Anti-proliferative effect of *Euphorbia stenoclada* in human airway smooth muscle cells in culture

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Received 3 February 2006; received in revised form 6 July 2006; accepted 16 July 2006
Available online 21 July 2006

Abstract

The ethanolic extract of a Malagasy species *Euphorbia stenoclada* (ES) (Euphorbiaceae), traditionally used as a herbal remedy against asthma and acute bronchitis, was tested to evaluate possible anti-proliferative activity on human airway smooth muscle cells (HASMC).

The ES ethanolic extract totally abolished the interleukin-1β (IL-1β) induced proliferation of HASMC (IC50 = 0.73 ± 0.08 μg/mL). No cytotoxic effect was observed up to 20 μg/mL. A bioassay-guided fractionation of the ethanolic extract was performed by reversed-phase (RP) flash chromatography, giving five fractions (FA to FE) where fraction FE was the only active one (IC50 = 0.38 ± 0.02 μg/mL). The purification of this bioactive fraction FE was carried out by RP-HPLC affording six sub-fractions 1–6, and only sub-fraction 5 kept the anti-proliferative activity. Its major constituent was identified as quercetin (IC50 = 0.49 ± 0.12 μg/mL) by means of HPLC/UV/MS and co-elution with the authentic standard.

Quercetin was also identified in the fraction FE but was inactive. A structure–activity relationship with flavonols determined that methylation reduced the anti-proliferative activity whereas glycosylation abolished it.

The present study shows that the anti-proliferative properties of *Euphorbia stenoclada* are mediated through the presence of quercetin that may explain the traditional use of this plant as a remedy against asthma.

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Keywords: Asthma; Proliferation; Airway smooth muscle; Quercetin; Flavonoids; *Euphorbia stenoclada* (Euphorbiaceae)

1. Introduction

In the framework of our research on bioactive principles from Malagasy species (Rakotoarison et al., 2003; Um et al., 2003), we examined the endemic species *Euphorbia stenoclada* Baill. (Euphorbiaceae), locally known as ‘famata’ or ‘hamatse’. It is a spiny shrub belonging to the xerophytic vegetation grown in South-East of Madagascar (Tulear area). It belongs to the genus *Euphorbia* or spurge, the largest genus of Euphorbiaceae with about 1600 species characterized by the presence of white milky latex. The compounds isolated from this genus include flavonoids, triterpenoids, alkanes, amino acids and alkaloids (Singla and Pathak, 1990). Flavonoids from Euphorbiaceae family are well documented for their various activities such as anti-tumour (Bomser et al., 1996), anti-inflammatory (Bani et al., 2000), antioxidant (Lin et al., 2002), anti-diuretic (Yoshida et al., 1988), anti-diarrheic (Agata et al., 1991) or anti-malaric (Tona et al., 1999). The genus *Euphorbia* has been subject of intense phytochemical examination, because of its medicinal uses for the treatment of numerous diseases including skin diseases, gonorrhoea, migraine, intestinal parasites and wart cures. But neither phytochemical nor pharmacological studies have been conducted on *Euphorbia stenoclada* yet.

*Euphorbia stenoclada* is traditionally used by the Malagasy population as an infusion of the aerial parts to treat respiratory diseases such as acute bronchitis and asthma. Bronchial asthma is a rather widespread disease in Madagascar, characterized...
as an hyper-reactive airway disorder associated with recurrent and reversible wheezing episodes, and respiratory symptoms such as cough and shortness of breath (Peat and Mellis, 2002; Szefer, 2002; Wood, 2002). Asthma is also associated with fibrosis, and increased thickness of the smooth muscle layer (for review: Joubert and Hamid, 2005).

Therefore, our study aimed at evaluation of potential anti-proliferative effects on the airway smooth muscle of *Euphorbia stenoclada* (ES) aerial parts. Hence, we treated human airway smooth muscle cells in primary culture (HASMC) with interleukin-1β (IL-1β), a pro-inflammatory cytokine present in large quantities in asthmatic airways and known for its proliferative effect on these cells (De et al., 1993, 1995), with or without ES extracts pre-treatment. This anti-proliferative assay was also used to perform a bioassay-guided fractionation assay to identify the active compounds of ES.

2. Material and methods

2.1. Plant material and extraction

Aerial parts of ES were collected in summer 2003 from the Tulear region, in the South Eastern part of Madagascar, and authenticated by A. Rakotozafy (Ethnobotany department, IMRA, Madagascar). A voucher specimen (ref. 4768) was deposited in the herbarium of the botanical and zoological park of Tsimbazaza (Antananarivo, Madagascar). Eleven grams of a mixture of leaves and stems were finely grounded and macerated three times in 96% ethanol (EtOH) at room temperature during 24 h. The filtered extracts were combined and evaporated under reduced pressure, resuspended in water, defatted with cyclohexane (three times liquid/liquid partitions), and dried to afford a 1.46 g of ethanolic extract (13.2% w/w) (Fig. 1).

![Image](90x152 to 330x330)

Fig. 1. Scheme of the bioassay-guided fractionation of the ethanolic extract from ES aerial parts. After ethanolic extraction and defatting with cyclohexane, the extract was fractionated by C18-Flash chromatography into five fractions FA to FE. The active fraction FE was further fractionated by the same way into six sub-fractions 1–6, and subjected to the anti-proliferative assay.

2.2. Fractionation, isolation and structure/activity relationship (SAR)

Fractionation of the bioactive ethanolic extract of ES was carried out by a bio-guided approach (Fig. 1). About 10 µg of the ethanolic residue was dissolved in 1 mL of 50% EtOH/water for evaluation of the anti-proliferative property on human airway smooth muscle cell in culture (HASMC). One gram of the defatted extract was dissolved in methanol, and fractionated on an octadeyl silica gel column (flash chromatography, 40 mm × 150 mm, Biotage®, Dyax Corp. Company) using a stepwise elution with water/methanol from 10% to 100% methanol (flow rate = 40 mL/min). Thirty-seven fractions were collected and analysed by means of TLC (thin layer chromatography) (silica gel plates F254, 5554, Merck) with EtOAc/formic acid/acetic acid/water, 100:11:11:27 (v/v/v/v) as the eluent system. Spots were detected at 254 and 366 nm wavelength, and revealed with NP/PEG (1% methanol diphenylboric acid/β-ethanolamino ester/polyethylene glycol) reagent. Similar fractions were combined and afforded five fractions (FA to FE). These five fractions were dissolved in 50% EtOH/water at a 1% final concentration for assessment of the anti-proliferative activity. The active fraction (fraction FE) was purified by a semi-preparative reversed-phase HPLC (250 mm × 21 mm, Nucleodur®, Macherey-Nagel) eluted with 0.01 M H3PO4 (phase A) and MeOH (phase B) in the following conditions: from 95% to 50% for 10 min (A), from 50% to 30% for 25 min (A) and to 100% for 5 min (B), followed by washing and reconditioning of the column. Fractions were monitored at 370 nm (115 UV detector, Gilson) (Fig. 2). The purification afforded six sub-fractions, controlled by analytical reversed-phase HPLC (9010 pump and Prostar photodiode array detector, Varian) as mentioned above, dried and dissolved in 50% EtOH/water as the most suitable solvent for ES fractions for less cytotoxic side-effects on HASMC as compared with DMSO or ethanol alone (data not shown).

The constituents of the active fraction FE were identified by means of reversed-phase HPLC/UV/MS (HPLC, Agilent; Polaris column; Bruker 3000+ mass spectrometer) and co-eluted with the respective standards in the previously reported elution conditions (Escarpa and Gonzalez, 2000). Mass spectra were obtained in negative and positive modes.

Structure/activity relationship (SAR) was studied by comparing the anti-proliferative activity of quercetin and its methylated (3′-methylquercetin and pentamethylquercetin) and glycosylated derivatives (hyperoside, quercitrin, and isoquercitrin) (all standards supplied by Chromadex) at 10 and 20 µg/mL.

2.3. Primary culture of human airway smooth muscle cells

Human bronchial smooth muscle cells (HASMC) were cultured from human bronchial smooth muscle obtained from healthy lung transplant donors after sudden death (Centre for Biological Resources (CRB), Dr. N. Martinet, Nancy, France). The smooth muscle cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM/F12), supplemented with 10% foetal bovine serum (FBS), penicillin (50 U/mL), streptomycin...
Fig. 2. RP-HPLC profiles (280 nm) of ES infusion extract, ES ethanolic extract and active fraction FE. The comparison of the profiles of the active fraction FE with ES ethanolic extract from which it was extracted, along with the infusion (aqueous extract) of ES as traditionally used in asthma treatment by Malagasy patients.

(50 µg/mL), non-essential amino acids (1:100), l-glutamine (2 mM) (all products from Invitrogen), and insulin (5 µg/mL) (Lilly, St Cloud, France) in a humidified chamber (37 °C, 5% CO2) with the medium changed every other day (all products supplied by Invitrogen, Cergy Pontoise, France). Cells were used for experiments at passage 7.

2.4. Cell treatment

Cells were treated with human IL-1β (10 U/mL, R and D Systems, Lille, France) or its solvent for 4 days with medium changed daily. Cells were pre-treated with the ES ethanolic extract, the five fractions FA to FE, the six sub-fractions 1–6 or with ethanol (1% in culture medium) as a blank for 1 h before IL-1β treatment, every day for 4 days. Concentration–response curves to the aforementioned compounds were performed in order to calculate its potency expressed as inhibitory concentration 50 (IC50).

Cell proliferation was measured by the XTT assay (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide, “Cell Proliferation kit II XTT”, Roche Diagnostics, Mannheim, Germany), according to the manufacturer’s instructions. Briefly, cells were seeded in 96-well culture plates (3000 cells per well) in low-FBS (0.3%), insulin-free, DMEM-F12 medium, allowed to adhere for 3 h, and were then treated for 4 days with medium changed everyday. Cells were then exposed to XTT (1 mg/mL) for 3 h and absorbance measured at 450 nm.

2.5. Expression of results and statistical analysis

Proliferation studies were performed three times in triplicate. Results are expressed as a percentage of proliferation compared with controls and are presented on graphs as means ± S.E.M. (standard error of the mean). Data were analysed by a two-tailed Student’s t-test, a Student–Newman–Keuls test when comparing more than two variables, or a Dunnett test when comparing dose–response data, at a p < 0.05 level of significance. IC50 are expressed as means ± S.D. (standard deviation), based on concentration-dependent curves performed three times in triplicate.

3. Results

3.1. Anti-proliferative effect of ES ethanolic extract and bio-guided fractionation

IL-1β-induced proliferation of HASMC was maximum at 10 U/mL (49.2 ± 2.3% increase over baseline proliferation, p < 0.001; data not shown), and this concentration was used in the following experiments. IL-1β-induced proliferation of HASMC (51.6 ± 1.9% increase over baseline proliferation, p < 0.001) was totally abolished by ES ethanolic extract (102.9 ± 2.1% inhibition at 10 µg/mL; p < 0.001), with an IC50 of 0.73 ± 0.08 µg/mL. Fractions FA to FD did not show any inhibitory effect on IL-1β-induced proliferation of HASMC. Fraction FE was the only active fraction, and totally abolished IL-1β-induced proliferation of HASMC (101.6 ± 1.9% increase over baseline proliferation, p < 0.001) was totally abolished by ES ethanolic extract (102.9 ± 2.1% inhibition at 10 µg/mL; p < 0.001), with an IC50 of 0.73 ± 0.08 µg/mL. Analysis by HPLC-UV-DAD of this active fraction revealed the exclusive presence of flavonols. These results suggest that the bioactive compound(s) of ES exhibiting anti-proliferative activity is (are) only present in fraction E and has (have) a flavonolic structure.

Fraction E was subsequently fractionated into six sub-fractions, named 1–6, using a semi-preparative reversed-phase HPLC. Each of these sub-fractions contained a unique product, identified by means of reversed-phase HPLC/UV/MS. Sub-fraction 5 only displayed the ability to totally abolish proliferation of HASMC induced by IL-1β (105.7 ± 1.2%
inhibition; \( p < 0.001 \) with an IC\(_{50}\) of 0.49 ± 0.12 \( \mu \)g/mL. The five other sub-fractions (1–4 and 6) did not show any anti-proliferative effect. The solvent itself (50% EtOH/water at a 1% final concentration) did not modify IL-1\( \beta \)-induced proliferation of HASMC (data not shown).

### 3.2. Identification of the bioactive compound

To identify the compound isolated from sub-fraction 5 that displays the anti-proliferative activity towards IL-1\( \beta \)-induced proliferation of HASMC, the whole fraction FE was analysed by RP-HPLC-DAD-MS (reversed-phase-high performance liquid chromatography-diode array detector-mass spectrometry). The major compound of fraction FE was determined as quercitrin contained in sub-fraction 1 (retention time \( R_t \) of 19.52 min, UV maximal absorption \( \lambda_{max} \) at 253 and 349 nm and molecular weight (MW) of 447.1). Sub-fractions 2–4 and 6 were eluted at \( R_t \): 20.93, 22.78, 24.7 and 32.48 min, respectively, and had a flavonolic UV profile. Quercitrin was therefore not the active compound of FE. A second flavonol was detected in FE, identified as quercetin. It was contained in sub-fraction 5 (\( R_t = 27.07 \) min, \( \lambda_{max} \): 254 and 368 nm, and MW: 301.0) (Fig. 3). Confirmation of the structure was done by co-elution with authentic standards.

![Fig. 4. Structure of flavonols used in the SAR study.](image)

**Fig. 4.** Structure of flavonols used in the SAR study. The flavonol concentrations used in SAR activity are 10 (10) and 20 (20) \( \mu \)g/mL. Q, quercetin; QC, quercitrin (or quercetin 3-O-rhamnoside); IQ, isoquercetin (or quercetin 3-O-glucoside); H, hyperoside (or quercetin 3-O-galactoside); MQ, 3'-methyl quercetin; PMQ, pentamethylquercetin.
3.3. Structure/activity relationship

Since quercetin and querctin only differ in nature of the substituent in position C-3 (position 3 of the C ring), we conducted a study of structure/activity relationship, by comparing the anti-proliferative activity of quercetin with glycosylated or methylated derivatives (Fig. 4). None of the querctin hetero-sides displayed any activity on the IL-1β-induced proliferation of HASMC, suggesting that the hydroxy group in position 3 of the C ring needs to be kept unsubstituted. Studies conducted with two methylated derivatives showed that the flavonol with one hydroxyl function of the B ring (also called catechol moiety) substituted with a methoxy in position 3′(3′-methylquerctin) was no longer active at 10 μg/mL; however, its anti-proliferative effect was restored at higher concentrations. In contrast, the anti-proliferative properties of the methoxylated querctin derivative (pentamethylquerctin) were lost, even at higher concentrations. These results then suggest that all free hydroxyl groups of querctin are necessary to its anti-proliferative activity, and that any substitution, whether methylation or glycosylation, lowers if not totally abolishes this effect.

4. Discussion and conclusions

The present study provides evidence that an ethanolic extract of Euphorbia stenoclada inhibits IL-1β-induced proliferation of the human airway smooth muscle, and identifies querctin as the major anti-proliferative compound of Euphorbia stenoclada. Euphorbia stenoclada aerial parts are used in Madagascar as an infusion to treat respiratory disorders such as acute bronchitis and asthma. Other Euphorbia spp. are also traditionally used to treat these diseases in other countries, as for instance Euphorbia hirta L. in India (Singh et al., 2005) or Euphorbia lunulata Bunge in South China (Nishimura et al., 2005). However, only few studies have been conducted in order to understand their traditional uses including identification of their active components and their mechanism of action.

Although terpenes of Euphorbiaceae have been shown to exhibit anti-inflammatory (Corea et al., 2005), antinociceptive (Ahmad et al., 2005) or anti-tumour (Ferreira et al., 2005) properties, the active compounds isolated in this study from Euphorbia stenoclada were polyphenols and not terpenes. Flavonoids isolated from other Euphorbia spp. have also been studied for their anti-ulcer (Lin and Yuan, 1988), antibacterial (Vijaya et al., 1995) and antiviral properties (Ahn et al., 2002). A recent study described the effect of gallic acid and querctin isolated from Euphorbia lunulata Bunge on several cell lines. These phenolic acid and flavonol could mimick some effects of IL-10, a cytokine with some anti-inflammatory effects in asthma (Nishimura et al., 2005). Helioscinpin-A, another polyphenol isolated from Euphorbia helioscopia L., exerts an inhibitory effect of leukotirole D4-induced tracheal contraction in rats and also on antigen-induced bronchial constriction in an experimental asthma model in the guinea pig (Park et al., 2001). Thus, it has been observed from this study that a polyphenolic constituent of Euphorbia stenoclada can inhibit the airway smooth muscle cell proliferation induced by inflammatory agents such as IL-1β.

Our bio-guided fractionation based on HASMC proliferation showed that the compound that supports the anti-proliferative activity of the ethanolic extract prepared from ES aerial parts was exclusively querctin. This anti-proliferative effect on the human airway smooth muscle has never been reported earlier. However, this result is in accordance with numerous studies showing the anti-proliferative activity of querctin in other cell types, such as in cancer cell lines (for reviews: Kanadaswami et al., 2005; Lambert et al., 2005). In addition, various experimental studies have shown benefits of querctin in the treatment of asthma: this flavonol has been shown to inhibit bronchial obstruction and airway hyperresponsiveness in the guinea pig (Dorsch et al., 1992), to display in vitro relaxant effects on guinea pig trachea pre-contracted with histamine, carbachol or KCl (Ko et al., 1999, 2002, 2003) or to inhibit the release of histamine in vitro from rat peritoneal mast cells (Haggag et al., 2003). The present study extends its properties as an inhibitor of airway smooth muscle proliferation thus adds knowledge about possible potential anti-asthmatic properties of querctin in vivo.

The structure–activity relationship conducted in this study also provides some new information on querctin that was the most potent anti-proliferative compound among other substituted flavonols. It seems that, on one hand, a methyl substitution in the B ring induces a 50% decrease in the activity of querctin whereas a total methylation of the querctin’s hydroxyl groups totally abolished it. Therefore, the presence of free hydroxyl groups on the B ring seems to be necessary for the anti-proliferative activity of querctin. On the other hand, 3-glycosylation by (rhamnose, glucose or galactose) completely abolished the activity. This result confirms the importance of the 3-OH position substitution of querctin as a cornerstone in the activity. Other works in the literature showed that querctin derivatives having one or more methyl substitution were more active than querctin itself to relaxation of isolated guinea pig trachea contracted with histamine, carbachol or KCl (Ko et al., 1999). These authors reported further that 3-O-methylquerctin isolated from Rhamnus nakaharai (Hayata) Hayata, a species used as folk medicine in Taiwan for the treatment of inflammation and asthma, exhibited relaxant activity on the guinea pig trachea in vitro by inhibition of phosphodiesterases (Ko et al., 2002). Recently, the same group reported that 3-O-methylquerctin is active in vivo in mice, inhibiting inflammation and airway hyper-responsiveness in a murine model of asthma (Ko et al., 2004).

In conclusion, we have clearly shown that Euphorbia stenoclada displays anti-proliferative activity on the human airway smooth muscle, due to the presence of querctin. Even though further studies are needed to confirm these properties of Euphorbia stenoclada in vivo, and in particular validated in a murine model of asthma, the present work opens new perspectives for asthma treatment based on ethnopharmacological studies.

References


