gastrointestinal smooth muscle does not explain the inhibitory effects of ACD on gastrointestinal transit as described herein.

Since contractions induced by Ca^{2+} addition in electromechanical and pharmacomechanical coupling were similarly inhibited with a same ACD concentration, it is also unlikely that ACD may be acting merely as a voltage operated Ca^{2+} channel blocker. The experiments showed in Fig. 4 were designed to use duodenal tissues under Ca^{2+}-free conditions in presence of a typical voltage-gated Ca^{2+} channel blocker such as verapamil [46], which was used at a concentration that completely inhibit contractions induced by high K^+ solutions (data not shown). So, the contractions observed after Ca^{2+} addition in combination with carbachol may be attributed to Ca^{2+} influx through receptor-operated pathways [47]. Because such contractions were as inhibited as that using K^+ as contractile stimulus, it is reasonable to conclude that ACD impairs with similar efficacy contractions of duodenal smooth muscle mediated by either voltage- or receptor-gated Ca^{2+} channels.

The role of store operated Ca^{2+} channels in the inhibitory effects of ACD was also investigated. It is well known that Ca^{2+} depletion from its intracellular stores located mainly in sarcoplasmic reticulum can produce opening of Ca^{2+}-permeable ion channels that are named store-operated channels [48]. Then, after complete depletion, Ca^{2+} restoration promotes vigorous contractions even in the absence of a contractile agent as observed in Fig. 5. Since such contractions were significantly reduced after treatment of intestinal tissues with ACD, it is reasonable to conclude that this phytotherapic compound is also able to inhibit store- mediated contractions with a same efficacy that it inhibited contractions induced by voltage- or receptor-operated pathways.

At this point, it should be considered that all pathways discussed until now (voltage-, receptor- or store-gated channels) have a common feature: they are structurally important plasmalemmal components in smooth muscle cells [49]. Thus, if ACD showed similar efficacy to inhibit contractile events that are mainly located in the plasmalemma, it is reasonable to conclude that its inhibitory properties may reflect an ability to depress intestinal smooth muscle contraction at some stage distal to the structural components found in cell membrane surface.

To address such hypothesis, we investigated the inhibitory effects of ACD on the contractile response mediated by the Ca^{2+} release from its intracellular stores. Duodenal tissues were initially maintained in Ca^{2+}-free conditions (in solution without $CaCl_2$ containing a high concentration of EGTA to assure the reduction in extracellular Ca^{2+} levels [50]. Under such conditions, phasic contractions are also observed after the addition of acetylcholine, which are due to inositol triphosphate (IP_3)-mediated Ca^{2+} release from sarcoplasmic reticulum in response to activation of M_2/M_3 muscarinic receptors [51]. Such phenomenon is feasible since no significative Ca^{2+} amounts are available to enter cytosol from the EGTA-enriched extracellular medium. It is noteworthy that repeated stimuli applied to smooth muscle preparations maintained in Ca^{2+}-free conditions show decreased responses that may be explained by the gradual depletion of the internal stores [52]. As tissues treated with ACD showed decreased responses after acetylcholine in Ca^{2+}-free medium, we can conclude that ACD is able to inhibit contractile events mediated intracellularly.

Since nitric oxide is an important inhibitory mediator causing myorelaxant effects in gastrointestinal system [41], some experiments were designed in presence of the nitric oxide synthase inhibitor L-NAME. However, the myorelaxant effects of ACD did not suffer significative changes since neither IC_{50} nor maximal relaxation for reversion of the carbachol- induced contraction were changed in tissues treated conjunctly with L-NAME and ACD. Thus, the relaxant effects of ACD appear independent of the nitric oxide pathway in rat duodenum.

In general, the present findings reveal that the phytotherapic compound ACD can inhibit gastrointestinal transit in mice, which is probably due to its direct myorelaxant actions. Such properties were confirmed in rat intestinal smooth muscle and appear to be caused by its ability to act in intracellular pathways in smooth muscle cells. At least for *M. chamomilla*, a recent report of [53] described inhibitory actions against cAMP-dependent phosphodiesterase. The cyclic nucleotide cAMP is involved in the intracellular regulation of the gastrointestinal smooth muscle behavior causing relaxation. Thus, a putative inhibition of phosphodiesterase by ACD is a good candidate to be one of the pathways involved in the

inhibitory effects of ACD on gut, hypothesis that deserves further studies to be confirmed.

5. Conclusion

The ACD is a standardized liquid formulation containing *M. recutita, F. vulgare* and *G. lutea*, that seems provide a multi-target phytotherapic for the treatment of FGIDs. It exhibited significant inhibitory effect on emesis and nausea, reduced intestinal transit and affected the contractions of duodenal smooth muscle mediated by either voltage- or receptor-gated Ca^{2+} channels with the same efficacy that it is also able to inhibit the contractile response mediated by the release from its intracellular store. The positive effect of ACD on castor oil-induced diarrhea seems not related to peristaltic action. Taken together, ACD might have great potential as an herbal medicine useful in the treatment of disorders of the FGIDs.

Declaration of Competing Interest

The authors declare that there is no conflict of interest to disclose.

Acknowledgements

This work was supported by Na-tional Coun-cil for Sci-en-tific and Techono-log-i-cal De-vel-op-ment (CNPq Process: 551122/ 2007-2 and 552761/ 2010-9).

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