Spasmolytic Action of Diplotropin, a Furanoflavan from *Diplotropis ferruginea* Benth., Involves Calcium Blockade in Guinea-Pig Ileum

Julianeli T. Lima[a], Jackson R. G. S. Almeida[a], José Maria Barbosa-Filho[a], Temilce S. Assis[a,b], Marcelo S. Silva[a], Emídio V. L. da-Cunha[a,c], Raimundo Braz-Filho[d], and Bagnólia A. Silva[a]

[a] Laboratório de Tecnologia Farmacêutica, Universidade Federal da Paraíba, P.O. Box 5009, 58051-970, João Pessoa, Paraíba, Brazil
[b] Departamento de Fisiologia e Patologia, Universidade Federal da Paraíba, 58051-970, João Pessoa, Paraíba, Brazil
[c] Departamento de Farmácia, Universidade Estadual da Paraíba, 58100-000, Campina Grande, Paraíba, Brazil
[d] Setor de Química de Produtos Naturais, LCQUI-CCT, Universidade Estadual do Norte Fluminense, 28015-620, Campos, Rio de Janeiro, Brazil

Reprint requests to Dr. J. M. Barbosa-Filho or Dr. B. A. Silva. Tel/Fax: +55-83-216-7364.
E-mail: jbarbosa@ltf.ufpb.br or bagnolia@ltf.ufpb.br

Z. Naturforsch. **60b,** 1093 – 1100 (2005); received June 6, 2004 / August 15, 2005

Diplotropis ferruginea Benth. (Fabaceae) is a tree popularly known in Northeastern Brazil as "sucupira-preta". In the present work, the isolation, identification and pharmacological activity of a furanoflavan-type flavonoid (2,3-*trans*-3,4-*trans*)-3,4,5,8-tetramethoxy-(6,7,2",3")-furanoflavan, which received the trivial name diplotropin is reported. The structure was determined by means of spectroscopic techniques, especially EIMS and 1D and 2D NMR. Diplotropin ($10^{-8} - 3 \cdot 10^{-4}$ M) inhibited the phasic contractions induced by both acetylcholine ($IC_{50} = 4.6 \pm 0.8 \cdot 10^{-5}$ M) and histamine ($IC_{50} = 2.3 \pm 1.1 \cdot 10^{-5}$ M) in guinea-pig ileum. Diplotropin relaxed the ileum pre-contracted with KCl ($EC_{50} = 3.9 \pm 1.1 \cdot 10^{-6}$ M), acetylcholine ($EC_{50} = 3.7 \pm 1.6 \cdot 10^{-6}$ M) and histamine ($EC_{50} = 4.4 \pm 1.4 \cdot 10^{-5}$ M) in a concentration-dependent manner. As the maintenance of tonic contraction induced by these contractile agents involves Ca^{2+} influx through voltage-dependent Ca^{2+} channels, it is suggestive that this relaxation may be due to the blockade of Ca^{2+} influx through those channels. This hypothesis was confirmed by the observation that diplotropin antagonized ($pD'_2 = 4.83 \pm 0.37$) $CaCl_2$ induced contractions in Ca^{2+}-free depolarizing medium ($IC_{50} = 1.5 \pm 0.8 \cdot 10^{-5}$ M).

Key words: Diplotropis ferruginea, Spasmolytic Action, Guinea-Pig Ileum

Introduction

The Leguminosae or Fabaceae is a large family of herbs, shrubs and trees. It is comprised by approximately 700 genera and 17,000 species showing a cosmopolitan distribution in tropical, subtropical and temperate zones [1]. This family is subdivided in three subfamilies: Mimosoideae, Caesalpinioideae and Papilionoideae, and the last is constituted of about 400 genera and 12,000 species [2]. Within these genera, the genus *Diplotropis* is included, totaling 22 species.

Few studies are found about the genus *Diplotropis*. There are only three papers describing the isolation of quinolizidine alkaloids from *Diplotropis martiusii* [3], flavonoids, steroids and triterpene from *Diplotropis pupurea* [4], benzenoid and triterpene from *Diplotropis ferruginea* [5]. The species *Diplotropis ferruginea* Benth. (Fabaceae) is a tree, 10 – 12 m tall and is popularly known in Northeastern Brazil as "sucupira-preta", where it is used in folk medicine to treat symptoms of rheumatism, arthritis and diabetes [6]. The tree is widely distributed in the states of Bahia and Rio Grande do Norte, Brazil.

A search on NAPRALERT database (NAtural PRoducts ALERT) and Web of Science did not show any reference to the species *D. ferruginea* Benth. However, previous studies from this research group have shown that the crude ethanol extract (CEE) obtained from the stem-barks of this plant exhibited non-selective spasmolytic effects in all tissues tested (rat aorta and uterus, guinea-pig ileum and trachea) [7].

There have been papers demonstrating that flavonoids have effects on intestinal motility both *in vitro* and *in vivo*. For example, it was reported that quercetin and other flavonoids inhibited guinea-pig ileum induced contractions [8, 9]. Capasso *et al.* [10] screened 13 flavonoids (apigenin, catechin, crysin, flavone, hesperetin, kaempferol, morin, myricetin, naringenin, naringin, phloridzin, quercetin and taxifolin) on contractions in guinea-pig ileum induced by PGE_2, LTD_4, acetylcholine and $BaCl_2$. These spasmolytic effects of flavonoids may have been due to a nonspecific action since they were also found to inhibit acetylcholine and $BaCl_2$ induced contractions. These authors concluded that the spasmolytic effects displayed by the flavonoids were related to an inhibition of the calcium influx and/or calcium release from intracellular stores.

Thus, given the importance of the pharmacological actions of other flavonoids in isolated guinea-pig ileum and knowing that no pharmacological study has been done using this species and that other species belonging to Fabaceae family have ethnomedicinal use to the treatment of intestinal cramps and chronic diarrhea [6]. The aim of the present study was to report the isolation and identification of a new furanoflavan-type flavonoid (diplotropin) as well as to investigate the effect of diplotropin in isolated guinea-pig ileum and to verify the mechanism of action of this flavonoid.

Material and Methods

Plant material

The stem-bark of *D. ferruginea* Benth. was collected in September 2002 near the city of Caraúbas in the state of Rio Grande do Norte – Brazil and identified by the botanist, Dr. Maria de Fátima Agra, from the Setor de Botânica of the Laboratório de Tecnologia Farmacêutica of the Universidade Federal da Paraíba. A voucher specimen (Agra 5559) was authenticated and deposited in the Herbarium Prof. Lauro Pires Xavier (JPB), Departamento de Sistemática e Ecologia, Universidade Federal da Paraíba, João Pessoa, Paraíba, Brazil.

General procedures

Melting points were determined on a REICHERT, model R3279 "Kofler" apparatus, with a temperature range of 0 – 350 °C and are uncorrected. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) were recorded at room temperature with a Jeol Eclipse 400 spectrometer with an 5 mm multinuclear probe. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.00 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm, and the coupling constant J (in Hz). The two dimensional (2D) experiments were acquired and processed with the Delta software provided by Jeol. Mass spectra were measured on a Shimadzu spectrometer.

Extraction and isolation

The crude ethanol extract (CEE) was obtained according to Almeida *et al.* (2003) [5]. The dried and powdered stem-barks (10 kg) of *D. ferruginea* were extracted with EtOH (95%), yielding, 413 g of crude ethanol extract (CEE). The CEE was suspended in H_2O and partitioned with hexane and $CHCl_3$. The hexane extract (55.6 g) was dissolved in hot MeOH and left in a freezer for 24 h, yielding a yellow precipitate, which after recrystallization from hexane yielded (**1**) (2.35 g).

*(2,3-*trans*-3,4-*trans*)-3,4,5,8-Tetramethoxy-(6,7,2",3")-furanoflavan*

Yellow crystals, $[\alpha]_D^{23} + 47°$($CHCl_3$, c 0.425); melting point 114 – 115 C; IR ν_{max} (KBr): 1300, 1285, 1271 and 1250 cm^{-1}. EIMS, *m/z* 370 ([M]$^+$); ^{1}H and ^{13}C data (Table 1).

Animals

Adult guinea-pigs (*Cavia porcellus*, 300 – 500 g) obtained from the Thomas George Biotery, Laboratório de Tecnologia Farmacêutica of the Universidade Federal da Paraíba, were used. The animals were submitted the following conditions: 12 h light : 12 h dark cycle, ventilation and temperature controlled (27 ± 2 °C) and received water and food pellets *ad libitum*. All sets of experiments were carried out in the period of 07:00 – 14:00 h.

Study of spasmolytic activity of diplotropin

Tissue preparation

To perform *in vitro* studies, the guinea-pig ileum was prepared according to Daniel *et al.* [11]. The guinea-pigs were killed by cervical dislocation, the abdomen opened and the ileum was immediately removed. Only the distal portions, with 3 cm length, were used. The tissues were placed in 6 ml isolated

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR for **1** in $CDCl_3$ as solvents and residual $CDCl_3$ used as internal references (δ_H 7.24 and δ_C 70.00), compared with data described in the literature for linear (**1**) and its angular isomer.[a,b] Chemical shifts (δ, ppm) and coupling constants (J, Hz, in parenthesis).*

	1 (this paper)				Literature data		Angular isomer	
	δ_C	δ_H	$^{2}J_{CH}$	$^{3}J_{CH}$	δ_H	δ_C	δ_H	δ_C
C								
5	147.29	–		H-4; MeO-5	–	147.6	–	148.6
6	113.74	–	H-3”	H-2”	–	114.1	–	133.9
7	148.53	–		H-2”; H-3”	–	148.9	–	148.3
8	129.62	–		MeO-8	–	130.0	–	114.2
9	144.99	–		H-2; H-4	–	145.4	–	143.0
10	111.02	–	H-4	H-3	–	111.4	–	110.6
1’	138.79	–	H-2	H-3; H-3’/H-5’	–	139.1	–	138.4
CH								
2	80.45	5.02 (d, 6.6)		H-2’/H-6’; H-4	5.02 (d)	80.5	5.15 (d, 6.5)	70.70
3	82.84	3.90 (dd, 6.6, 4.4)	H-2; H-4	MeO-3	4.10 – 3.90 (dd)	82.9	3.91 (dd, 6.5, 4.7)	82.0
4	74.47	4.79 (d, 4.4)	H-3	H-2; MeO-4	4.80 – 4.78 (d)	74.5	4.66 (d, 4.7)	74.7
2’, 6’	126.56	7.46 (dd, 8.0, 1.4)		H-2; H-4’	7.50 – 7.28 (m)	126.6	7.39 (m)	126.5
3’, 5’	128.19	7.36 (t, 8.0)			7.50 – 7.28 (m)	128.2	7.39 (m)	128.2
4’	127.72	7.30 (dd, 8.0, 1.4)		H-2’/H-6’	7.50-7.28 (m)	127.7	7.39 (m)	127.7
2”	143.51	7.50 (d, 2.6)	H-3”		7.52 (d, 2.0)	143.5	7.51 (d, 2.2)	143.7
3”	104.80	6.86 (d, 2.6)	H-2”		6.84 (d, 2.0)	104.8	6.82 (d, 2.2)	104.4
CH_3								
MeO-3	58.32	3.29 (s)		H-3	3.38 (s)	58.3	3.28 (s)	58.6
MeO-4	56.71	3.35 (s)		H-4	3.44 (s)	56.7	3.38 (s)	57.0
MeO-5	60.55	4.06 (s)			4.08 (s)	60.6	4.07 (s)	60.9
MeO-6	–	–	–	–	–	–	3.98 (s)	61.6
MeO-8	61.33	4.04 (s)			4.04 (s)	61.3	–	–

[a] Number of hydrogens bound to carbon atoms deduced by comparative analysis of HBBD- and APT-^{13}C NMR spectra. Homonuclear 2D ^{1}H-^{1}H-COSY spectrum was also used in these assignments. Chemical shifts and coupling constants (J) obtained from 1D ^{1}H NMR spectrum. [b] Chemical shift in disaccord with the prevision based in the comparative analysis of these furanoflavan.

organ baths containing modified Krebs solution with the following composition (mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.3, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11 dissolved up in distilled water and bubbled continuously with a 95% O_2 and 5% CO_2 gas mixture and maintained at 37 °C. The pH was adjusted to 7.4 with HCl 1 N. Tension changes were recorded through an isometric force transducer (7003) counterbalanced by 1 g loading, connected with a polygraph (Gemini 7070), both from Ugo Basile (Italy). The phasic contractions were recorded using isotonic levers coupled to kymographs and smoked drums.

At the beginning of each experiment, the reactivity of the tissue preparations was tested with KCl (40 mM). After washout and 30 min recovery in modified Krebs solution, contractions were evoked by adding KCl (40 mM), acetylcholine or histamine (10^{-6} M).

Effect of diplotropin on histamine- or acetylcholine-induced contractions in guinea-pig ileum

Two simple concentration-response curves were obtained for both histamine and acetylcholine. Diplotropin was then added and after an incubation period of 15 min (time required to produce maximum effects), a third concentration-response curve was constructed in the presence of diplotropin. The tissue was washed when the agonist responses had returned to resting level. The procedure was repeated in the presence and absence of various concentrations of diplotropin. Inhibition was measured by comparing the response before and after addition of the furanoflavan in the organ bath, and IC_{50} values were obtained graphically from simple concentration-response curves.

Mechanism of action of diplotropin on guinea-pig ileum: Effect of diplotropin on tonic contractions induced by histamine, acetylcholine or KCl

After stabilization of the preparations, an isometric contraction was elicited to a concentration of histamine (10^{-6} M), acetylcholine (10^{-6} M) or KCl (40 mM), which gave a response at 60 – 75% of the maximum (determined from preliminary experiments). Histamine, acetylcholine or KCl remained in contact with the preparation until a plateau of contraction was reached (approximately 8 min), after that time

the tissue was washed. After a further 30 min, the process was repeated and at the plateau of contraction, diplotropin was added cumulatively. Subsequent concentrations were added only after the response to the previous concentration became stable. The relaxant effect induced by diplotropin was expressed as the percentage of relaxation (100% when baseline was reached) of the maximal contractile tension induced by histamine, acetylcholine or KCl.

Effect of diplotropin on Ca^{2+}-induced contractions in Ca^{2+}-free depolarizing solution

The strips were prepared as described before. To access the effect of diplotropin on the influx of Ca^{2+} through the voltage-dependent Ca^{2+} channels [12, 13], the strips were bathed for 30 min. in modified Krebs solution and then exposed for 45 min to high-K^+ Ca^{2+}-free depolarizing solution at the following composition (mM): NaCl 51.7, KCl 70, $MgSO_4$ 1.3, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11. In general, two cumulative concentration-response curves to $CaCl_2$ were obtained at 60 min intervals in each preparation [14]. After obtaining the first curve, washing and complete relaxation, different concentrations of diplotropin were added to the bath and left in contact with the tissue for 15 min. Then, a second cumulative concentration–response curve to $CaCl_2$ was obtained in the presence of the furanoflavan. The maximal contraction obtained with the first concentration-response curve to $CaCl_2$ was taken as 100%, and all contractions were calculated as a function of this value. Each preparation was exposed to only one concentration of diplotropin. Inhibition of $CaCl_2$ responses was calculated by comparing the response in the absence or presence of the diplotropin. The IC_{50} values were calculated as described before.

Statistical analysis

Values were expressed as means ± S. E. M. and "n" refers to the number of animals used in each set of experiments. Statistical analyses were performed using the GraphPad Prism® 3.03 software (GraphPad Software Inc., San Diego CA). The EC_{50}, IC_{50} and pD'$_2$ values were determined by non-linear regression from concentration-response curves [15, 16]. Differences between the means were statistically compared using Student's test and/or one-way ANOVA followed by Bonferroni's test, as appropriate, and were considered to differ significantly when $p < 0.05$.

Results

Structural determination of compound **1**

The EIMS spectrum of diplotropin (**1**) revealed a molecular ion peak at *m/z* 370 ($[M]^+$) which together with the ^{1}H (1D and 2D ^{1}H-^{1}H-COSY), ^{13}C (HBBD and APT) and ^{1}H-^{13}C-COSY-$^nJ_{CH}$ ($n = 1$, HMQC; $n = 2$ and 3, HMBC) NMR spectral data (Table 1) led to deduction of the molecular formula $C_{21}H_{22}O_6$ (11 degrees of insaturation) compatible with a flavan skeleton. The 1D ^{1}H and ^{13}C (HBBD and APT) together with the 2D ^{1}H-^{13}C-COSY-$^nJ_{CH}$ ($n = 1$, HMQC; $n = 2$ and 3, HMBC) NMR spectra led to the elucidation of a highly methoxylated flavan with a furan ring bonded to ring A and having an unsubstituted ring B. The ^{1}H NMR spectrum revealed the presence of a phenyl group by signals at $\delta_H = 7.46$ (dd, $J = 8.0$ and 1.4 Hz, H-2'/H-6'), 7.36 (t, $J = 8.0$ Hz, H-3'/H-5') and 7.30 (dd, $J = 8.0$ and 1.4 Hz, H-4'), which showed correlations in the HMQC with ^{13}C signals at $\delta_C = 126.56$ (CH-2'/CH-6'), 128.19 (CH-3'/CH-5') and 127.72 (CH-4'). The location of this phenyl group at the carbinolic carbon atom CH-2 [δ_H/δ_C: 5.02 (d, 6.6 Hz)/80.45] was deduced by heteronuclear long-range spin-spin interactions between C-1' ($\delta_C = 138.79$) with H-3'/H5' ($\delta_H = 7.36$, $^3J_{CH}$), H-2 ($\delta_H = 5.02$, $^2J_{CH}$) and H-3 ($\delta_H = 3.90$, $^3J_{CH}$) as well as by correlations of the ^{13}C signal of the CH-2 ($\delta_C = 80.45$) with both the ^{1}H signals at $\delta_H = 7.46$ (H-2'/H-6', $^3J_{CH}$) and 4.79 (H-4, $^3J_{CH}$) observed in the HMBC spectrum. The homonuclear spin-spin couplings of H-3 ($\delta_H = 3.90$) with both H-2 ($\delta_H = 5.02$) and H-4 ($\delta_H = 4.79$) were revealed by the 2D ^{1}H-^{1}H-COSY spectrum, which also showed the interaction of H-2" ($\delta_H = 7.50$, d, $J = 2.6$ Hz) and H-3" ($\delta_H = 6.86$, d, $J = 2.6$ Hz) of the furan ring. The direct heteronuclear correlations of these ^{1}H signals of the heterocyclic (ring C) and furan ring with the corresponding ^{13}C signals was deduced through cross-peaks observed in the HMQC spectrum: δ_H/δ_C: 5.02/80.45 (CH-2), 3.90/82.84 (CH-3), 4.79/74.47 (CH-4), 7.50/143.51 (CH-2") and 6.86/104.80 (CH-3"). The identification of the ^{1}H signals of the MeO-3 and MeO-4 was based in the HMBC spectrum, which showed spin-spin interaction ($^3J_{CH}$) of the CH-3 ($\delta_C = 82.84$) with MeO-3 ($\delta_H = 3.29$) and CH-4 ($\delta_C = 74.47$) and MeO-4 ($\delta_H = 3.35$), as shown in Table 1. The presence of a methoxy group at carbon C-5 ($\delta_C = 147.29$) was established by long-range interaction of the signal at $\delta_C = 147.29$ (C-5) with both H-4 ($\delta_H = 4.79$, $^3J_{CH}$)

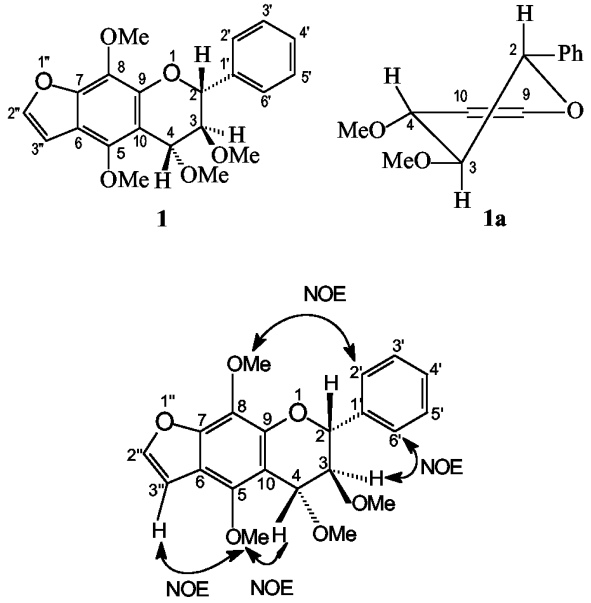

Fig. 1. Important ^{1}H-^{1}H NOESY correlations for diplotropin (**1**).

and MeO-5 ($\delta_H = 4.06$, $^{3}J_{CH}$). The dipolar-dipolar interaction revealed by NOE effect (Fig. 1) between the hydrogen atoms of the MeO-5 ($\delta_H = 4.06$) and H-3" ($\delta_H = 6.86$) was used to locate the carbon atoms C-6 and C-7 of the flavan skeleton in the furan ring (**1**). Consequently, the remaining methoxy group (δ_H/δ_C: 4.04/61.33) was located at C-8, which was confirmed by the long-range coupling of C-8 ($\delta_C = 129.62$) with the hydrogens of the MeO-8 ($\delta_H = 4.04$, $^{3}J_{CH}$). The ^{13}C chemical shifts of the methoxyl groups MeO-5 ($\delta_C = 60.55$) and MeO-8 ($\delta_C = 61.33$) are in accordance with their localizations in hindrance positions. A methoxy group linked to sp^2 carbon atom and located at a position without steric hindrance reveals ^{1}3C signals about $\delta_C = 56$ ppm.

The relative stereochemistry of (**1**) was determined from the coupling constants revealed by ^{1}H signals corresponding to H-2, H-3 and H-4 (Table 1) and by NOE effects observed ^{1}H-^{1}H-NOESY spectrum (Fig. 1). The values corresponding to the *vicinal* interaction ($^{3}J_{H,H}$) between the hydrogens H-2 and H-3 ($J = 6.6$ Hz) and H-3 (dd, $J = 6.6$ and 4.4 Hz) with both H-2 and H-4 ($J = 4.4$ Hz) are consistent with the relative configuration showed in (**1**) and **(1a)**.

Consistent with these deductions, the NOESY spectrum showed cross peaks attributed to dipolar interaction (space proximity, Fig. 1), which was also used to confirm the location of the methoxyl groups: MeO-8 (δ_H 4.04) with H-2'/H-6' ($\delta_H = 7.46$); MeO-5 ($\delta_H = 4.06$) with both H-3" ($\delta_H = 6.86$) and H-4 ($\delta_H = 4.79$); H-3 ($\delta_H = 3.90$) with H-2'/H-6' ($\delta_H = 7.46$) and H-2 ($\delta_H = 5.02$) with H-4 ($\delta_H = 4.79$).

All the data discussed above led to deduce the relative configuration (2,3-*trans*-3,4-*trans*)-3,4,5,8-tetramethoxy-(6,7,2",3")-f uranoflavan (**1**). Despite the great distribution of flavonoids in nature, this type of structure was only found in *Derris araripensis* and *Lonchocarpus subglaucescens* [17]. The NOE effect between MeO-8 and H-2'/H-6' (Fig. 1) points to the possibility of an induced deshielding in the H-2 of the isomer (2,3-*trans*-3,4-*trans*)-3,4,5,6-tetramethoxy-(7,8:2",3")-f uranoflavan (angular isomer) [17] by an anisotropic effect, in accordance with the difference observed in the chemical shifts of the H-2 and H-4 of

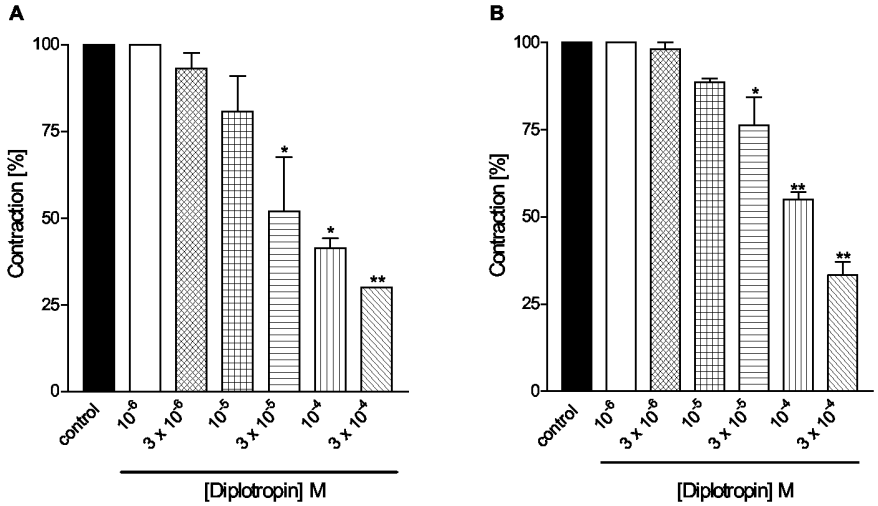

Fig. 2. Effect of diplotropin on phasic contractions induced by 10^{-6} M histamine (A) and acetylcholine (B) in guinea-pig ileum. The columns and bars represent the means and S.E.M., respectively ($n = 4$). Significant differences are indicated by $^{*}p < 0.05$ and $^{**}p < 0.001$ (control × diplotropin; Student's test).

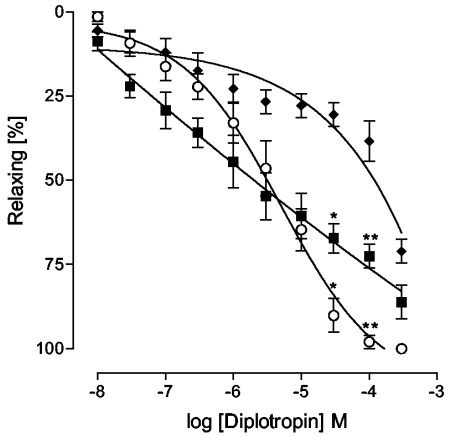

Fig. 3. Effect of different concentrations of diplotropin on the 10^{-6} M histamine (♦)-, 10^{-6} M acetylcholine (■)- and 40 mM KCl (○)-induced tonic contractions in guinea-pig ileum. The symbols and vertical bars shown on the figure represent the means and S.E.M., respectively ($n = 6$). $^{*}p < 0.05$ and $^{**}p < 0.001$ (KCl × acetylcholine, Student's test).

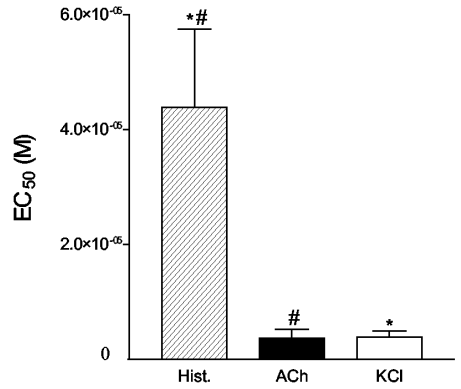

Fig. 4. EC_{50} values of diplotropin on the histamine-, acetylcholine- or KCl-induced tonic contractions in guinea-pig ileum. The columns and bars represent the means and S.E.M., respectively ($n = 6$). One-way ANOVA followed by Bonferroni's test. Significant differences are indicated by $^{*,\#}p < 0.05$. (histamine × acetylcholine or histamine × KCl).

the linear (**1**) and angular isomers, without neglecting the possibility of a small conformational change. Thus, this angular isomer revealing signals of H-2, H-3 and H-4 with approximately same values of coupling constants observed in the linear isomer (*e.g.* **1**) may also represent relative configuration (2,3-*trans*-3,4-*trans*).

Effect of diplotropin on histamine- or acetylcholine-induced contractions in guinea-pig ileum

Diplotropin ($10^{-8} - 3 \cdot 10^{-4}$ M) inhibited the phasic contractions induced by both histamine or acetylcholine (Fig. 2A and B, respectively). The corresponding values of IC_{50} obtained graphically were 2.3 ± 1.1 and $4.6 \pm 0.8 \cdot 10^{-5}$ M for histamine and acetylcholine, respectively.

Mechanism of action of diplotropin on guinea-pig ileum: Effect of diplotropin on tonic contractions induced by histamine, acetylcholine or KCl

Cumulative addition of diplotropin during the development of the tonic component of the contractions resulted in a concentration-dependent relaxation in the guinea-pig ileum that had been precontracted with histamine (10^{-6} M), acetylcholine (10^{-6} M) or KCl (40 mM) (Fig. 3). The threshold concentration of diplotropin was 10^{-8} M for histamine, acetylcholine or KCl induced contractions, but maximal relaxation occurred at $3 \cdot 10^{-4}$ M for all tested contractile agents (Fig. 3). The EC_{50} values (Fig. 4) were $4.4 \pm 1.4 \cdot 10^{-5}$ M, $3.7 \pm 1.6 \cdot 10^{-6}$ M and $3.9 \pm 1.1 \cdot 10^{-6}$ M for histamine, acetylcholine and KCl, respectively.

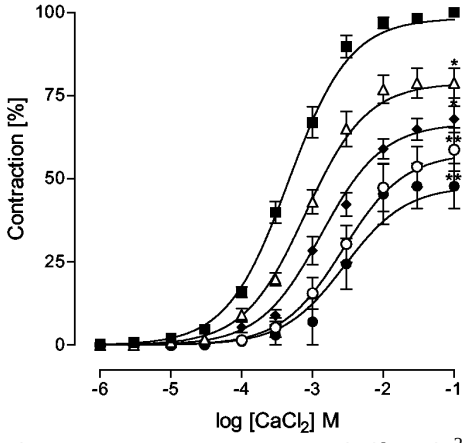

Fig. 5. Concentration-response curves to $CaCl_2$ in Ca^{2+}-free and high-K^+ depolarizing solution in guinea-pig ileum in the absence, control (■) and presence of diplotropin [M]: 10^{-5} (△); $3 \cdot 10^{-5}$ (♦); 10^{-4} (○) and $3 \cdot 10^{-4}$ (●). Contractions expressed as percentage of the maximum control responses. Values are mean ± S.E.M. for 5 experiments. One-way ANOVA followed by Bonferroni's test. Significant differences are indicated by $^{*}p < 0.05$ (control × diplotropin [M]: 10^{-5}; $3 \cdot 10^{-5}$) and $^{**}p < 0.001$ (control × diplotropin [M]: 10^{-4}; $3 \cdot 10^{-4}$).

Effect of diplotropin on Ca^{2+}-induced contractions in Ca^{2+}-free depolarizing solution

Fig. 5 shows the mean cumulative concentration-response curves for $CaCl_2$ alone and in the presence of

different concentrations of diplotropin (10^{-5}, $3 \cdot 10^{-5}$, 10^{-4} and $3 \cdot 10^{-4}$ M). Diplotropin produced a non parallel and concentration-dependent rightward shift of the concentration-response curve to $CaCl_2$, significantly reducing the maximal response ($IC_{50} = 1.5 \pm 0.8 \cdot 10^{-5}$ M). Analysis of the data showed a linear regression and the value of linear correlation coefficient (r^2) was 0.7. The pD'_2 and Schild slope values were 4.83 ± 0.37 and -0.62 ± 0.23, respectively, indicating a non-competitive blockade.

Discussion

In the present study, we have investigated the effects of diplotropin on intestinal smooth muscle, and the most important finding in this work is the demonstration that diplotropin exerts a nonselective spasmolytic action, and that this effect is due in part to the inhibition of Ca^{2+} influx through the voltage-dependent Ca^{2+} channels.

Since diplotropin inhibited the contractions induced by different contractile agents in smooth muscle of guinea-pig ileum (Figs 2 and 3), it would be reasonable to affirm that diplotropin has no selectivity to the contractile agents tested. Moreover, as the IC_{50} and EC_{50} values were not significantly different, it can be suggested that diplotropin may be acting by a similar pathway in the tissue studied.

In the guinea-pig ileum, the phasic component is reached at 15 sec after the contact of the stimulus with the tissue, while the tonic component is reached after 8 min under the same conditions [18].

In order to verify whether diplotropin acts on Ca^{2+} influx across the membrane, we evaluated its effect on the tonic component of the contractile response induced by acetylcholine, histamine or KCl in isolated guinea-pig ileum. As shown in Fig. 3, diplotropin relaxed in a concentration-dependent manner the precontracted ileum with the previously mentioned contracturing agents. Independently of the contraction being evoked by either pharmacomechanical or electromechanical coupling, the maintenance of the tonic component involves activation of voltage-dependent Ca^{2+} channels [19]. Therefore we can postulate that diplotropin may be acting by the blockade of Ca^{2+} influx through these channels to produce nonselective spasmolytic effects.

The confirmation of this hypothesis resulted from the observation that diplotropin inhibited in a concentration-dependent manner the $CaCl_2$-induced contractions in guinea-pig ileum in Ca^{2+}-free depolarizing solution (Fig. 5). Whether the flavonoid inhibits the Ca^{2+} influx across the membrane acting indirectly on voltage-dependent Ca^{2+} channels, blocking protein kinase C or opening potassium channels, these are questions that need to be answered.

Flavonoids are capable of modulating the activity of many enzymes and have a remarkable spectrum of biochemical and pharmacological activities [20]. Furthermore, the behavior of many cell systems, suggest that these substances may possess significant gastrointestinal, antihepatotoxic, antiallergic, antiinflammatory, antiosteoporotic and even antitumor activities [21]. Many of the biological actions of flavonoids have been attributed to their antioxidant properties; however the precise mechanisms by which flavonoids exert their beneficial or toxic actions remain unclear.

In conclusion, we have shown that diplotropin produces spasmolytic effect in guinea-pig ileum and that this effect is due in part to the inhibition of Ca^{2+} influx through voltage-dependent Ca^{2+} channels. However, we do not discard other possible mechanisms that have not been studied yet.

Acknowledgements

The authors thank Vicente Carlos O. Costa, Raimundo Nonato S. Filho, and José Crispim Duarte for providing technical assistance.

This work was supported by CNPq, CAPES and IMSEAR (Brazil) for grants and fellowships.

[1] V. H. Heywood, in V. H. Heywood (ed.): Flowering plants of the world, p. 149 – 152, B. T. Batsford Ltd., London (1996).
[2] W. S. Judd, C. S. Campbell, E. A. Kellog, P. F. Stevens, Plant Systematics: A Phylogenetic Approach, p. 282 – 288, Sinauer Associates, Inc. USA (1999).
[3] A. D. Kinghorn, M. F. Balandrin, L. J. Lin, Phytochemistry **21**, 2269 (1982).
[4] R. Braz-Filho, O. R. Gottlieb, S. L. V. Pinho, F. J. Q. Monte, A. I. Rocha, Phytochemistry **12**, 1184 (1973).
[5] J. R. G. S. Almeida, E. V. L. Da-Cunha, M. S. Silva, P. F. Athayde-Filho, R. Braz-Filho, J. M. Barbosa-Filho, Rev. Bras. Farmacogn. **13**, 44 (2003).
[6] M. P. Corrêa, Dicionário das plantas úteis do Brasil e das exóticas cultivadas, p. 149, IBDF, Ministério da Agricultura, Rio de Janeiro (1984).

[7] J. T. Lima, F. S. Claudino, F. A. Cavalcante, J. R. G. S. Almeida, J. M. Barbosa-Filho, B. A. Silva, Braz. J. Pharm. Sci. **39**, 158 (2003).
[8] M. J. Fanning, P. Macander, G. Drzewiecki, E. Middleton, Int. Arch. Aller. A. Imm. **71**, 371 (1983).
[9] P. J. Macander, Prog. Clin. Biol. Res. **213**, 489 (1986).
[10] A. Capasso, A. Pinto, N. Mascolo, G. Autore, F. Capasso, Phytother. Res. **5**, 85 (1991).
[11] E. E. Daniel, C. Y. Kwan, L. Janssen, J. Pharmacol. Toxicol. **45**, 159 (2001).
[12] G. B. Weiss, in G. B. Weiss (ed.): New perspective on Calcium Antagonists, p. 47 – 57, American Physiological Society, Bethesda, Maryland (1981).
[13] T. Godfraind, R. Miller, M. Wibo, Pharmacol. Rev. **38**, 321 (1986).
[14] J. M. Van-Rossum, Arch. Int. Pharmacod. T. **143**, 299 (1993).
[15] H. O. Schild, Br. J. Pharmacol. **2**, 189 (1947).
[16] D. H. Jenkinson, E. A. Barnard, D. Hoyer, P. P. A. Humphrey, P. Leff, N. P. Shankley, Pharmacol. Rev. **47**, 255 (1995).
[17] A. F. Magalhães, A. M. G. A. Tozzi, B. H. L. N. Sales, E. G. Magalhães, Phytochemistry. **42**, 1459 (1996).
[18] B. A. Silva, V. L. A. Nouailhetas, J. Aboulafia, Am. J. Physiol. **277**, C739 (1999).
[19] C. M. Rembold, in: M. Bárány (ed.): Biochemistry of smooth muscle contraction, p. 227 – 239, Academic Press, San Diego (1996).
[20] G. Rusak, H. O. Gutzeit, J. Ludwig-Müller, Food Technol. Biotech. **40**, 267 (2002).
[21] G. Di Carlo, N. Mascolo, A. A. Izzo, F. Capasso, Life Sci. **65**, 337 (1999).