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Antispasmodic and antioxidant activities of fractions and bioactive constituent davidigenin isolated from *Mascarenhasia arborescens*

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ABSTRACT

Ethnopharmacological relevance: Mascarenhasia arborescens A. DC. (Apocynaceae) is used in traditional medicine in the North of Madagascar to treat intestinal disorders, intestinal spasms and diarrhoea.
Aim of the study: The main objective of this work was to evaluate the antispasmodic activity of the crude methanolic extract of *Mascarenhasia arborescens* and of its four partitions and to identify the effective compound responsible for this effect.
Materials and methods: Isolation and structure elucidation techniques were performed in order to identify the bioactive constituent of *Mascarenhasia arborescens* and HPLC analysis was used for its quantification. Total phenolic content (TPC) of crude extracts and partitions were determined using the Folin–Ciocalteu method. Crude methanolic extract, partitions and the bioactive compound were investigated for their spasmolytic activity on several isolated organs. Their antiradical activity was also investigated by the DPPH test.
Results: Bioassay-guided fractionation using isolated guinea pig ileum pre-contracted with histamine 3×10^{-6} M led to the isolation of davidigenin (DG), a dihydrochalcone, as the main active constituent from the most promising methylene chloride partition (McP). This partition was effective on isolated guinea pig ileum pre-contracted with 3×10^{-6} M histamine, with a median effective concentration (EC_{50}) of 41.19 ± 3.74 μg/ml. The DG content of this partition was shown to be 26.5% by HPLC. DG induced a concentration-dependent relaxation of the histamine pre-contracted guinea pig ileum with an EC_{50} of 8.04 ± 0.81 μg/ml and a concentration-dependent relaxation of the acetylcholine pre-contracted rat duodenum with an EC_{50} of 9.35 ± 0.30 μg/ml. It inhibited in a non-competitive manner histamine-induced isolated ileum contraction and the acetylcholine-induced isolated duodenum contraction. Moreover, DG does not have any antiradical activity.
Conclusions: We demonstrated for the first time antispasmodic and antioxidant effects of *Mascarenhasia arborescens*. This study supports its use in traditional medicine. Furthermore, we highlighted the crucial role of davidigenin in the antispasmodic activity of this plant.

1. Introduction

Mascarenhasia arborescens A. DC. (Apocynaceae) is a tree growing in the East of Africa and Madagascar (Markgraff, 1976). The genus *Mascarenhasia* is also represented in Madagascar by seven endemic species (Schatz, 2001). In Madagascar, *Mascarenhasia arborescens* is popularly known as *Barabanja* or *Gidroa*. According to our ethnobotanical investigations in groups of women and traditional healers in the region of Ambilobe, Ambanja and Sadjoavato, this plant is traditionally used to treat intestinal disorders, intestinal spasms and diarrhoea. Infantile diarrhoeal diseases are still widespread in Madagascar and are a major cause of infant deaths along with malnutrition, respiratory infections and malaria (Metz, 1994). They may be caused by different pathogens, including bac-

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teria (enteropathogens *Escherichia coli* and *Campylobacter jejuni*), viruses (enteroviruses and rotaviruses), or parasites (*Ascaris lumbricoïdes*, *Strongyloides stercoralis*, *Entomoeba hystolitica*, *Schistosoma mansoni*, *Hymenolepis nana*, *Taenia saginata* or *Taenia solium*) (Ravaoarinoro et al., 1986; Cassel-Beraud et al., 1990a, 1990b; Boisier et al., 2001; Buchy, 2003). Diarrhoea is often accompanied by intestinal spasms that cause abdominal cramps and pain. It is on account of these data that we investigated *Mascarenhasia arborescens* for antispasmodic activity, and tried to identify the most likely candidate for the biological activity. Prior to our work, no phytochemical or biological studies have been reported on this species. The present paper describes the antispasmodic activity on two isolated organs of the crude methanolic extract (CME) of *Mascarenhasia arborescens* and of its four partitions (hexane (HeP), methylene chloride (McP), ethyl acetate (EaP) and aqueous (AqP)), and the isolation and identification of the bioactive compound. We tested also several crude extracts, partitions and the pure compound for their antiradical activities. Moreover, we quantified by HPLC the bioactive compound in crude methanolic extract (CME) and its four partitions.

2. Materials and methods

2.1. Plant material

Mascarenhasia arborescens was collected by Odile Désiré in June 2006 in Ankingameloka, Madagascar, located 33 km South of the Ambanja district on road RN6. This species was identified by Armand Rakotozafy (botanist, IMRA) by comparison with an authentic specimen deposited at the Department of Botany, Botanical and Zoological Park of Tsimbazaza (No. 325), Antananarivo, Madagascar. A voucher specimen was deposited at the Faculty of Sciences of Antsiranana, Madagascar, No. APO/Masc/02.

2.2. Extraction and isolation of the bioactive constituent

The plant material (5 kg) of *Mascarenhasia arborescens* was kept at room temperature (25–30 °C) for air drying (3 weeks). The air-dried plant material (500 g), a mixture of leaves and stems, was powdered and extracted by repeated maceration with methanol at room temperature (3× 1 l, 72 h each). The filtered solvent was evaporated under vacuum and lyophilised to afford a crude methanolic extract (CME) (54.4 g, 10.9% w/w). The residue (45 g) was suspended in water and was partitioned by successive extractions three times with different solvents of increasing polarity to yield hexane (HeP, 0.03 g), methylene chloride (McP, 0.27 g), ethyl acetate (EaP, 1.81 g) and aqueous (AqP, 14.16 g) partitions. The CME and its four partitions were stored in dark bottles at 4 °C after solvent evaporation and freeze-drying.

The methylene chloride partition (McP) showing strong antispasmodic activity on guinea pig ileum pre-contracted with histamine was subjected to further purification. Using thin layer chromatography (TLC), McP was loaded on preparative glass plate (20 cm × 20 cm, 0.2 mm, SiO_2 F_{254}, Macherey–Nagel) and run seven times using 200 ml CH_2Cl_2/MeOH (98/2) as developing solvent. Nine fractions (F-1 to F-9) were obtained. Three fractions (F-1 to F-3) were three different pure compounds. These nine fractions were dried and dissolved in DMSO (0.1% final concentration) for assessment of their antispasmodic activity on guinea pig ileum pre-contracted with histamine. The compound F-1 was the most active. Using LC–MS, 1D and 2D NMR spectroscopic analysis, the bioactive compound F-1 (22 mg) was identified as davidigenin (DG), a dihydrochalcone (Fig. 1).

In order to realize the different pharmacological tests, a second batch of McP was subjected to a column chromatography (silica gel 60 H) eluting with CH_2Cl_2/EtOAc (95/5 to 0/100), followed by increasing concentrations of MeOH to give several fractions. Fraction F-120 to F-130 contained pure DG (40 mg).

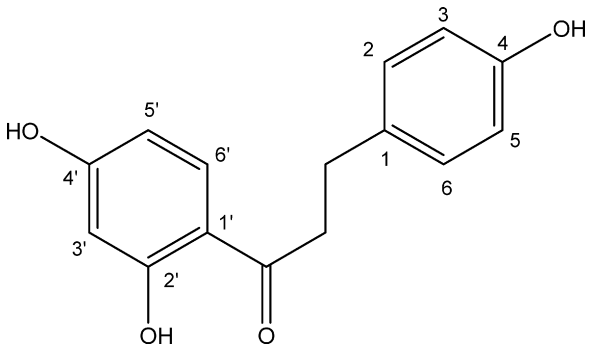

Fig. 1. Chemical structure of davidigenin (DG).

The ESI-mass spectrometry (ESI-MS) of compound 1 gave an $[M+H]^+$ at *m*/*z* 259 with the positive ion mode indicating a mass 258 compatible with the molecular formula $C_{15}H_{14}O_4$. The structure of DG was confirmed by comparison with previously published spectral data (Jensen et al., 1977).

Davidigenin. White powder (62 mg), UV (DCM/MeOH) λ_{max} nm ($\log \varepsilon$) 196, 212, 280, 312; IR (KBr) ν_{max} 3442 (OH), 1626 (C=O), 1597 (aromatic rings) cm^{-1}; ESI-MS *m*/*z* 259 $[MH]^+$ ($C_{15}H_{14}O_4$ requires 258); ^1H NMR (400 MHz, MeOD): δ 7.72 (1H, *d*, *J* = 8.7 Hz, H-6′), 7.07 (2H, *d*, *J* = 8.4 Hz, H-2 and 6), 6.70 (2H, *d*, *J* = 8.4 Hz, H-3 and 5), 6.34 (1H, *dd*, *J* = 8.7, 1.8 Hz, H-5′), 6.26 (1H, *s*, *J* = 1.8 Hz, H-3′), 3.18 (2H, *t*, *J* = 7.7 Hz, H-α), 2.91 (2H, *t*, *J* = 7.7 Hz, H-β) and ^{13}C NMR (50 MHz, MeOD): δ 204 (C=O), 165 (C-2′), 164.9 (C-4′), 155.3 (C-4), 132.3 (C-6′), 131.8 (C-1), 129.0 (C-2, 6), 114.8 (C-3, 5), 112.6 (C-1′), 107.7 (C-5′), 102.2 (C-3′), 29.62 (C-α), 29.36 (C-β).

2.3. Synthesis of davidigenin

Davidigenin was prepared as described by Jensen et al. (1977), in order to quantify it in crude methanolic extract and partitions of *Mascarenhasia arborescens* by HPLC. DG was obtained by mixing isoliquiritigenin (Alfa Aesar, France, 500 mg) with Pd/C 10% (Alfa Aesar, France, 25 mg) in methanol and stirring the solution in a flask under atmospheric pressure of H_2 gas at room temperature for 3 h. Yield obtained was 97%. The structure of the synthesized derivative was found in accordance with the one of the previously described dihydrochalcone DG (Jensen et al., 1977).

2.4. Analysis of davidigenin in crude methanolic extract and partitions of Mascarenhasia arborescens by RP-HPLC with DAD

2.4.1. Chemicals and general procedures

HPLC grade acetonitrile was purchased from Scharlau Chemie SA (Spain). Deionized water was prepared by a Milli-Q Water Purification System (Millipore, France). Before utilization, acetonitrile and water were filtered through 45 μm nylon 66 membrane filter (Supelco) and 0.22 μm cellulosic filter (GE Water & Process Technologies, France) respectively. Davidigenin was obtained by synthesis from isoliquiritigenin (Alfa Aesar, France) as indicated in Section 2.3.

2.4.2. Chromatographic conditions and instrumentation

HPLC analysis was performed on a Hewlett Packard Series 1100 HPLC system, equipped with G1311A quaternary gradient pump, G1315A diode array detector (DAD), G1322A degasser, G1313A autosampler, G1316A column communication, and ChemStation software. Chromatography was carried out on a Hypersil Gold C18

column (250 mm × 4 mm, 5 μm particle size) and a precolumn from ThermoScientific® (France). At room temperature and a flow rate of 1 ml/min using solvent A (water) and solvent B (acetonitrile) with a linear gradient elution: 80% A and 20% B to 100% B in 30 min and equilibration in 10 min. Flow rate and injection volume were 1.0 ml/min and 10 μL. The UV spectrum of DG standard dissolved in the mobile phase was obtained by DAD. The wavelength of 280 nm was selected for detection of target analyte since it showed maximum absorption and good sensitivity of the target peaks.

2.4.3. Preparation of sample solution

A crude methanolic extract (CME) and subsequent partitions (HeP, McP, EaP and AqP) were obtained from *Mascarenhasia arborescens* according to the protocol described in Section 2.2. Each extract (5 mg/ml) was dissolved in HPLC grade acetonitrile for quantitative determination of DG. After filtering through a 0.45 μm membrane nylon filter (Supelco), the extract was injected directly.

2.4.4. Preparation of standard solution and calibration curves

DG obtained by synthesis was accurately weighed and then dissolved in an appropriate volume of HPLC grade acetonitrile to produce a standard stock solution of DG at concentration of 5 mg/ml. Working standard solutions for calibration curve were prepared by diluting the stock solution of DG with HPLC grade acetonitrile at seven concentrations in the range of 0.0025–1.5 mg/ml. The stock and working solutions were kept at 0 °C until use. The calibration curve was plotted after linear regression of the peak areas versus concentrations. The linear regression equation for DG was expressed as $y = bx + c$, where x is the concentration, y is the peak area of the standard, and b and c are constants.

2.4.5. HPLC method validation

The selectivity of the method was determined by analysis of standard compound and samples. The peak of DG was identified by comparing its retention time and UV spectrum with those of the standard. The limits of detection (LOD) and limits of quantification (LOQ) were defined at the lowest concentrations of analyte in a sample that can be detected and quantified. These limits were determined on the basis of the signal-to-noise ratios of 3:1 and 10:1 respectively. The determination of LOD and LOQ values of DG was carried out by diluting standard solution of the corresponding compound sequentially. The accuracy of the method was evaluated by analyzing the recovery percentage of DG in CME and different partitions. The recovery percentage was calculated using the ratio of contents detected (actual) to those added (theoretical).

2.5. Determination of total polyphenol content

Total polyphenol content (TPC) was measured by the Folin–Ciocalteu method previously described by Lixiang et al. with slight modification (Lixiang et al., 2009). Different crude extracts, methanolic (CME), aqueous (CAE) and hydro-acetonic (60/40) (CHAE), were prepared in order to compare their TPC. Briefly, an aliquot of 0.5 ml of sample solution (with appropriate dilution to obtain absorbance in the range of the prepared calibration curve) was mixed with 2.5 ml of Folin–Ciocalteu reagent (10 times dilution). After 2 min, 2.0 ml of saturated Na_2CO_3 solution (145 g/L) was added. The mixture was allowed to react at 50 °C for 5 min, then to cool and stand 5 min before the reading of absorbance of the reaction mixture at $\lambda = 760$ nm. A calibration curve of gallic acid (ranging from 0.01 to 0.10 mg/ml) was prepared, and TPC was standardised against gallic acid and expressed as mg gallic acid equivalent per gram of sample on a dry weight (DW) basis.

2.6. Pharmacological experiments

2.6.1. Drugs and solubility

Acetylcholine chloride, histamine dihydrochloride, pyrilamine maleate (stamine) were purchased from Sigma (USA), atropine sulphate from Prolabo (France). All other chemicals used were of analytical grade available. All of the salts (NaCl, KCl, $CaCl_2$, $MgSO_4$, KH_2PO_4 and $NaHCO_3$) as well as D-glucose used to prepare Tyrode's solution were purchased from Prolabo. The crude methanolic extract and various partitions of *Mascarenhasia arborescens*, davidigenin and drugs, were dissolved in DMSO (0.1% final concentration). Control experiments demonstrated that the DMSO used to dissolve drugs or plant extract or fractions did not affect the various parameters of contractile responses of the isolated tissues at their final bath concentrations.

2.6.2. Animals

Guinea pigs and Wistar rats in the animal house of IMRA (Institut Malgache de Recherches Appliquées) were used. All the animals were kept in air-conditioned rooms, controlled lighting (12 h:12 h light-darkness cycle) with free access to normal food and water. All experiments were conducted in accordance with the internationally accepted principles for laboratory animal use and care and with institutional guidelines.

2.6.3. Guinea pig ileum

Guinea pigs of either sex, weighing between 250 and 350 g, were anesthetized and sacrificed by cervical dislocation followed by exsanguination. A piece of ileum, 10–20 cm long, about 15 cm proximal to the ileo-cecal junction, was removed from the abdominal chamber. The proximal ileum was washed and placed in Tyrode's solution and the mesenteric residues and adherent fat were eliminated. The segments of ileum about 2 cm in length were hung in a 20 ml tissue bath containing Tyrode's solution, continuously bubbled with a mixture of 95% oxygen and 5% carbon dioxide (carbon gas) and maintained at 37 °C, pH 7.4. The composition of Tyrode's solution was as follows (mM): KCl 2.68, NaCl 136.9, $MgCl_2$ 1.05, $NaHCO_3$ 11.90, NaH_2PO_4 0.42, $CaCl_2$ 1.8 and glucose 5.55 (Emendörfer et al., 2005). Each segment was hooked to an isotonic transducer (Type Ugo Basile 7006) combined with a two-channel amplifier (Ugo Basile Type GEMINI 7070, APELEX, France). A tension of 0.5 g was applied to each tissue and kept constant throughout the experiment. The strips were allowed to equilibrate for 1 h and rinsed every 15 min with Tyrode's solution. After equilibration, the segments were stimulated with histamine and then rinsed three times after attaining the maximum concentration.

2.6.4. Effect of extracts, partitions, fractions and davidigenin on the pre-contracted guinea pig ileum

Crude extracts, partitions, fractions, davidigenin (F-1) and pyrilamine were dissolved in DMSO and serial dilutions were then made, with a final concentration of DMSO of less than 0.1% in the organ bath. After equilibration and stimulation, the strips were contracted with 3×10^{-6} M histamine. When the contraction plateau was reached, crude extracts and partitions were tested at a concentration of 250 μg/ml, fractions at 100 μg/ml and pyrilamine at 10 nM. In order to determine the EC_{50}, 250–1000 μg/ml of the crude methanolic extract (CME) were cumulatively injected every 4 min, which is the required time to reach the plateau of the relaxant effect. Similarly, the methylene chloride and ethyl acetate partitions (McP and EaP) and davidigenin (DG) were tested using concentrations ranging from 30 to 100 μg/ml and 4 to 12.5 μg/ml respectively and their EC_{50} were also calculated.

2.6.5. Effect of crude methanolic extract and davidigenin on the spasmodic activity of histamine

After washing, the tissues were pretreated with CME or DG and cumulative concentration–response curves were recorded, isotonically in the organ bath. Concentration–response curves for histamine (10^{-9} to 10^{-5} M) were tested in the absence or presence of CME (0.25 or 0.5 mg/ml) or DG (5, 10 or 12.5 μg/ml). The effect was allowed to reach a steady state at each concentration every 4 min. Pyrilamine maleate was used as a positive control.

2.6.6. Rat duodenum

Wistar rats of either sex, weighing between 200 and 300 g, were anesthetized and killed by cervical dislocation and exsanguination (Ghosh, 1984). A small piece of duodenum, 2 cm long, was dissected out of the abdominal cavity of rat duodenum, cleaned and mounted in 20 ml tissue baths containing Tyrode's solution (see Section 2.6.3) aerated with a mixture of 95% oxygen and 5% carbon dioxide and maintained at 37 °C. Each segment was hooked to an isotonic transducer (Type Ugo Basile 7006) combined with a two-channel amplifier (Ugo Basile Type GEMINI 7070, APELEX, France). A tension of 1 g was applied to each tissue and kept constant through the length of the experiment. The strips were equilibrated for 1 h and rinsed every 15 min with the survival solution. After equilibration, the segments were stimulated with acetylcholine chloride and then rinsed three times after attaining the maximum concentration.

2.6.7. Effect of davidigenin on the pre-contracted rat duodenum

Davidigenin was dissolved in DMSO and serial dilutions were then made, the concentration of DMSO never exceeding 0.1% in the organ bath. After equilibration and stimulation, the strips were contracted with 10^{-5} M acetylcholine (ACH). In order to determine EC_{50}, when the plateau of the contraction was reached, 7.5–12.5 μg/ml of DG was cumulatively injected every 4 min, the required time to reach the plateau of the relaxant effect.

2.6.8. Effect of davidigenin on the spasmodic activity of acetylcholine

The concentration–responses of the ACH were studied in the absence and in the presence of DG. To this purpose, 10^{-9} to 10^{-4} M ACH was cumulatively injected in the organ bath every 3 min, and its concentration–response curve was subsequently constructed. After three rinses, 7.5, 10 or 12.5 μg/ml DG was introduced in the organ bath 5 min before injection of 10^{-9} to 10^{-4} M ACH. Atropine was used as a positive control.

2.7. Antiradical activity: DPPH assay

The antiradical test was performed as described by Aquino et al. (2001). Crude extracts, partitions and davidigenin with isoliquiritigenin and rutin as positive control (Rivière et al., 2009) were tested for antiradical activity using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH°). An aliquot (50 μL) of the MeOH solution containing an extract or the positive control was added to 2.5 ml of freshly prepared DPPH° solution (25 μg/ml in methanol). An equal volume (50 μL) of the vehicle alone (MeOH) was added to control tubes ($DPPH^{\circ}_0$). Absorbance at λ 515 nm was measured on an Uvikon 933 spectrophotometer after 20 min incubation. Crude extracts, partitions, products and positive control were tested at a concentration range of 40–0.4 μg/ml, in order to determine IC_{50} values.

A calibration curve was obtained after linear regression of DPPH° at concentrations ranging from 1 to 50 μg/ml. The percentage of remaining DPPH° ($DPPH^{\circ}_{REM}$) was calculated as follows:

$$\%\,DPPH^{\circ}_{REM} = \frac{[DPPH^{\circ}_{20\,min}]}{[DPPH^{\circ}_0]} \times 100$$

All experiments were carried out in triplicate.

Table 1
Effect of different crude extracts, partitions and fractions of *Mascarenhasia arborescens* on the histamine pre-contracted guinea pig ileum.

Test fractions[a]	Percentage of inhibition at 250 μg/ml for crude extracts and partitions, at 100 μg/ml for fractions, and at 10 nM for pyrilamine[b]	CE_{50} (μg/ml)[c]
CME	19.5 ± 5.4 (*n* = 8)	629 ± 111 (*n* = 8)
CAE	6.6 ± 0.5 (*n* = 10)	ND
HeP	23.9 ± 0.9 (*n* = 10)	ND
McP	97.6 ± 2.6 (*n* = 10)	41.2 ± 3.7 (*n* = 10)
EaP	58.0 ± 5.4 (*n* = 10)	173 ± 14 (*n* = 10)
AqP	17.6 ± 1.7 (*n* = 10)	ND
Fraction F-1 (DG)	99.6 ± 0.6 (*n* = 8)	8.04 ± 0.81 (*n* = 8)
Fraction F-2	39.2 ± 3.5 (*n* = 8)	ND
Fraction F-3	43.4 ± 3.2 (*n* = 8)	ND
Fraction F-4	57.2 ± 5.4 (*n* = 8)	ND
Fraction F-5	56.9 ± 5.2 (*n* = 8)	ND
Fraction F-6	13.9 ± 1.8 (*n* = 8)	ND
Fraction F-7	52.3 ± 5.9 (*n* = 8)	ND
Fraction F-8	56.5 ± 5.9 (*n* = 8)	ND
Fraction F-9	63.7 ± 6.7 (*n* = 8)	ND
Pyrilamine	42.1 ± 1.3 (*n* = 8)	ND

[a] Fractions come from chromatography fractionation (TLC) of the McP.
[b] Mean ± S.D. (*n* = number of experiments).
[c] Activity was expressed as concentration producing 50% relaxation (CE_{50}) with respect to histamine (3×10^{-6} M) evoked contraction. Mean ± S.D. (*n* = number of experiments).

2.8. Statistical analysis

All statistical calculations were carried out with GraphPad Prism 4. The results are expressed as the mean ± standard error of mean (S.E.M.) of (*n*) independent experiments with individual values. Unpaired Student's *t*-test was used for statistical comparison; *p* values less than 0.01 or 0.05 were considered as significantly different from the control.

3. Results

3.1. Effect of the methanolic crude extract of Mascarenhasia arborescens on isolated guinea pig ileum pre-contracted with histamine

Due to the complicated constituents and various pharmacological activities of plant materials and because of ethnobotanical data, we opted for an *in vitro* bioassay-guided fractionation. The antispasmodic activity of the crude extracts and partitions of *Mascarenhasia arborescens* on the histamine pre-contracted ileum are shown in Table 1. According to the traditional use (decoction in water), we also prepared a crude aqueous extract (CAE) and compared this activity to those of the crude methanolic extract (CME). The CME was used for the study because its percentage of inhibition at 250 μg/ml was higher (19.5 ± 5.4% (*n* = 8)) than that of the CAE (6.6 ± 0.5% (*n* = 10)).

The overall effect of the CME of leaves and stems of *Mascarenhasia arborescens* on guinea pig ileum contractions induced by histamine is shown in Fig. 2. The extract exhibited an antispasmodic effect at both concentrations (250 and 500 μg/ml) used whereas DMSO (solvent) had only a marginal effect. The extract produced a significant response with a percentage of inhibition of 36.2 ± 1.8% obtained at 500 μg/ml. The CME was also tested at

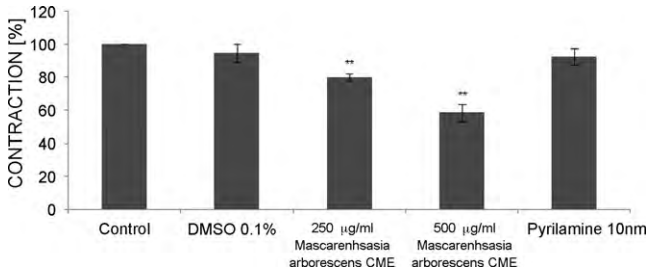

Fig. 2. Antispasmodic effect of two concentrations of *Mascarenhasia arborescens* crude methanolic extract (250 and 500 µg/ml) on histamine-induced guinea pig ileum contractions. Data are normalized to the effect of 3 µM histamine alone (control: 100%). Data represents mean ± S.E.M. of 3 (pyrilamine), 4 (DMSO), 20 (histamine alone) and 8 (for the other curves) independent experiments. $^{**}p < 0.01$ versus contractions induced by histamine alone. Pyrilamine (10 nM) is the positive control. DMSO (0.1%) is the negative control.

750 and 1000 µg/ml (data not shown). The EC_{50} of this extract was determined as 629 ± 111 µg/ml ($n = 8$). Pyrilamine was used as antagonistic control. This compound induced a percentage of inhibition of 40.7 ± 1.6% at 10 nM.

The histamine-dependent contraction curve of guinea pig ileum in the absence or presence of crude methanolic extract of *Mascarenhasia arborescens* is shown in Fig. 3. Both extract concentrations (250 and 500 µg/ml) shifted the histamine–response curve to the right and down. The EC_{50} of histamine alone was determined as $3.35 \pm 0.35 \times 10^{-7}$ M ($n = 20$) and its E_{max} was obtained with 5×10^{-6} M. In the presence of the lower concentration of the extract (250 µg/ml), the EC_{50} of histamine was $7.51 \pm 0.47 \times 10^{-7}$ M ($n = 6$) and its E_{max} was reduced to 79.84 ± 2.36%. These differences are considered significant ($p < 0.01$). In the presence of 500 µg/ml of the extract, the EC_{50} of histamine was found to be $45.5 \pm 22.1 \times 10^{-7}$ M ($n = 6$). At this concentration, the extract reduced the maximal histamine-induced contraction to 58.36 ± 5.05% ($p < 0.01$). In the presence of 10 nM pyrilamine maleate, the EC_{50} of histamine was increased significantly to $3.11 \pm 0.29 \times 10^{-6}$ M ($n = 3$) ($p < 0.01$) but its E_{max} (92.38 ± 4.99%) was not significantly reduced ($p > 0.2$). These results demonstrate that CME significantly inhibited, in a non-competitive and concentration-dependent manner, the contractile response elicited by histamine on guinea pig ileum.

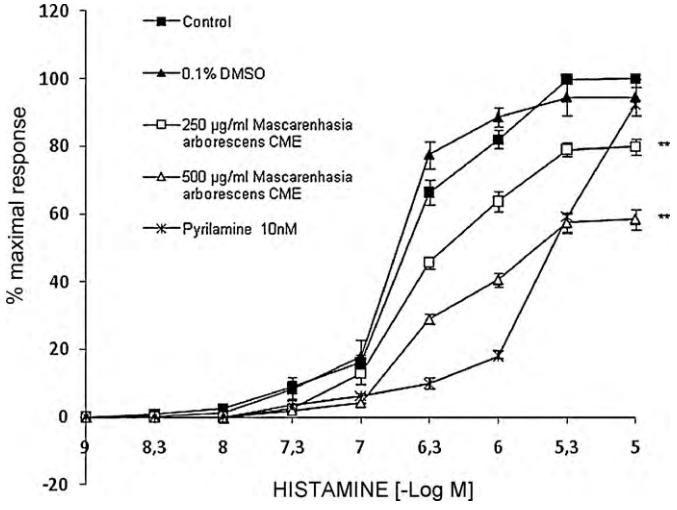

Fig. 3. Histamine contraction–response curves on the isolated guinea pig ileum in the absence or in the presence of crude methanolic extract (250 or 500 µg/ml), 0.1% DMSO or 10 nM pyrilamine maleate. Data represents mean ± S.E.M. of 3 (pyrilamine), 4 (DMSO), 20 (histamine alone) and 6 (for the other curves) independent experiments. $^{**}p < 0.01$ versus contractions induced by histamine alone. Pyrilamine (10 nM) is the positive control. DMSO (0.1%) is the negative control.

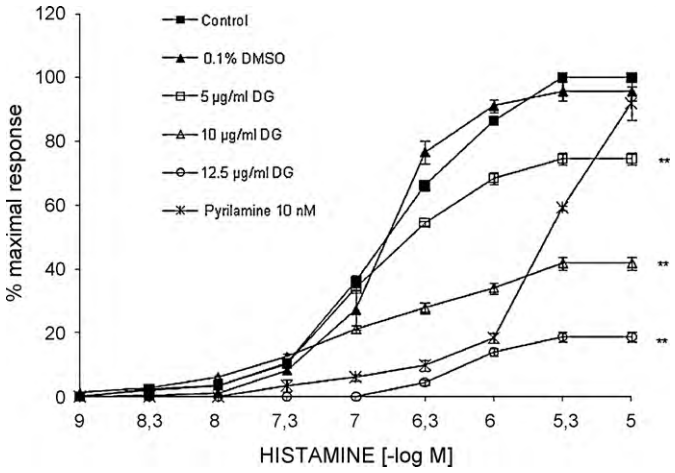

Fig. 4. Histamine contraction–response curves on the isolated guinea pig ileum in the absence or in the presence of davidigenin DG (5, 10 or 12.5 µg/ml), 0.1% DMSO and 10 nM pyrilamine maleate. Data represents mean ± S.E.M. of 3 (pyrilamine), 4 (DMSO), 30 (histamine alone) and 8 (for the other curves) independent experiments. $^{**}p < 0.01$ versus contractions induced by histamine alone. Pyrilamine (10 nM) is the positive control. DMSO (0.1%) is the negative control.

3.2. Effect of bioassay-directed fractions and sub-fractions from Mascarenhasia arborescens on isolated guinea pig ileum pre-contracted with histamine and identification of the bioactive compound

The plant extract was tested by different identification reactions for the presence of different groups of chemical constituents and it was found to contain saponins, steroids, flavonoids, tannins, phenols, while it tested negative for the presence of alkaloid compounds. On isolated guinea pig ileum pre-contracted with histamine, McP was more active (EC_{50} = 41.2 ± 3.7 µg/ml, $n = 10$) than the CME (EC_{50} = 629.6 ± 110.9 µg/ml, $n = 8$), suggesting the presence of a strong antispasmodic agent in this fraction (Table 1). EaP was more active than CME (EC_{50} = 173.5 ± 14.2 µg/ml, $n = 10$). The other fractions (HeP and AqP) are much less active than McP and EaP.

Of the nine fractions (F-1 to F-9) obtained from preparative TLC separation of the McP, three fractions (F-1 to F-3) were three different pure compounds. F-1 was found to be the most active fraction (Table 1). At 100 µg/ml, F-1 produced a significant inhibition of the contraction induced by histamine at 3 µM on guinea pig ileum, with 99.6 ± 0.6% ($n = 8$), whereas pyrilamine at 10 nM caused 42.1 ± 1.3% inhibition ($n = 8$). The other fractions (F-2 to F-9) showed a similar or higher percentage of inhibition of histamine-induced contraction on guinea pig ileum compared to pyrilamine tested at 10 nM. We continued the study with the most active pure compound F-1, identified as davidigenin (DG), a dihydrochalcone. The EC_{50} of this natural product was determined as 8.04 ± 0.81 µg/ml ($n = 8$).

3.3. Effect of davidigenin on the histamine-induced contractions in the isolated guinea pig ileum

The concentration–response curves of histamine in the absence and in the presence of 5, 10 and 12.5 µg/ml of DG are shown in Fig. 4. The three concentrations of DG shifted the histamine–response curve to the right and down. The EC_{50} of histamine alone was determined as $2.30 \pm 0.14 \times 10^{-7}$ M ($n = 30$) and its E_{max} was obtained with 5×10^{-6} M. In the presence of the lower concentration of DG (5 µg/ml), the EC_{50} of histamine was increased to $5.37 \pm 0.47 \times 10^{-7}$ M ($n = 8$) and its E_{max} was reduced to 74.56 ± 1.90%. These differences are considered to be significant ($p < 0.01$). At the concentration of 10 µg/ml, DG reduced

Table 2
Validation data from calibration curve of davidigenin in the crude methanolic extract and partitions of *Mascarenhasia arborescens*.

Compound	Linearity range (μg/ml)	Regression equation	LOD (μg/ml)	LOQ (μg/ml)	Correlation coefficient (r)
Davidigenin	2.5–1500	$y = 4 \times 10^{-5}x + 0.00005$	0.05	0.25	0.9999

the histamine-induced contraction to 41.78 ± 2.08% ($p < 0.01$). In the presence of 12.5 μg/ml of davidigenin, the E_{max} was significantly reduced to 18.76 ± 1.47% ($p < 0.01$). In the presence of 10 nM pyrilamine maleate, the EC_{50} of histamine was significantly increased to $3.11 \pm 0.29 \times 10^{-6}$ M ($n = 3$) ($p < 0.01$) but its E_{max} (92.38 ± 4.99%) was not significantly reduced ($p > 0.2$). The results presented demonstrate that DG significantly inhibits, in a non-competitive and concentration-dependent manner, the contractile response elicited by histamine on guinea pig ileum.

3.4. Effect of davidigenin on the acetylcholine-induced contractions in the isolated rat duodenum

DG is also active on isolated rat duodenum pre-contracted with acetylcholine (ACH). This active compound induced, in a non-competitive manner, a concentration-dependent relaxation of the rat duodenum with an EC_{50} of 9.35 ± 0.30 μg/ml ($n = 8$).

The concentration–response curves of ACH in the absence and in the presence of 7.5, 10 and 12.5 μg/ml of DG are shown in Fig. 5. The three concentrations (5, 10 and 12.5 μg/ml) of DG shifted the ACH–response curve to the right and down. The EC_{50} of ACH alone was determined as $9.59 \pm 0.50 \times 10^{-7}$ M ($n = 30$) and its E_{max} was obtained with 5×10^{-5} M. In the presence of the lower concentration of the dihydrochalcone (7.5 μg/ml), the EC_{50} of ACH was found to be $1.49 \pm 0.23 \times 10^{-5}$ M ($n = 8$). The two lower concentrations of DG (7.5 and 10 μg/ml) reduced the ACH-induced contraction to 68.3 ± 4.80% ($n = 8$) and 43.41 ± 1.65% ($n = 8$) respectively. These differences are highly significant ($p < 0.01$). In the presence of 12.5 μg/ml of DG, the E_{max} was significantly reduced to 20.65 ± 0.80 ($p < 0.01$). In the presence of 100 nM atropine, used as antagonistic control, the EC_{50} of ACH was significantly increased to $7.82 \pm 1.02 \times 10^{-5}$ M ($n = 3$) ($p < 0.01$) but its E_{max} (110.72 ± 1.41%) was not significantly reduced ($p > 0.01$). The results presented demonstrate that DG significantly inhibited, in a non-competitive and concentration-dependent manner, the contractile response elicited by ACH on rat duodenum.

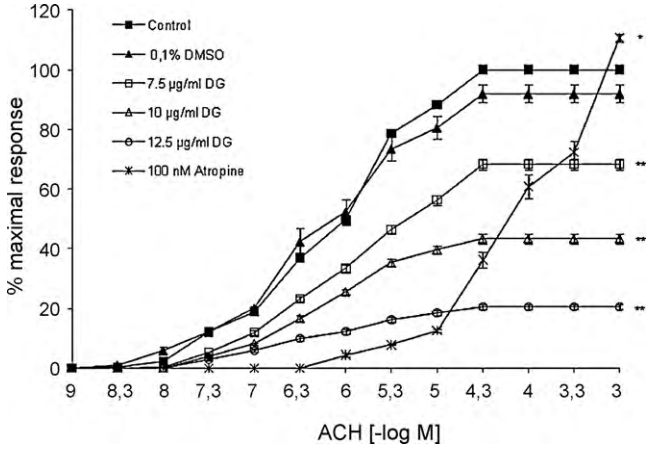

Fig. 5. Acetylcholine (ACH) contraction–response curves on the isolated rat duodenum in the absence or in the presence of davidigenin DG (7.5, 10 or 12.5 μg/ml), 0.1% DMSO and 100 nM atropine. Data represents mean ± S.E.M. of 3 (atropine), 4 (DMSO), 30 (ACH alone) and 8 (for the other curves) independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus contractions induced in the presence of ACH alone. Atropine (100 nM) is the positive control. DMSO (0.1%) is the negative control.

3.5. Antiradical activities and total polyphenol content

In the present study, the total phenolic content (TPC) of crude extracts and partitions were determined using the Folin–Ciocalteu method. Different crude extracts, methanolic (CME), aqueous (CAE) and hydro-acetonic (60/40) (CHAE), were prepared in order to compare their TPC and their antiradical activities. As results, TPC of the extracts and partitions decreased in the following order, as shown in Table 4: EaP > AqP > CAE > CHAE > CME > McP. The TPC of EaP was higher than those of crude extracts and the other partitions. Ethyl acetate seems to be the best solvent to concentrate phenolic substances of intermediate polarity (Lixiang et al., 2009).

DPPH free radical-scavenging activities of crude extracts, partitions, DG and isoliquiritigenin are also shown in Table 4. For each sample, seven concentrations (mg/ml) were tested. In order to quantify the antioxidant activity, the IC_{50} was calculated. EaP and AqP exhibited the higher DPPH radical-scavenging activities, and this trend was similar to that observed for the TPC. By comparing crude extracts and partitions, the free radical-scavenging activities decreased in the order of EaP > AqP > CAE > CME > CHAE > McP. The free radical-scavenging activity of crude extracts was less than those of EaP and AqP, which may result from the concentration of the active components, such as some polyphenols, in these partitions during the solvent–solvent partitioning processes. For example, flavonoids are well known scavengers of oxygen free radicals (Di Carlo et al., 1999). DG isolated from McP and the corresponding chalcone, isoliquiritigenin, were also tested. These two compounds are inactive. The antiradical activity of *Mascarenhasia arborescens* is not due to DG but to other unidentified molecules.

3.6. Analysis of davidigenin in Mascarenhasia arborescens by RP-HPLC-UV detection

In the study, DG in the CME and different partitions (HeP, McP, EaP and AqP) of *Mascarenhasia arborescens* leaves and stems was quantified using a developed and validated HPLC with DAD method. These results indicate that this method is fast, sensitive and suitable to quantitative assessment in DG of *Mascarenhasia arborescens*.

The proposed chromatographic method was validated to determine the LOD, LOQ, linearity and accuracy. The calibration curve of davidigenin was linear in the range of 2.5–1500 μg/ml. Regression equation and coefficient of correlation (>0.999) revealed a good linearity response for developed method and are presented in Table 2. The LOD was 0.05 μg/ml for DG and the LOQ for the same analyte was 0.25 μg/ml (Table 2). This indicated that the proposed method exhibits a good sensitivity for the quantification of DG. The recovery was found to be in the range of 98.98–102.56% for crude methanolic extract and different partitions. These results are summarized in Table 3.

Table 3
Davidigenin contents of the crude methanolic extract and partitions of *Mascarenhasia arborescens*.

Crude extract and fractions	Content (%)	R.S.D. (%)	Recovery
CME	0.50	0.86	102.56
HeP	0.023	0.75	98.98
McP	26.48	0.38	100.12
EaP	1.80	0.13	99.87
AqP	Trace	Trace	Trace

Table 4
Determination of total polyphenol content of crude extracts and partitions using the Folin–Ciocalteu method, and antiradical activities of extracts, partitions and pure compounds using the DPPH assay.

	Total polyphenol content expressed as milligram of gallic acid equivalent per gram of sample on a dry weight basis (%)	Antiradical activity IC_{50} (μg/ml)
CME	13.6 ± 0.2 (*n* = 3)	7.25 ± 0.14 (*n* = 4)
CHAE	22.8 ± 0.2 (*n* = 3)	11.40 ± 1.56 (*n* = 3)
CAE	35.8 ± 0.3 (*n* = 3)	6.16 ± 0.39 (*n* = 3)
HeP	ND	>40 (*n* = 3)
McP	11.3 ± 0.4 (*n* = 3)	13.34 ± 0.26 (*n* = 3)
EaP	42.7 ± 0.2 (*n* = 3)	2.97 ± 0.21 (*n* = 4)
AqP	37.2 ± 0.2 (*n* = 3)	4.26 ± 0.36 (*n* = 3)
Davidigenin		>40 (*n* = 3)
Isoliquiritigenin		>40 (*n* = 3)
Rutine		2.91 ± 0.18 (*n* = 4)

Mean ± S.D. (*n* = number of experiments).

The content of DG was subsequently determined by the corresponding regression equation shown in Table 2. The content of DG in CME, HeP, McP and EaP was shown to be 0.5%, 0.023%, 26.48% and 1.8% respectively (Table 3).

4. Discussion

Diarrhoeal diseases still affect many children in Madagascar and are a major cause of infant deaths (Metz, 1994). Several mechanisms are found to be responsible for diarrhoea. This pathology is often accompanied by intestinal spasms (Modigliani, 1989). Beyond the etiologic treatment of the cause of diarrhoea and the rehydration, relief of abdominal pain and cramp is sought. It is on this component of acute intestinal disease that this plant is supposed to act. So, we evaluated *Mascarenhasia arborescens* for its antispasmodic activity.

Our results demonstrated that the CME of the aerial part of this species significantly inhibited, in a non-competitive and concentration-dependent manner, the contractile response elicited by histamine on guinea pig ileum. This effect was reproduced by davidigenin, a dihydrochalcone, extracted from the most promising partition (McP). According to the quantification by HPLC, this most active partition appears to be the richest in DG (26.48%). DG was also active on acetylcholine-induced duodenal contractions. The shape of the dose–response curves showed non-competitive effects on histamine and ACH receptors (Figs. 4 and 5).

Phenolics have been previously described as having an effect on intestinal smooth muscle (Harborne and Williams, 2000). Some flavonoids were demonstrated to have effects on intestinal motility both *in vitro* and *in vivo* (Di Carlo et al., 1999). Quercetin and other flavonoids were described for their spasmolytic effect on gastrointestinal transit. For example, apigenin, quercetin and kaempferol inhibited guinea pig ileum induced contractions. Some authors concluded that the spasmolytic effect displayed by the flavonoids was related to interference with calcium influx and/or calcium release from intracellular stores. They found that pretreatment with a calcium blocker (verapamil) increased the inhibitory actions of the flavonoids (Capasso et al., 1991a, 1991b). Other authors showed that some flavonoids seem to inhibit castor-oil-induced diarrhoea and intestinal motility and secretion. These effects would be mediated by α-2 adrenergic receptors and calcium (Di Carlo et al., 1993). Nitric oxide may be also involved in quercetin-induced gastrointestinal effects (Di Carlo et al., 1996). Vitexin also produced a spasmolytic effect on rat duodenum (Ragone et al., 2007). Few studies exist on the antispasmodic activity of chalcones and dihydrochalcones. However, recently, the literature underlined a potent inhibition of carbamylcholine-induced contraction of mouse jejunum of isoliquiritigenin, the corresponding chalcone of davidigenin (Sato et al., 2007). Some authors show that isoliquiritigenin plays a dual role in regulating gastrointestinal motility, both spasmogenic and spasmolytic. The spasmogenic effect may involve the activating of muscarinic receptors, while the spasmolytic effect predominantly due to blockade of the calcium channels (Chen et al., 2009). Another recent study underlined the relaxant activity of the hexane extract of *Syzygium samarangense* (Blume) Merr. & L.M. Perry and its constituent flavonoids, including three chalcones, mediated by blockade of calcium influx (Ghayur et al., 2006). Our data confirmed those of literature that show DG acts also as a potent antispasmodic agent on mouse jejunum with an EC_{50} value of 5.07 ± 1.63 μM (Sato et al., 2007). Our results illustrate that this compound acts on whole intestinal area. The results may explain, at least partially, the traditional use of *Mascarenhasia arborescens* in intestinal spasms and diarrhoea.

DG has been isolated and identified in other species such as *Viburnum davidii* Franch., Caprifoliaceae (Jensen et al., 1977; Wang et al., 2008), *Artemisia dracunculus* L., Asteraceae (Logendra et al., 2006) or *Euphorbia portlandica* L., Euphorbiaceae (Madureira et al., 2004). This compound has also been chemically synthesized (Severi et al., 1998). DG is known to possess different activities. DG induces apoptosis in human lung fibroblasts (Liu et al., 2002), inhibits the aldose reductase ALR2 activity, a key enzyme involved in diabetic complications (Severi et al., 1998; Logendra et al., 2006; Ribnicky et al., 2009).

Phenolic compounds such as DG, contribute to the anti-allergic effects of *Saiboku-to*, a traditional Chinese herbal remedy, in patients with bronchial asthma through suppression of release of leukotrienes (LTB4 and LTC4) from human polymorphonuclear leukocytes (Homma et al., 2000; Niitsuma et al., 2001). DG, unlike liquiritigenin (LG), is contained neither in *Saiboku-to* nor in constituent herbs but is a metabolite of LG identified in the urine of patients receiving *Saiboku-to* (Homma et al., 1997; Li et al., 1998). In patients with bronchial asthma, an antitussive effect of a Japanese herbal remedy, *Bakumondo-to*, whose main constituent is *Glycyrrhizae radix*, was also observed in subjects with a high urinary excretion of LG and DG (Tamaki and Niitsuma, 1999; Kamai et al., 2005). DG is known as a hydrogenated metabolite of liquiritigenin, a flavanone, which effect on mouse jejunum is considerably weaker (Li et al., 1998; Sato et al., 2007). DG is a reductive metabolite formed via hydrogenative cleavage of the liquiritigenin C-ring by intestinal bacterial flora at the absorption stage (Homma et al., 1997). This conversion of liquiritigenin into davidigenin occurs in the lower gastrointestinal system in guinea pigs (Kamai et al., 2005). A study using the human intestinal cell line Caco-2 suggests excellent absorption of liquiritigenin and davidigenin through the human intestinal epithelial cell line, as opposed to liquiritin and liquiritin apioside, glycosides of liquiritigenin (Asano et al., 2003). In a recent study, the metabolism of isoliquiritigenin by human liver microsomes was investigated, and seven phase 1 metabolites were identified including DG as a minor metabolite (Guo et al., 2008).

Smooth muscles contraction, in response to agonists such as histamine or ACH, is initiated by the increase of the cytoplasmic-free Ca^{2+} concentration. (Karaki and Weiss, 1988). These agonists, by activating phospholipase C produce inositol triphosphate which in its turn stimulates sarcoplasmic reticulum to release the internal Ca^{2+} stores (Hashimoto et al., 1985). This released Ca^{2+} stimulates the entrance of the external Ca^{2+} by the Ca^{2+}-induced Ca^{2+} release pathway (Berridge and Irwin, 1989; Berridge, 1991). Preliminary tests performed on aorta (data not shown) underlined the calcium channel blocking activity of DG when it decreased the Ca^{2+} dose–response curves, constructed in a Ca^{2+}-free medium, in a competitive manner similar to verapamil, a standard calcium antagonist. The shape of the dose–response curves shows a competitive inhibition of calcium-induced contractions, suggesting that

the latter might be the prime mechanism of action for the antispasmodic effect of DG. Our preliminary results on aorta are very promising for this compound that seems to be bioavailable according to the literature. Activity as a calcium antagonist might have important consequences for the treatment of hypertension and other cardiovascular diseases whose prevalence is increasing in the Indian Ocean region because increasing morbid obesity (Mauny et al., 2003).

The results of this paper underline the pharmaceutical potential of *Mascarenhasia arborescens* and DG on intestinal area. Our preliminary tests on aorta show that the antispasmogenic activity of DG could be attributed to inhibition of calcium ions entry. Moreover, some fractions of this plant, the most polar, are very antioxidant. This activity does not depend on DG but probably on others polyphenols of this plant.

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