

Spasmolytic effect of galetin 3,6-dimethyl ether, a flavonoid obtained from *Piptadenia stipulacea* (Benth) Ducke

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Abstract

Piptadenia stipulacea (Benth) Ducke is a tree of the Caatinga, in Northeast Brazil, popularly known as “Jurema-branca”, “Jurema malícia-da-serra”, “Carcará” and “Calumbi”. In folk medicine, a decoction or tincture of its bark and leaves are used to treat wounds and as healing agents. Galetin 3,6-dimethyl ether (FGAL) is a flavonoid isolated from the aerial components of *Piptadenia stipulacea* (Benth) Ducke. We decided to investigate a possible FGAL spasmolytic effect on preparations of both the guinea pig ileum and trachea, the rat uterus and the male rat aorta. FGAL inhibited oxytocin ($IC_{50} = 2.2 \pm 0.4 \times 10^{-5}$ M) and carbachol (CCh)-induced ($IC_{50} = 7.7 \pm 1.3 \times 10^{-5}$ M) phasic contractions in the rat uterus, but was more effective in the inhibition of the oxytocin-induced contractions. In the guinea pig ileum, FGAL equipotently inhibited CCh ($IC_{50} = 2.8 \pm 0.4 \times 10^{-5}$ M) and histamine-induced ($IC_{50} = 2.3 \pm 0.5 \times 10^{-5}$ M) phasic contractions. FGAL equipotently and concentration-dependently relaxed guinea pig trachea preparations pre-contracted with CCh, both in the absence ($EC_{50} = 0.8 \pm 0.1 \times 10^{-5}$ M) and presence ($EC_{50} = 1.0 \pm 0.1 \times 10^{-5}$ M) of a functional epithelium. FGAL also relaxed preparations of the rat aorta pre-contracted with phenylephrine in both the absence ($EC_{50} = 5.0 \pm 1.1 \times 10^{-6}$ M) and presence ($EC_{50} = 5.4 \pm 1.2 \times 10^{-6}$ M) of a functional endothelium. FGAL shows a non-selective spasmolytic effect on each of the smooth muscle preparations we have tested, but with a greater effect on those from the rat aorta. The relaxant effect on preparations of both the guinea pig trachea and the rat aorta seems to not involve the epithelium or endothelium-derived relaxing factors.

Key words: *Piptadenia stipulacea*, spasmolytic effect, smooth muscle, galetin 3,6-dimethyl ether

Introduction

The genus *Piptadenia* (Fabaceae) comprises about 80 species (Cardozo, 2006) of trees, shrubs and vines (Jobson and Luckow, 2007). The species of this genus contain various types of secondary metabolites such as tannins (Zelada and Coni, 1915), steroids (Miyachi *et al.*, 1976), alkaloids (Yamataso *et al.*, 1972) and flavonoids (Alves *et al.*, 2003; Cardozo, 2006).

Flavonoids exhibit several pharmacological effects: antioxidant and anti-inflammatory (Hämäläinen *et al.*, 2007), antitumor (Izzo, 1996), vascular protective (Beretz and Cazenave, 1988) and spasmolytic on the guinea pig ileum (Macander, 1986; Lima *et al.*, 2005) and trachea (Lima *et al.*, 2011) and the rat uterus (Lima, 2008).

The species *Piptadenia stipulacea* (Benth) Ducke is a tree of the Caatinga, in Northeast Brazil (Albuquerque and Andrade, 2002), popularly known as “Jurema-branca” (Fabricante and Andrade, 2007), “Jurema malícia-da-serra”, “Carcará” and “Calumbi” (Florentino *et al.*, 2007). In folk medicine, a decoction or tincture of its bark and leaves are used to treat wounds (Albuquerque and Andrade, 2002), and as healing agents (Bezerra, 2008). Ethyl acetate and aqueous fractions of this species showed antinociceptive and anti-inflammatory effects in mice (Queiroz *et al.*, 2010).

A flavonoid named galeitin 3,6-dimethyl ether (FGAL) (Fig. 1) was isolated from the aerial parts of *Piptadenia stipulacea* (Benth) Ducke and identified using NMR, with both ^1H and ^{13}C (uni and bidimensional) (Lira *et al.*, 2009). This flavonoid showed antinociceptive and anti-inflammatory effects in mice (Queiroz *et al.*, 2010).

Considering that there are no reports in the literature of the spasmolytic effects of FGAL and that many flavonoids show spasmolytic activity, we decided to investigate whether FGAL has a spasmolytic effect on preparations of the guinea pig ileum and trachea, the rat uterus and the male rat aorta.

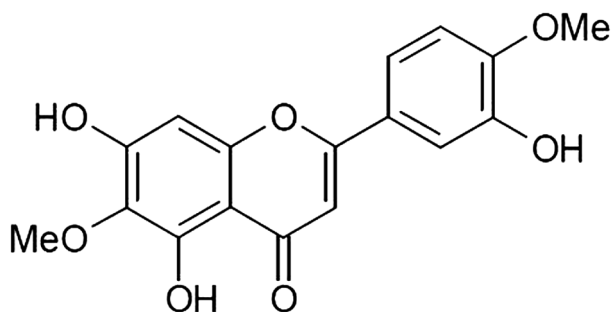


Fig. 1. Chemical structure of galeitin 3,6-dimethyl ether (FGAL) (Lira *et al.*, 2009).

Material and Methods

Plant material

Piptadenia stipulacea (Benth.) Ducke was collected in Serra Branca municipality, Paraíba, in April 2005. The botanical material was identified by Maria de Fátima Agra (PhD) of the Department of Botany of LTF and a voucher specimen is deposited in the Herbarium Prof. Lauro

Pires Xavier (JPB), UFPB under the identification code AGRA *et al.* 3331 (JPB).

Extraction and isolation of galetin 3,6-dimethyl ether (FGAL)

Aerial components from the species were milled and extracted at room temperature with ethanol, obtaining a crude ethanol extract which separated into the hexane, chloroform, ethyl acetate and methanol phases after liquid/liquid extraction with organic solvents. The chloroform phase was subjected to CC-normal phase providing three flavonoids: (1) santin, (2) demethoxycentaureidin and (3) galetin 3,6-dimethyl ether (FGAL). The structures were identified with both and ^{13}C (uni and bidimensional) ^1H NMR and by comparison with literature data (Lira *et al.*, 2009).

Animals

Experiments were performed with trachea rings and ileum segments from guinea pigs (*Cavia porcellus*) of both sexes weighing between 300 and 500 g. Aortic rings from male rats and the uterus from virgin rats (*Rattus norvegicus*) were collected from animals weighing between 250–350 g and 150–250 g, respectively. The animals were maintained in a 12 h light-dark cycle (lights on: 06:00–18:00 h) under controlled temperature ($21 \pm 1^\circ\text{C}$) and with free access to food (Purina[®], Brazil) and water. All experimental procedures were performed in accordance with guidelines approved by the Animal Research Ethics Committee (CEPA) of LTF/UFPB (protocol CEPA/LTF: 0105/10).

Drugs and salts

Magnesium sulphate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), calcium chloride bihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), potassium chloride (KCl) and magnesium chloride (MgCl_2) were purchased from Vetec Química Fina Ltda. (Duque de Caxias, RJ, Brazil). Monosodium phosphate-1-hydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) and monopotassium phosphate (KH_2PO_4) were purchased from Nuclear (São Paulo, Brazil). Glucose and sodium bicarbonate (NaHCO_3) were purchased from Dinâmica (São Paulo, Brazil). Sodium chloride (NaCl) was purchased from Fmaia (São Paulo, Brazil).

Histamine, Cremophor EL[®], diethylstilbestrol, oxytocin, acetylcholine (ACh), arachidonic acid and phenylephrine were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Ethanol PA was purchased from Reagen Ltda. (Colombo, PR, Brazil). Carbamylcholine hydrochloride (CCh) was purchased from Merck & Co., Inc. (Whitehouse Station, NJ, USA). All substances were dissolved in distilled water except FGAL, which was solubilized in Cremophor EL[®] (3%), dissolved in distilled water to a concentration of 10^{-2} M, and re-diluted in distilled water as needed for each experimental protocol. The final Cremophor EL[®] concentration in the organ-bath never exceeded 0.01% (v/v).

Organ preparation

The animals were euthanized following the principles of laboratory animal care in accordance with the guidelines of the bioethics committee. Female rats were pretreated with diethylstilbestrol (1 mg/kg sc) 24 h prior to the experiment to induce estrus, after which time they were sacrificed by cervical dislocation. The trachea and ileum from guinea-pigs, and the male rat

aorta and rat uterus were immediately removed, immersed in Krebs (aorta and trachea), modified Krebs (ileum) and Locke Ringer (uterus) solutions bubbled with carbogen mixture (95% O₂ and 5% CO₂). The solutions composition (mM) were: Krebs: NaCl (118), KCl (4.55), MgSO₄ (5.7), KH₂PO₄ (1.1), CaCl₂ (2.52), NaHCO₃ (25), glucose (11); modified Krebs: NaCl (117), KCl (4.7), MgSO₄ (1.3), NaH₂PO₄ (1.2), CaCl₂ (2.5), NaHCO₃ (25), glucose (11); Locke Ringer: NaCl (154), KCl (5.63), MgCl₂ (2.1), CaCl₂ (2.16), NaHCO₃ (5.95), glucose (5.55). The pH was adjusted to 7.4 by addition of a few drops of a 1N HCl solution. The segment of uterus was cut longitudinally into strips that were 1 to 2 cm in length and about 1 mm wide, and were immersed in organ baths at 32°C (Crankshaw, 2001). The ileum segment was cut transversally into 2 to 3 cm lengths (Daniel *et al.*, 2001), the trachea was divided into segments containing 3–4 cartilage rings each and the aorta cut into 3 mm wide rings. The preparations were immersed in 37°C organ baths with 5 mL of solution, bubbled with carbogen mixture. To register isometric contractions, both aorta and trachea segments were suspended from steel rods and connected to a force transducer (FORT-10) attached to an amplifier (TMB4M), both from World Precision Instruments (Sarasota, FL, USA), the latter connected to an A/D converter into a PC running Biomed® software (BioData, Brazil). Isotonic contractions of the segments of both the uterus and ileum were suspended by cotton yarn and recorded on smoked kymograph drums using levers. The ileum was stabilized for 30 min, the uterus for 40 min and both the trachea and aorta for 60 min at a preload tension of 1 g (baseline). During the organ resting phase the solution in the bath was changed every 15 min to avoid the accumulation of metabolites.

Experimental procedures

1. Effect of FGAL on oxytocin and CCh-induced phasic contractions in the rat uterus

After the stabilization period, two similar dose-response curves were obtained with submaximal concentrations of oxytocin (10⁻² IU/mL) and CCh (10⁻⁵ M) (control). The contraction process was repeated until a stable response to oxytocin or CCh was obtained. FGAL effects were then determined by preincubating the uterine strips for 15 min with a single concentration in independent experiments before adding oxytocin or CCh. IC₅₀ values were expressed as the mean ± S.E.M. of individual IC₅₀ values and assessed with nonlinear regression.

2. Effect of FGAL on histamine and CCh-induced phasic contractions in guinea pig ileum

After the stabilization period, two similar dose-response curves were obtained with submaximal concentrations of CCh and histamine (10⁻⁶ M) (control). The contraction process was repeated until a stable response to CCh or histamine was obtained. FGAL effects were then determined by preincubating the ileum segments for 15 min with a single concentration in solutions of different concentrations in independent experiments before adding CCh or histamine. IC₅₀ values were expressed as the mean ± S.E.M. of individual IC₅₀ values and assessed with nonlinear regression.

3. Effect of FGAL on CCh-induced tonic contractions in guinea pig trachea in both absence and presence of functional epithelium

After the resting period, the trachea rings were contracted with CCh (10⁻⁶ M) and the isometric tension was recorded. When a stable contraction was attained (15–20 min), arachidonic acid (10⁻⁴ M) was added to the organ bath to confirm the presence of an epithelium (Tschirhart *et*

al., 1987). Epithelium functionality was confirmed by the presence of arachidonic acid-induced relaxation (above 50% of maximal tension). In some trachea rings, the luminal surface was gently rubbed with Krebs-wet cotton to remove the epithelial layer. The absence of epithelium was confirmed when arachidonic acid-induced relaxation was absent or when the relaxation was less than 10% of maximal tension. During the tonic phase of a second response to CCh, FGAL was cumulatively added in an attempt to obtain dose-relaxation curves in both the absence and presence of epithelium. The relaxant effect induced by FGAL was expressed as the reverse percentage of the initial contraction force elicited by CCh.

4. Effect of FGAL on phenylephrine-induced tonic contractions in rat aorta in both absence and presence of functional endothelium

After the resting period, the aortic rings were contracted with phenylephrine (0.3 μM) and the isometric tension was recorded. When a stable contraction was attained (15–20 min), acetylcholine (1 μM) was added to the organ bath to confirm the presence of a functional endothelium (Furchgott and Zawadzki, 1980). Endothelium functionality was confirmed by the presence of acetylcholine-induced relaxation (above 50% of maximal tension). In some aortic rings, the luminal surface was gently rubbed with Krebs-wet cotton to remove the endothelial layer. The absence of functional endothelium was confirmed by the absence of acetylcholine-induced relaxation or when the relaxation was inferior to 10% of maximal tension. During the tonic phase of a second response to phenylephrine, FGAL was cumulatively added as an attempt to obtain dose-relaxation curves in both the absence and presence of a functional endothelium. The relaxant effect induced by FGAL was expressed as the reverse percentage of the initial contraction force elicited by phenylephrine.

Statistical analysis

Results were statistically analyzed using the Student's *t*-test and a one-way analysis of variance (ANOVA), followed by Bonferroni's test when appropriate. Differences between values were considered to be significant when a calculated *P* was less than 0.05.

Concentrations producing half-maximal response (EC_{50}) and inhibiting half-maximal effect by an agonist (IC_{50}) were calculated with nonlinear regression, used here to represent the spasmolytic potency, and are presented as the mean \pm standard error of the mean (S.E.M.) in all experiments.

All data were analyzed with GraphPad Prism[®] software version 5.01 (GraphPad Software Inc., San Diego CA, USA).

Results

Effect of FGAL on oxytocin and CCh-induced phasic contractions in the rat uterus

FGAL inhibited phasic contractions induced by 10^{-2} IU/mL oxytocin ($\text{IC}_{50} = 2.2 \pm 0.4 \times 10^{-5}$ M) and 10^{-5} M CCh ($\text{IC}_{50} = 7.7 \pm 1.3 \times 10^{-5}$ M) in the rat uterus (Fig. 2), suggesting a selective effect related to receptors, due to IC_{50} values showing a significant difference, with FGAL being more potent in inhibiting oxytocin-induced contractions. The FGAL inhibitory effect was reversed 1 h after its removal from the organ baths (data not shown).

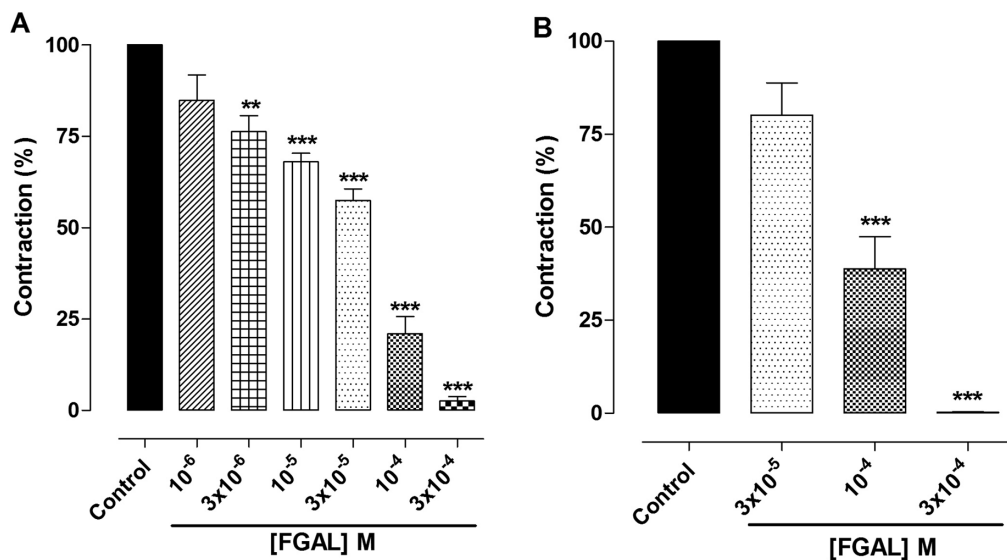


Fig. 2. Effect of FGAL on the phasic contractions induced by 10^{-2} UI/mL of oxytocin (A) and by 10^{-5} M of CCh (B) in the rat uterus. The results are expressed as mean + S.E.M. **, $P < 0.05$ and ***, $P < 0.001$ (control \times FGAL) ($n = 5$).

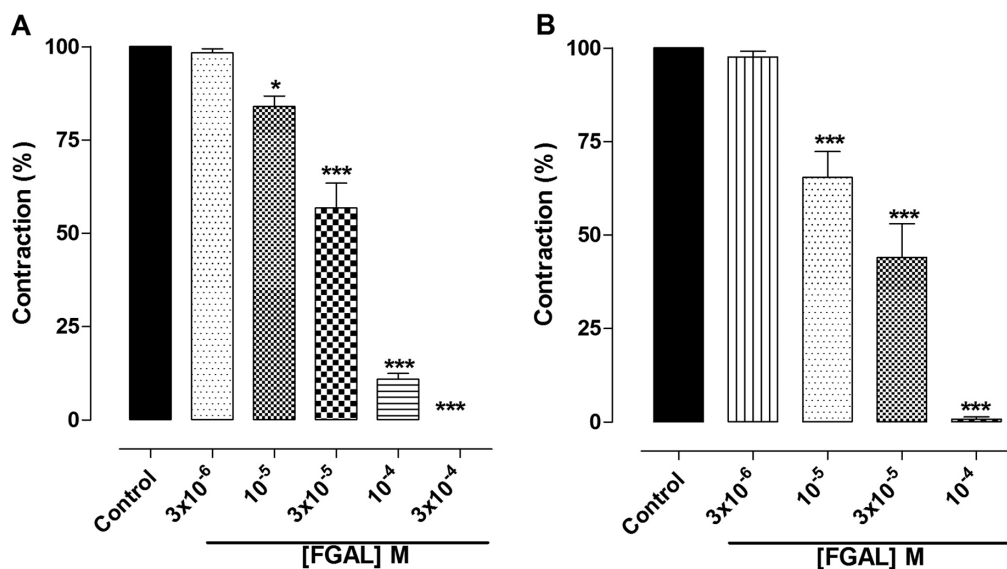


Fig. 3. Effect of FGAL on the phasic contractions induced by 10^{-6} M of CCh (A) and by 10^{-6} M of histamine (B) in the guinea-pig ileum. The results are expressed as mean + S.E.M. *, $P < 0.05$ and ***, $P < 0.001$ (control \times FGAL) ($n = 5$).

Effect of FGAL on histamine and CCh-induced phasic contractions in guinea pig ileum

FGAL inhibited phasic contractions induced by 10^{-6} M histamine ($IC_{50} = 2.3 \pm 0.5 \times 10^{-5}$ M) and 10^{-6} M CCh ($IC_{50} = 2.8 \pm 0.4 \times 10^{-5}$ M) in the guinea pig ileum (Fig. 3), suggesting an “agonist-

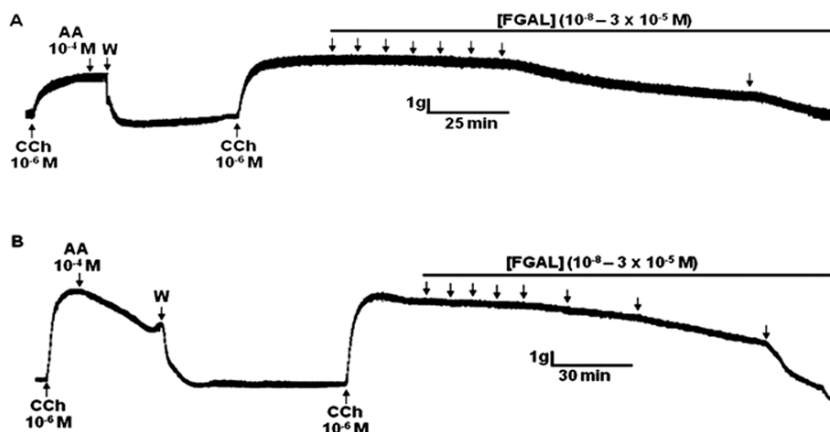


Fig. 4. Original record of the relaxant effect of FGAL on tonic contractions induced by 10^{-6} M CCh in guinea pig trachea rings, in the absence (A) and presence (B) of epithelium. The arrows after the induction of second contraction represent the cumulative addition of FGAL in the concentrations of 10^{-8} , 3×10^{-8} , 10^{-7} , 3×10^{-7} , 10^{-6} , 3×10^{-6} , 10^{-5} and 3×10^{-5} M, respectively. W = wash.

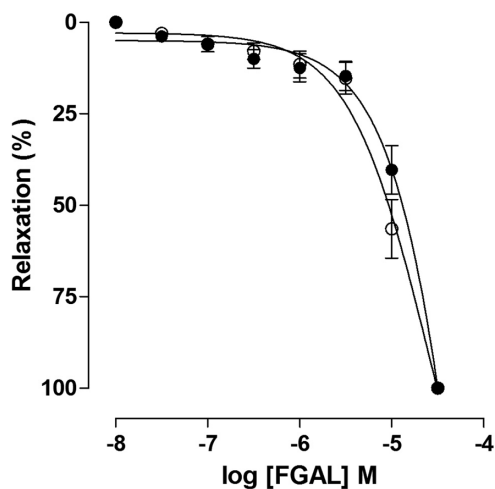


Fig. 5. Effect of FGAL on the tonic contractions induced by CCh in both the absence (\circ) and presence (\bullet) of epithelium in the guinea pig trachea ($n = 5$). Symbols and vertical bars represent the mean \pm SEM, respectively.

related non-selective effect", due to IC_{50} values showing no significant statistical difference. The FGAL inhibitory effect was reversed 1 h after its removal from the organ baths (data not shown).

Effect of FGAL on CCh-induced tonic contractions in guinea pig trachea in the absence and presence of functional epithelium

FGAL (10^{-8} – 3×10^{-5} M) concentration-dependently relaxed trachea rings pre-contracted with CCh (10^{-6} M) in both the absence ($EC_{50} = 0.8 \pm 0.1 \times 10^{-5}$ M) and presence ($EC_{50} = 1.0 \pm$

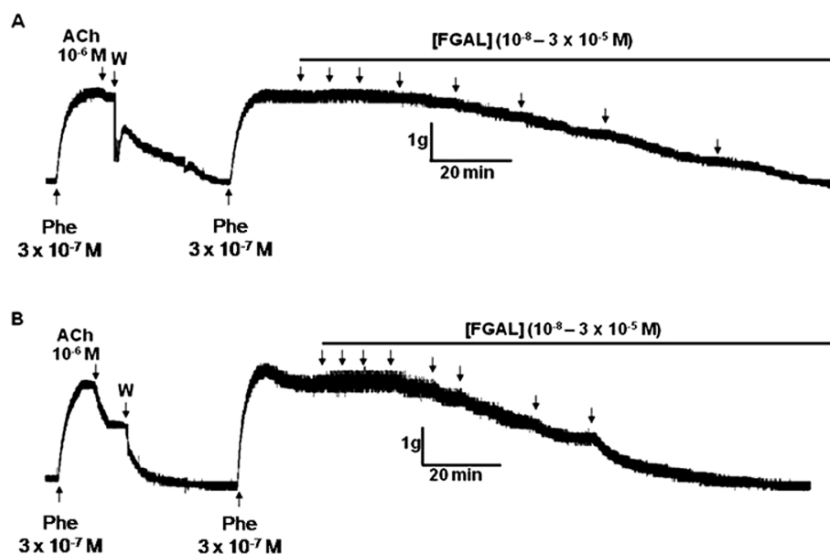


Fig. 6. Original record of the relaxant effect of FGAL on tonic contractions induced by phenylephrine in rings of the male rat aorta, in both the absence (A) and presence (B) of endothelium. The arrows after the induction of second contraction represent the addition of cumulative FGAL in concentrations of 10^{-8} , 3×10^{-8} , 10^{-7} , 3×10^{-7} , 10^{-6} , 3×10^{-6} , 10^{-5} and 3×10^{-5} M, respectively. W = wash.

0.1×10^{-5} M) of an epithelium (Figs. 4 and 5). There was no significant difference between the FGAL EC_{50} values. The FGAL relaxant effect was reversed two hours after removing the flavonoid from the tracheal rings (data not shown).

Effect of FGAL on phenylephrine-induced tonic contractions in rat aorta in the absence and presence of an endothelium

FGAL (10^{-8} – 3×10^{-5} M) dose-dependently relaxed aorta rings pre-contracted with phenylephrine (3×10^{-7} M) in both the absence ($EC_{50} = 5.0 \pm 1.1 \times 10^{-6}$ M) and presence ($EC_{50} = 5.4 \pm 1.2 \times 10^{-6}$ M) of a functional endothelium (Figs. 6 and 7). The FGAL relaxant effect was reversed two hours after removing the flavonoid from the aorta rings (data not shown).

Discussion

This study has demonstrated that the flavonoid galetin 3,6-dimethyl ether (FGAL) isolated from *Piptadenia stipulacea* (Benth) Ducke shows nonselective spasmolytic effect in the guinea pig ileum and trachea, the male rat aorta and the rat uterus.

Smooth muscle is the main tissue responsible for controlling most hollow organs of the body. Smooth muscle cells are present in the walls of various organs (stomach, intestines, bladder, pulmonary airway, uterus) and vessels in the body. Thus, there is great interest in investigating drugs which act on smooth muscle that are either obtained directly from plants or that are derivatives of these. The regulation of smooth muscle contraction has an important role in many

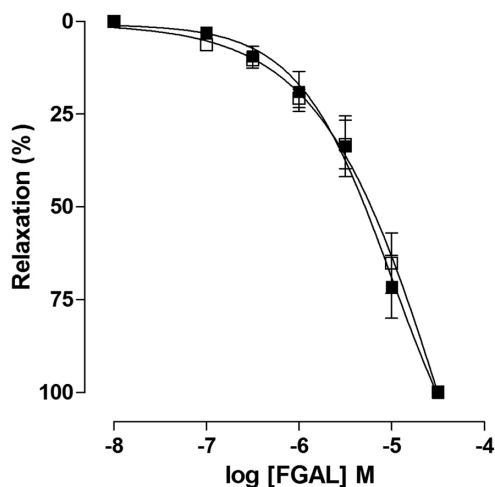


Fig. 7. Effect of FGAL on the tonic contractions induced by phenylephrine in rings of the male rat aorta in both the absence (■) and presence (□) of endothelium ($n = 5$). Symbols and vertical bars represent the mean \pm S.E.M., respectively.

pathophysiological processes where the abnormal contraction of smooth muscle is important, such as in hypertension, cerebral and coronary vasospasm, bronchial asthma, erectile dysfunction and childbirth complications (Webb, 2003).

The mechanism of smooth muscle contraction requires an increase in $[Ca^{2+}]_i$ with the main source of Ca^{2+} being the extracellular fluid. This enters the cytoplasmic compartment during membrane depolarization or after agonist stimuli (Wray *et al.*, 2005).

Substances that act on the uterine smooth muscle producing spasmolytic effect can be used to treat diseases that affect this organ. The investigation of FGAL spasmolytic effects has shown that this flavonoid inhibits CCh ($IC_{50} = 7.7 \pm 1.3 \times 10^{-5}$ M) and oxytocin-induced ($IC_{50} = 2.2 \pm 0.4 \times 10^{-5}$ M) phasic contractions in the rat uterus. Significant differences were observed among the IC_{50} values suggesting that, in the rat uterus, FGAL acts at the receptor level (Fig. 2).

The intestinal smooth muscle model is an important mean of investigating the mechanisms involved in pathophysiological processes such as diarrhea and intestinal cramps. In the guinea pig ileum, FGAL concentration-dependently inhibited CCh ($IC_{50} = 2.8 \pm 0.4 \times 10^{-5}$ M) and histamine-induced ($IC_{50} = 2.3 \pm 0.5 \times 10^{-5}$ M) phasic contractions, so equipotent, suggesting that FGAL is not acting at the receptor level in the guinea pig ileum, but probably at a site common of the pathway these agonists (Fig. 3).

One of the most important cell types involved in pulmonary airway diseases are smooth muscle cells, which are implicated in the development of asthma. The tracheal epithelium has an important role in modulating responses to several agonists in the pulmonary airway smooth muscle. The airway epithelial cells modulate the basal tone and reactivity of smooth muscle by releasing epithelium derived relaxant factors (EDRF) (Hashiba *et al.*, 1999), such as nitric oxide (NO) (Nijkamp *et al.*, 1993) and prostaglandins (Farmer *et al.*, 1987). The FGAL relaxant effect in the guinea pig trachea pre-contracted with CCh is concentration-dependent and occurs in both the

absence ($EC_{50} = 0.8 \pm 0.1 \times 10^{-5}$ M) and presence ($EC_{50} = 1.0 \pm 0.1 \times 10^{-5}$ M) of the epithelium (Figs. 4 and 5). This suggests that the FGAL relaxant effect in the guinea pig trachea does not involve epithelium-derived relaxing factors.

Recently, several substances and channels have been described as endothelium-derived hyperpolarizing factors (EDHF): small conductance Ca^{2+} -activated K^{+} channels (SK_{Ca}), intermediate conductance Ca^{2+} -activated K^{+} -channels (IK_{Ca}), nitric oxide (NO), prostacyclin and epoxyeicosatrienoic acids. EDHF pathways are compromised in hypertension and diabetes (Edwards *et al.*, 2010). Consequently, drugs which act on vascular smooth muscle represent an alternative in treating these diseases. The FGAL relaxed rat aorta pre-contracted with phenylephrine in both the absence ($EC_{50} = 5.0 \pm 1.1 \times 10^{-6}$ M) and presence ($EC_{50} = 5.4 \pm 1.2 \times 10^{-6}$ M) of a functional endothelium (Figs. 6 and 7). This effect is concentration-dependent and equipotent, suggesting that the FGAL vasorelaxant effect in the rat aorta does not involve endothelium-derived relaxing factors.

The spasmolytic potency of FGAL in the guinea pig ileum was similar to that obtained with the flavonoid (2,3-*trans*-3,4-*trans*)-3,4,5,8-tetramethoxy-(6,7,2'',3'') furanoflavan which inhibited the phasic contractions induced by both acetylcholine ($IC_{50} = 4.6 \pm 0.8 \times 10^{-5}$ M) and histamine ($IC_{50} = 2.3 \pm 1.1 \times 10^{-5}$ M) (Lima *et al.*, 2005). But, in the guinea pig trachea, the potency of FGAL was higher than the flavonoid 5,6-dimethoxy-7-phenyl-6,7-dihydro-5H-furo [3,2-g] chromen-4,9-dione in the absence ($EC_{50} = 2.7 \pm 0.4 \times 10^{-5}$ M) or presence ($EC_{50} = 4.2 \pm 0.6 \times 10^{-5}$ M) of epithelium (Lima *et al.*, 2011). The pharmacological potency of FGAL was higher in the rat aorta, both in the absence ($EC_{50} = 5.0 \pm 1.1 \times 10^{-6}$ M) and presence ($EC_{50} = 5.4 \pm 1.2 \times 10^{-6}$ M) of the endothelium. So, FGAL is an interesting candidate as a pharmacological tool in medical practice. However further studies are needed to clarify the mechanism of action of FGAL.

Therefore, we conclude that FGAL shows a nonselective spasmolytic effect in the guinea pig ileum and trachea, the rat uterus and the rat aorta. In both the guinea pig trachea and rat aorta, the relaxant effect seems to not involve epithelium and endothelium-derived relaxant factors, respectively.

Further studies are required to elucidate the mechanism of the spasmolytic action of FGAL in smooth muscles.

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