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Journal of Chemical and Pharmaceutical Research

J. Chem. Pharm. Res., 2011, 3(1):249-258

ISSN No: 0975-7384
CODEN(USA): JCPRC5

Selective spasmolytic effect of a new furanoflavoquinone derivative from diplotropin on guinea-pig trachea

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ABSTRACT

In previous general screening experiments was found a potent spasmolytic effect in guinea-pig ileum promoted by a furanoflavan-type flavonoid 3,4,5,8-tetramethoxy-(6,7,2",3")-furanoflavan named diplotropin, obtained from the stem-barks of Lonchocarpus araripensis Benth., which effect is due, in part, to the inhibition of Ca^{2+} influx through voltage-dependent Ca^{2+} channels. This study demonstrates for the first time in the chemistry literature a new furanoflavoquinone derivative 5,6-dimethoxy-7-phenyl-6,7-dihydro-5H-furo[3,2-g]chromen-4,9-dione, codified as DPTN-Sint. 1, obtained after chemoselective oxidation of natural prototype (diplotropin) and evaluation of its biological activity in rat aorta and uterus, guinea-pig ileum and trachea. The new derivative present a selective spasmolytic effect on guinea-pig trachea not found with the prototype molecule.

Keywords: *Lonchocarpus araripensis*, spasmolytic effect, diplotropin, guinea-pig trachea.

INTRODUCTION

Brazilian traditional medicine includes many plants for the treatment of different diseases. Plants belonging to the Fabaceae family are among the most used medicinal. Their main usage takes place in the folk treatment of symptoms of rheumatism, arthritis, diabetes, intestinal cramps, chronic diarrhea as well as respiratory complaints [1]. In addition to these usages, in some Brazilian regions the plants from the genus *Lonchocarpus* are traditionally used for the treatment of tumors, AIDS, headache, and skin diseases [2] as well as to relief of rheumatism, arthritis,

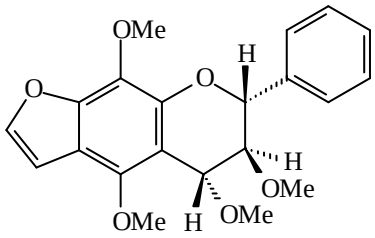
diabetes, inflammations, gastritis, peptic ulcer and general wounds. This genus is represented by approximately 100 species distributed in the tropical America, Africa and the Caribbean Islands [3]. Previous phytochemical investigations have proved that *Lonchocarpus* is a rich source of phenol compounds, including flavones, chalcones, flavonols, flavans, flavanones and aurones [4].

Lonchocarpus araripensis Benth. (Fabaceae) is a tree, 10-12 m tall, restricted to the Caatinga vegetation 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. The tree is widely distributed in hot and dry areas of the states of Bahia, Ceará and Rio Grande do Norte, Brazil.

Only few studies evaluating the therapeutic potential of this specie have been performed. The gastroprotective effect of a flavone isolated from this specie was demonstrated [5]. In a recent study, our group demonstrated that triterpenoid lupeol isolated from this specie attenuates the alterations characteristics of allergic airway inflammation. The investigation of mechanisms of action of this molecule may contribute for the development of new drugs for the treatment of asthma [6].

The chemical examination of this specie resulted in the isolation of a furanoflavan-type flavonoid, to which was given the trivial name diplotropin. Diplotropin presents spasmolytic activity on smooth muscles of guinea-pig ileum [7]. Compounds with the pharmacological profile of a smooth muscle relaxant have potential value for the treatment of diseases in which smooth muscle becomes hyperreactive to agonists [8].

Diplotropin (**1**) is highly functionalized on rings A and C with relative configuration (2,3-*trans*-3,4-*trans*)-3,4,5,8-tetramethoxy-(6,7,2",3")-furanoflavan. Furan moieties located at ring A in a linear or angular position, i.e. linked to either C-6/C-7 or C-7/C-8, respectively, are a common characteristic of the flavonoids exhibited by plants of the *Lonchocarpus* genus [4].



(**1**)

Fig. No. 1 Chemical structure of diplotropin

Pharmacological profile of its derivative on the respiratory, intestinal, uterine and circulatory tract has been subject of evaluation. Based on previous observations of the prototype diplotropin, and also in the folk usage of plants from this genus, we therefore speculated that this compound has the ability to inhibit smooth muscle contraction *in vitro*, as observed for diplotropin (**1**) in guinea-pig ileum smooth muscle [7], to confirm this hypothesis experimentally.

EXPERIMENTAL SECTION

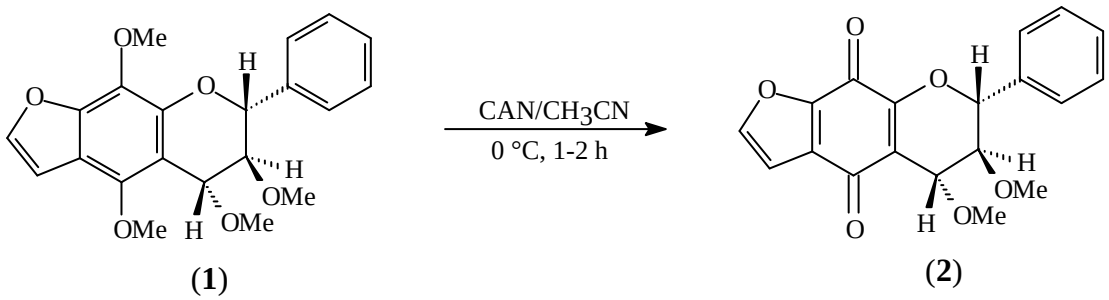
Extraction and isolation

The crude ethanol extract (CEE) was obtained according to Almeida et al. [9]. The dried and powdered stem-barks (10 kg) of *L. araripensis* were extracted with EtOH (95 %), yielding, 413 g

of CEE. The CEE was suspended in MeOH : H_2O (3 : 7 v/v) and partitioned with hexane, $CHCl_3$, AcOEt. 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**).

Derivatization reaction of diplotropin

Oxidative demethylation with ceric (IV) ammonium nitrate afforded the quinone compound after an aqueous workup and extracton with methylene chloride in experimental modified by Reed and Moore [10]. To a 0 ^oC 50 mg (0.135 mmol) of diplotropin **1** in 2 ml of acetonitrile and 1 ml of water was added a solution of 0.162 g of ceric (IV) ammonium nitrate in 1 ml of water. After TLC inspection (1-2 h at 0 ^oC), the mixture was partitioned between 10 ml of water and 20 ml of methylene chloride. The aqueous layer was extracted twice with 20 ml of methylene chloride, and the combined organic layers were washed with 10 ml of water, dried over magnesium sulfate, and the solvents were removed under reduced pressure to give the compound (**2**), codified as DPTN-Sint 1.



(**1**) (**2**)

Fig. No 2 Derivatization reaction of diplotropin (1)

(2,3-*trans*-3,4-*trans*)-3,4,5,8-tetramethoxy-(6,7,2",3")-furanoflavan (1)

Yellow crystals, melting point 114-115 ^oC; $IR_{\nu max}$ (KBr): 1300, 1285, 1271 and 1250 cm^{-1}. ^1H NMR ($CDCl_3$, 200 MHz) and ^{13}C NMR ($CDCl_3$, 50 MHz) (Table 1).

5,6-dimethoxy-7-phenyl-6,7-dihydro-5H-furo[3,2-g]chromen-4,9-dione (2)

Obtained in 56.6 % as orange amorphous solid, melting point 90-92 ^oC; $IR_{\nu max}$ (KBr): 1226, 1272, 1659 and 1688 cm^{-1}. ^1H NMR ($CDCl_3$, 200 MHz) and ^{13}C NMR ($CDCl_3$, 50 MHz) (Table 1).

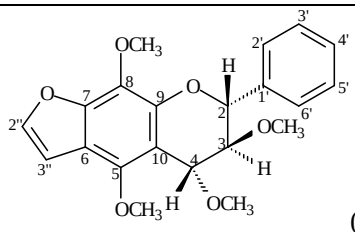
General Procedures

Melting points were determine on a REICHERT, model R3279 "Kofler" apparatus, with a temperature range of 0 to 350 ^oC and are uncorrected. ^1H NMR (200 MHz) and ^{13}C NMR (50 MHz) were recorded at room temperature with a Varian Mercury 200 spectrometer. The spectra were recorded in $CDCl_3$, and the solvent signals (7.24 and 77.00 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm, and the coupling constant *J* in Hz.

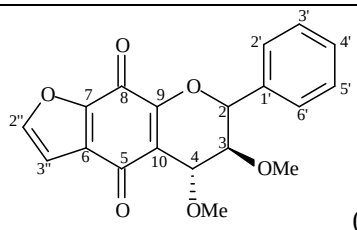
Animals

Adult guinea-pigs of both sexes (*Cavia porcellus*, 300-500 g), male and female Wistar rats (*Rattus norvegicus*, 200-350 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 (lights on: 06:00-18:00 h), filtered conditioned air, 23 ± 2 ºC, 50-70 % humidity, sterilized bed and fed with Purina® pellets and drinking sterilized water *ad libitum* were used. All sets of experiments were carried out in the period of 07:00-14:00 h.

Table No 1. ^{1}H NMR (200 MHz) and ^{13}C NMR (50 MHz) data for (1) and (2) in $CDCl_3$ as solvent. Chemical shifts (δ) in ppm and coupling constants (*J*) in Hz.



(**1**)



(**2**)

	δ_C	δ_H	δ_C	δ_H
C				
5	147.16	-	169.05	-
6	113.62	-	129.68	-
7	148.38	-	149.46	-
8	129.48	-	180.70	-
9	144.84	-	153.50	-
10	110.88	-	116.68	-
1’	138.66	-	135.43	-
CH				
2	80.35	4.79 (*d*, *J*=6.4)	78.26	5.55 (*d*, *J*=3.6)
3	82.71	3.66 (*dd*, *J*=6.4 e 4.4)	79.80	3.93 (*t*, *J*= 4.0)
4	74.35	4.55 (*d*, *J*=4.4)	70.06	4.41 (*dd*, *J*=4.0 e 1.6)
2’,6’	12.43	7.30 (*m*)	125.54	7.27 (*m*)
3’,5’	128.08	7.30 (*m*)	128.27	7.27(*m*)
4’	127.60	7.30 (*m*)	127.92	7.27(*m*)
2’’	143.39	7.25 (*d*, *J*=2.4)	149.02	7.70 (*d*, *J*=2.0)
3’’	104.71	6.61 (*d*, *J*=2.4)	108.53	6.84 (*d*, *J*=2.0)
CH_3				
MeO-3	58.25	3.26 (*s*)	58.14	3.24 (*s*)
MeO-4	56.67	3.32 (*s*)	57.92	3.38 (*s*)
MeO-5	60.49	3.81 (*s*)	-	-
MeO-8	61.27	3.79 (*s*)	-	-

Animal handling and procedures were performed according to international guidelines for the use and care of laboratory animals (National Research Council, 1996). The experimental procedure was evaluated and approved by the Ethic Committee in Animal Research (CEPA) of Universidade Federal da Paraíba (protocol CEPA/LTF # 0508/05).

Drugs and reagents

Carbamylcholine chloride (carbachol), acetylcholine chloride, arachdonic acid (AA), phenylephrine L (-) hydrochloride and cremophor were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All salts for the organ bath solutions and reagents were purchased from Vetec (Rio de Janeiro, RJ, Brazil). Stock-solutions of all the chemicals were prepared in distilled water, except for (**2**) that were dissolved in cremophor. All stock-solutions were stored at -20 °C, and the dilutions were made fresh on the day of the experiment.

Study of spasmolytic activity of (2)

Tissue preparation

Rat Aorta

Male Wistar rats (250-350 g) were killed by stunning and bleeding, their thoracic aorta was quickly removed and placed in Krebs’ solution with the following composition (mM): NaCl (118), KCl (4.6), $MgSO_4$ $7H_2O$ (5.7), NaH_2PO_4 H_2O (1.1), $CaCl_2$ (2.5), $NaHCO_3$ (25), glucose

(11) with pH adjusted to 7.4 with 1 M HCl solution, which was continuously bubbled with a O_2 95 % and CO_2 5 % gas mixture. Fat and connective tissues, surrounding the aorta, were removed carefully, and the aorta was cut into rings (3-4 mm length). The endothelium was removed from some rings when necessary, by gently inserting a cotton thread through the lumen of the rings. This treatment does not reduce the reactivity of smooth muscle [11]. The aortic rings were then suspended between two stainless steel wires in a organ chambers containing 6 ml of Krebs' solution. One of the wires was anchored in the organ chamber and the other was connected to a force-displacement transducer (Model 7003, Ugo Basile, Italy). Isometric tensions of arterial tissues were recorded on a polygraph. The removal of endothelium was functionally verified by the lack of relaxant response to acetylcholine (1 μM) in aortic rings precontracted with phenylephrine (1 μM) [12].

Effect of (2) on the tonic contractions induced by phenylephrine in the presence or absence of endothelium

Two submaximal tonic responses to phenylephrine (1 μM), which stabilized in 12-15 minutes, were obtained initially. Acetylcholine (1 μM) was added to the preparations during the tonic phase of the second response to check the presence of intact endothelium. During the tonic phase of a third response to phenylephrine, and (**2**) was added cumulatively in an attempt to obtain a concentration-inhibition curve, in the presence or absence of functional endothelium. Relaxation obtained as the reverse of initial contraction elicited by phenylephrine (1 μM), was expressed percentually.

Rat Uterus

Virgin female Wistar rats (200-280 g) estrogenized *s.c.* with 1 mg/kg of estradiol benzoate were used. The animals were killed by decapitation 24 h after injection and both uterine horns were extracted and cleaned of adherences. The longitudinal strips, approximately 2-3 cm, were placed vertically in 6-ml isolated organ baths containing Locke Ringer solution with the following composition (mM): NaCI (154), KCl (5.63), $CaCl_2$ (2.16), $MgCI_2$ (2.10), $NaHCO_3$ (5.95) and glucose (5.55). In the same way, Locke Ringer solution was used without calcium and bubbled continuously with a O_2 95 % and CO_2 5 % gas mixture and maintained at 32 °C. The pH was adjusted to 7.4 with HCl 1 N. Each uterine horn was subjected to a resting tension of 1 g and was allowed to equilibrate for at least 30-45 min before the drugs were added to the organ baths. The phasic contractions were recorded using isotonic levers coupled to kymographs and smoked drums (DTF, Brazil).

Effect of (2) on the phasic contractions induced by oxytocin or carbachol in the rat uterus

Strips from non-pregnant and estrogenized rat uterus were contracted with 10 mIU/ml of oxytocin or 10 μM carbachol and after 10 min. from washout a new contraction was elicited by each one of the agonists and the mean of this two contractions was considered as control (100 %). Before a third contraction, (**2**) (10^{-8} - 3 x 10^{-4} M) was pre-incubated for 10 min. in a single concentration per organ-bath. The inhibition was measured by comparing the response before and after addition of each agonist in the presence of (**2**). The drugs were subsequently washed-out and we observed the recovery from contractions elicited by each drug. In another set of experiments, the uterine strips were treated with corresponding volume of the vehicle used in the preparation of (**2**) solution.

Guinea-Pig Ileum

To perform *in vitro* studies, the guinea-pig ileum was prepared according to Daniel et al. [13]. Guinea-pigs (300-500 g) were killed by cervical dislocation, exsanguinated and the ileum was immediately removed. The terminal portions, 3 cm length, were used after discarding the 10 cm

portion close to the ileocaecal junction. The tissues were placed vertically in 6 ml isolated 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), bubbled with a O_2 95 % and CO_2 5 % gas mixture and maintained at 37 °C, pH 7.4. Tension changes were recorded through an isometric force transducer (7003) counterbalanced by 1 g loading, connected to a polygraph (Gemini 7070), both from Ugo Basile (Italy). Phasic contractions were recorded using isotonic levers coupled to kymographs and smoked drums (DTF, Brazil). The tissues were allowed to stabilize for 30 min. at a resting tension of 1 g. During the stabilization period the modified Krebs solution was changed every 10 min. to avoid accumulation of metabolites [14].

Effect of (2) on histamine- or acetylcholine-induced contractions in the guinea-pig ileum

At the beginning of each experiment, the reactivity of tissue preparations was tested with KCl 40 mM. After washout and 15 min.-recovery in modified Krebs solution, two simple concentration-response curves were obtained for either histamine or acetylcholine (1 μM). Various concentrations of (**2**) were added and after an incubation period of 15 min.; a third concentration-response curve was constructed in the presence of (**2**). The tissue was washed when the agonist responses returned to the resting level. Inhibition was measured by comparing the response before (100 %) and after addition of (**2**) in the organ bath. IC_{50} values were obtained graphically from simple concentration-response curves.

Guinea-Pig Trachea

Guinea-pigs of both sexes (300-500 g), were killed by cervical dislocation, the abdomen opened and the trachea was quickly removed and dissected free of adhering fat and connective tissue. From every trachea, four rings containing three to four cartilaginous segments were obtained. Each tracheal ring was hung between two hooks inserted into the lumen, and placed in a 6 ml organ bath containing Krebs' solution with the following composition (mM): NaCl (118), KCl (4.6), $CaCl_2$ (2.5), $MgSO_4$ (5.7), $NaHCO_3$ (25), KH_2PO_4 (1.1), glucose (11), which was maintained at pH 7.4, 37 °C and bubbled with a O_2 95 % and CO_2 5 % gas mixture. The tracheal preparations were allowed to stabilize for 60 min. at a resting tension of 1 g basal tension. At the beginning of each experiment, 60 mM KCl was added one time to evaluate the viability of the tissue. The KCl contraction was taken into account for normalization of all subsequent responses to the different agonists. After washout and 30 min. recovery in Krebs solution, contractions were evoked by adding carbachol (10^{-6} M). The integrity of the functional epithelium will be verified by the addition of arachidonic acid (AA) to the organ bath in the concentration of 10^{-4} M [15] during the tonic phase of the first contractile response induced by 10^{-6} M carbachol, tracheal rings that obtain superior relaxations to 50 % (in relation to force of initial contraction) they will be considered with epithelium. Tracheal rings without epithelium will be obtained through the removal of the same by attrition of the tracheal lumen surface with a cotton swab moistened with Krebs solution. The removal of the epithelium will be confirmed by the addition of AA to the organ bath, the absence of functional epithelium was confirmed by absence of AA-induced relaxation. This treatment does not reduce the reactivity of smooth muscle [11]. Isometric contractions were measured by a force transducer (FORT-10) coupled to an amplifier (TMB4M), both from World Precision Instruments (Sarasota, FL, USA) connected to a A/D converter into a PC running Biomed© software v.Rv2 (BioData, Brazil).

Effect of (2) (10^{-8} – 3 x 10^{-4} M) on the contractions induced by carbachol in the presence or absence of functional epithelium

After the resting period, the tracheal rings were contracted with carbachol (10^{-6} M) and the isometric tension was recorded. When a stable contraction was attained (15-20 min.) AA (10^{-}

^{4} M) was added to the organ bath to confirm the presence or absence of functional epithelium according to method described above. In a second carbachol-induced stable contraction, cumulative-concentrations of (**2**) (10^{-8} – 3 x 10^{-4} M) were added to the organ bath. In each preparation, a single concentration-relaxation curve was obtained. The relaxant effect induced by (**2**) was expressed as the reverse percentage of initial contraction force elicited by carbachol (100 % when baseline was reached) in tracheal rings with and without functional epithelium.

Statistical analysis

All the data are expressed as the mean ± S.E.M. and "n" refers to the number of animals used in each set of experiments. Statistical analyses were performed using the software Graph-Pad Prism© 4.0 (GraphPad Software Inc., San Diego CA). The concentration that produced a half-maximal response (EC_{50}) was determined by non-linear regression from concentration-response curves. Differences between the means were statistically compared using Student's test "t" and these differences were considered significant when *p* values were < 0.05.

RESULTS AND DISCUSSION

The IR spectrum of (**2**) showed absorptions at 1659 and 1688 cm^{-1} attributed to carbonyl groups. The remaining two methoxyl groups on downfield (3.24 and 3.38 ppm) after oxidation of the 1,4-dimethoxylated system of diplotropin in the ^{1}H-NMR spectrum confirms the remotion of this group. The formation of the quinone nucleus on **2** should be evidenced by signals at 169.05 and 180.70 ppm in ^{13}C-NMR spectrum, characteristic of absorption of carbonyl group in this class of compounds, as well as by the chemical shift of the two hydrogens H-2'' (7.70 ppm) and H-3'' (6.84 ppm), downfield showed in the ^{1}H-NMR spectrum, owing to withdrawing effect of electrons of two groups carbonyl, when compared with the parent compound diplotropin (**1**). The relative stereochemistry of (**2**) 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 in ^{1}H-^{1}H-NOESY spectrum. All data showed are consistent with the proposed structure **(2)** (Figure 2). For more details, see table 1.

After the oxidation reaction described above starting from natural prototype diplotropin and obtaining of the new derivative, codified as (**2**), the proposed experimental protocols were executed and we observed that (**2**) (3 x 10^{-5}, 10^{-4} and 3 x 10^{-4} M) in the same way that the diplotropin (**1**) don't promote relaxation of the isolated rat aorta in the presence or absence of endothelium but interestingly, unlike the natural prototype, compound (2) does not present spasmolytic activity in guinea-pig ileum and rat uterus (data not shown). In all experiments, (**2**) it was maintained in contact with the tissue for a period of 15 min. among each added concentration in the isolated organ bath.

According to the *in vitro* results obtained (Fig. 3 and 4), the quinone derivative (**2**) show satisfying spasmolytic activity in guinea pig trachea because (**2**) (10^{-8} to 3 x 10^{-4} M) relaxed the tracheal rings pre-contracted with carbachol (10^{-6} M) in a concentration-dependent manner in the presence (EC_{50} = 4,2 ± 0,6 x 10^{-5} M) and absence (EC_{50} = 2,7 ± 0,4 x 10^{-5} M) of functional epithelium (Fig. 3 and 4) without statistical significance between this values. In all experiments, the relaxant effect of (**2**) was reversible after 30 minutes washout with Krebs solution.

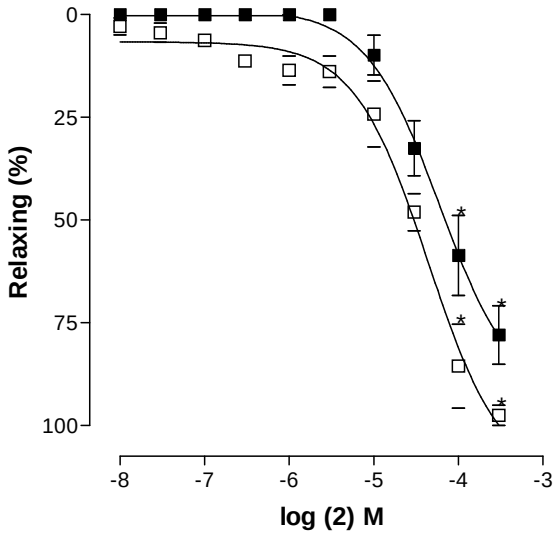


Fig. No 3 Effect of different concentrations of (2) on the 10^{-6} M carbachol-induced tonic contractions in guinea-pig trachea with (□) and without (■) functional epithelium.

The symbols and vertical bars shown on the figure represent the means and S.E.M. for 4 experiments. One-way ANOVA followed by Bonferroni's test. Significant differences are indicated by $^{*}p < 0.05$ (with x without epithelium)

In a previous study Lima et al. [7] showed that the furanoflavan-type flavonoid diplotropin (**1**) presents a non-selective spasmolytic activity in isolated guinea-pig ileum and rat uterus. Based on these results, we decided to modify the molecular structure of diplotropin and to carry out a study of the relationship between the structural modification and the effect of the synthetic derivative in different models of smooth muscles. These preliminary achievements point us to oxidize the aromatic 1,4-dimethoxy moiety of compound (**1**) to the quinone nucleus present in the compound (**2**) by a straightforward chemoselective procedure with CAN [10].

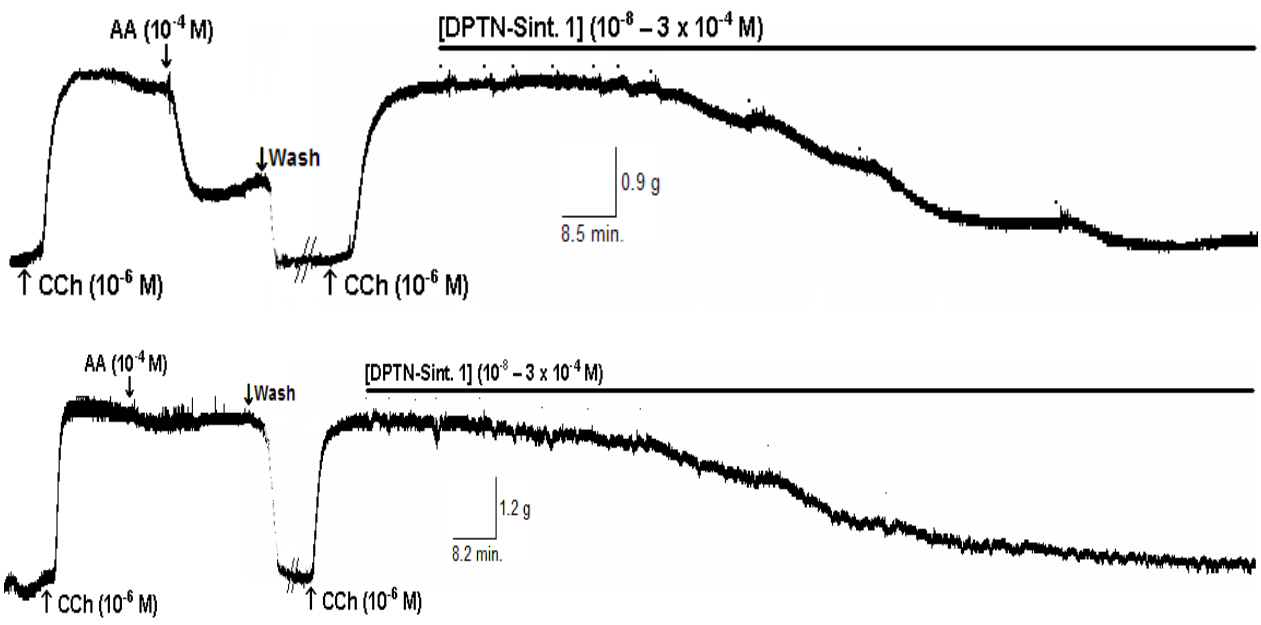


Fig. No 4 Typical tracings showing the effect of (2) (10^{-8} to 3 x 10^{-4} M) on guinea-pig trachea pre-contracted with carbachol (10^{-6} M) in the presence (A) and absence (B) of functional epithelium

In summary, we have reported on the synthesis and biological evaluation of a novel quinone derivative **(2)**. This compound is described here for the first time in chemistry literature. The results of the biological evaluation showed that **(2)** had a selective relaxant effect on guinea-pig trachea pre-contracted with carbachol (10^{-6} M) independently of the presence functional epithelium (Fig. 3, 4a and 4b). However it doesn't present relaxing effect in the other tested organs, unlike previously observed with his prototype, diplotropin, including the pre-contracted with carbachol (10^{-6} M) in the presence and the absence of functional epithelium and spontaneous tonus of guinea-pig trachea (Fig. 5a, 5b and 5c).

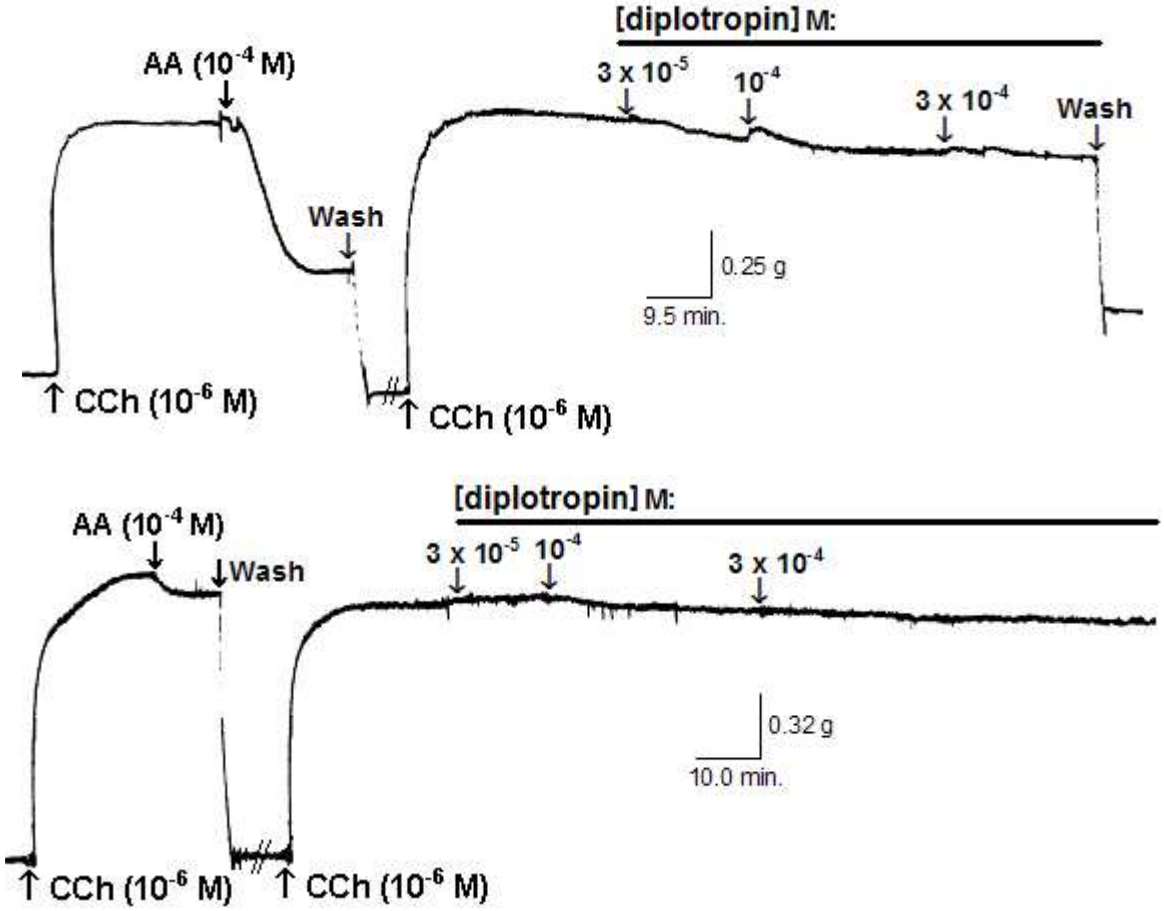


Fig. No 5 Typical tracings showing the effect of diplotropin (3 x 10^{-5}, 10^{-4} and 3 x 10^{-4} M) on spontaneous tonus (A) and pre-contracted guinea-pig trachea with carbachol (10^{-6} M) in the presence (B) and absence (C) of functional epithelium

Before observed, the introduction of the quinone group in (**2**) result in a significant modification in the selectivity of this derivative in relation to the natural product. Because carbachol contractile responses are largely dependent on extracellular Ca^{2+}, our results suggested that (**2**) might be acting as a partial blocker of Ca^{2+} influx of extracellular medium. Thus, more studies are necessary to confirm this hypothesis and to clarify the mechanisms underlying (**2**) exerts his relaxant effect.

Acknowledgements
The authors thank Vicente Carlos O. Costa, Raimundo Nonato S. Filho and José Crispim Duarte for providing technical assistance. The authors are grateful to Brazilian agencies CNPq and CAPES for financial support.

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