Biochemical Pharmacology 83 (2012) 514–523

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Paradoxical effects of polyphenolic compounds from Clusiaceae on angiogenesis

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ARTICLE INFO

Article history:
Received 10 September 2011
Accepted 2 December 2011
Available online 9 December 2011

Keywords:
Endothelium
Angiogenesis
Clusiaceae
Xanthone
Nitric oxide

ABSTRACT

Clusiaceae plants display high contents of xanthones and coumarins, the effects of which on endothelium, more particularly on angiogenesis, have not been assessed yet. We screened the capacity of six molecules from Clusiaceae – belonging to xanthones, coumarins and acid chromanes classes – to induce endothelium-dependent relaxation on mice aortic rings. Endothelial nitric oxide (NO) production was assessed in endothelial cell line using electron paramagnetic resonance technique. Then, the capacity of these molecules to induce capillary-like structures of endothelial cells was assessed. Cellular processes implicated in angiogenesis (adhesion, migration and proliferation) and Western blot analyses were then investigated. Among the tested molecules, isocalolongic acid (IA) and 2-deprenylrheediaxanthone (DRX) induced an endothelium-dependent relaxation of the aorta associated with an increase of NO production in endothelial cells. Using *in vitro* and *ex vivo* angiogenesis assays, it was shown that IA treatment promoted the formation of capillary-like network. In contrast, DRX prevented the ability of vascular endothelial growth factor (VEGF) to increase the formation of capillary-like network. IA increased endothelial cell proliferation while DRX decreased all cellular processes of angiogenesis. Western blot analysis showed that IA increased VEGF expression whereas DRX decreased ICAM-1 expression. Altogether, these data allowed identifying isolated molecules from Clusiaceae that exhibit a potential activity towards the modulation of endothelium-dependent relaxation involving NO release. Interestingly, they also highlighted paradoxical effects of the two compounds on cellular angiogenic processes, IA being pro-angiogenic and DRX anti-angiogenic.

1. Introduction

Natural dietary polyphenolic compounds present in a wide variety of plants are thought to protect against cardiovascular disease and cancer [1,2]. Polyphenols are a large family of natural compounds widely distributed in plants that include flavonoids and non-flavonoids. Flavonoids are the most studied polyphenols on cardiovascular system. Among non-flavonoids, xanthones, coumarins and acid chromanes are less investigated. Family Clusiaceae is known to biosynthesize the above polyphenolic compounds [3] and Clusiaceous species are widely distributed in tropical Asia, Africa, New Caledonia and Polynesia [4]. In the past few years, a large number of xanthones and coumarins have been identified from these species and they exhibit various biological activities such as antifungal, antimalarial, cytotoxic and antioxidant properties [5–8].

One of the therapeutic relevant effects of polyphenols on the cardiovascular system may be their ability to interact with the nitric oxide (NO)-generating pathway in vascular endothelium [9]. Xanthones and coumarins can act on the endothelium either by increasing NO release [10,11] or decreasing level of NO synthase inhibitors [12]. However, little information has been reported on biological activities of acid chromanes on endothelium.

Angiogenesis is a complex process characterized by the early degradation of extracellular matrix, essentially by matrix metalloproteinases, followed by migration and proliferation of endothelial cells and the maturation of the new blood vessel in response to local pro-angiogenic factors such as vascular endothelial growth factor (VEGF) [13].

The present study was designed to screen the effect of unrecognized polyphenolic compounds isolated from plants belonging to family Clusiaceae on the endothelium (Fig. 1). More precisely, we screened the capacity of six molecules belonging to xanthones, acid chromanes and coumarins classes to induce endothelium-dependent relaxation on isolated blood vessels and

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doi:10.1016/j.bcp.2011.12.002

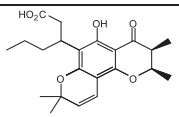
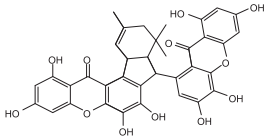
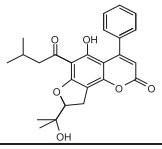
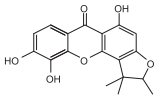
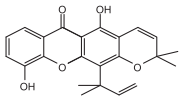
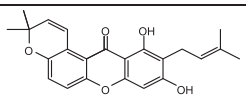
Compound	Chemical structure	From
Isocalolongic acid (IA)		*Calophyllum caledonicum*
Griffipavixanthone		*Garciniia virgatta*
Mammea A/AA cycloF		*Calophyllum dispar*
2-deprenylrheediaxanthone (DRX)		*Garcinia vieillardii*
Caloxanthone C		*Calophyllum caledonicum*
Calothwaitesixanthone		*Calophyllum caledonicum*

Fig. 1. Chemical structure and origin of tested polyphenolic compounds isolated from Clusiaceae plants.

NO release from endothelial cells. Since NO can act on the expression of protective genes of the cardiovascular system, including the regulation of angiogenesis, the molecules able to induce endothelium-dependent vasodilatation were then assessed on the different cellular processes implicated on angiogenesis: cell migration, proliferation, adhesion and the formation of capillary-like structures.

2. Material and methods

2.1. Reagents

The isolation and purification of isocalolongic acid, griffipavixanthone, mammea A/AA cycloF, 2-deprenylrheediaxanthone, caloxanthone C and calothwaitesixanthone have been previously described in detail [6,7,14,15]. For contractile and cellular experiments, all tested compounds were solubilized in pure DMSO (Sigma–Aldrich, Saint-Louis, MO) and then diluted in distilled water to reach a final concentration of DMSO of 1%. The residual concentration of DMSO never exceeded 0.01%, which was used as control condition for all experiments.

Trypsin-EDTA and culture *media* were obtained from Lonza (Basel, Switzerland). VEGF was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture

The EaHy 926 (American Type Collection, Manassas, VA) endothelial cell line was maintained at 37 °C in a humidified incubator gassed with 5% CO_2 in air and was cultured in growth medium (Dulbecco's modified Eagle's medium: Ham's F-12; Lonza) supplemented with 1% L-glutamine, 1% non-essential amino acids, 1% Na pyruvate, 1% streptomycin/penicillin (Lonza), 1% hypoxanthine, aminopterin, thymidine (Sigma–Aldrich) and 10% of heat-inactivated fetal bovine serum (FBS) (Invitrogen, Cergy Pontoise, France).

Also, freshly delivered umbilical cords were obtained from a nearby hospital. Human Umbilical Vein Endothelial Cells (HUVECs) were obtained as previously described [16] and grown on plastic flasks in MCDB 131 medium (Invitrogen) containing 1% L-glutamine, 1% streptomycin/penicillin, 500 ng/L epidermal growth factor, 2 μg/L basic fibroblast growth factor (PeproTech Inc., Rocky Hill, NJ), supplemented with 10% of heat-inactivated FBS. HUVECs were used at the second to fourth passage. Cells were grown for 24 h in the absence or presence of 0.25 μM, 8 μM or 25 μM tested compound, or VEGF (20 ng/mL).

2.3. Cell viability assay

Cell viability was determined by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma–Aldrich). Briefly, HUVECs were seeded at 10^4 cells/well in a 96-well plate, and 24 h after the culture medium (200 μL) was changed to the experimental medium supplemented with isocalolongic acid (IA) or 2-deprenylrheediaxanthone (DRX) from 0.25 μM to 25 μM. Following culture with IA or DRX for 24 and 48 h, 100 μL of medium were removed and 10 μL of MTT (5 mg/mL) was added in each well. After a 4-h incubation at 37 °C, 100 μL of 10% sodium dodecyl sulfate (SDS; Euromedex, Souffelweyersheim, France) was added and incubated overnight at 37 °C. The plate samples were then read at 570 nm on a microplate reader (Synergy HT, Biotek, Winooski, VT). IA and DRX concentrations were considered as no cytotoxic when cell viability was superior to 85% after 48 h stimulation. The concentrations either of IA or DRX required to induce biological response *in vitro* can be reached in the plasma (about 1–10 μM) for these chemical classes (xanthones or coumarins) and hence may act on the endothelium *in vivo*. We have chosen non-cytotoxic and physiologically relevant concentrations to perform angiogenesis assays [17–19].

2.4. NO radical determination by electron paramagnetic resonance (EPR)

Detection of NO production was performed using Fe^{2+} diethyldithiocarbamate (DETC, Sigma–Aldrich) as spin trap.

Briefly, cells were seeded on 6-well plates and used when 80–90% cell fluency were reached. Endothelial cells were either stimulated with the tested compounds (25 μM) or the reference thapsigargin 10 μM (TG, Sigma–Aldrich) for 10 or 30 min, respectively; the medium then was replaced with 250 μL of Krebs solution, then treated with 250 μL of colloid $Fe(DETC)_2$ and incubated for 45 min at 37 °C. Cells were then scrapped and frozen in plastic tubes. NO detection was measured *in situ* by EPR. NO measurement was performed on a table-top x-band spectrometer Miniscope (Magnettech, MS 200, Berlin, Germany). Recordings were made at 77 K, using a Dewar flask. Instrument settings were 10 mW of microwave power, 1 mT of amplitude modulation, 100 kHz of modulation frequency, 150 s of sweep time and 5 scans. Signals were quantified by measuring the total amplitude, after correction of baseline as done previously. Values are expressed as amplitude of signal per protein concentration (units/μg/μL of endothelial cell proteins).

2.5. Aortic preparation and mounting

All animal studies were carried out using approved local institutional protocols (no. 2009.8) and were conformed the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication no. 85-23, revised 1996). Male Swiss mice (6–8-week old) were killed by CO_2 asphyxia. The thoracic aorta was removed and carefully cleaned of adhering fat and connective tissue, then cut into rings (2 mm length). The rings were then mounted on a wire myograph filled with Krebs solution (composition in mM: NaCl 130, $NaHCO_3$ 14.9, KCl 3.7, KH_2PO_4 1.2, $MgSO_4{\cdot}7H_2O$ 1.2, $CaCl_2{\cdot}H_2O$ 1.6, glucose 11) (Sigma–Aldrich), maintained at 37 °C and continuously bubbled with a 95% O_2–5% CO_2 mixture. Resting tension was adjusted to 5 mN. Tension was measured with an isometric force transducer (Danish Myo Technology, Aarhus, Denmark). After an equilibration period of 90 min, the vessels were maximally contracted with U46619 (0.1 μM) in order to test their contractile capacity. In some experiments, the endothelium was removed by gently rubbing the intima surface with sterile nipple, as previously described [20]. The presence of functional endothelium was assessed in all preparations by determining the ability of acetylcholine (1 μM) to induce more than 50% relaxation of rings pre-contracted with U46619. Vessels were considered to be denuded of functional endothelium when there was no more 5% relaxation response to acetylcholine (1 μM).

2.6. Characterization of the relaxant effect of polyphenolic compounds

Aortic rings with and without functional endothelium were pre-contracted to the same tension with U46619 (30 nM, Sigma–Aldrich) (i.e. 80% of maximal response obtained in vessels with functional endothelium). When the contraction reached a steady state, increasing concentrations of polyphenolic compounds (2.5 nM–25 μM) were added cumulatively.

2.7. In vitro capillary network formation on ECM gel®

After 24 h of incubation with tested compounds or VEGF (20 ng/mL), HUVECs were detached with trypsin-EDTA. Cells were seeded with a density of 15×10^4 cells per well precoated with ECM gel® (Sigma–Aldrich). Briefly, 150 μL of ECM gel® substrate diluted with FBS-free medium (1:1 dilution) was added into a four-well plate and allowed to solidify for 1 h at 37 °C. Then, cells were incubated with medium containing 10% FBS and allowed to adhere for 1 h after which the different *stimuli* were added. Tube formation was examined by phase-contrast microscopy (MOTIC AE21; 100× magnification) after 4 and 24 h and was quantified using ImageJ software. The capillary length was counted in three randomly selected microscopic fields for each experiment. To determine pro- or anti-angiogenic properties of tested compounds, HUVECs were treated with tested compounds or with the combination of VEGF and tested compounds as described above and culture media were removed. Then, HUVECs were treated with each conditioned medium for 24 h, and tube formation was determined as described above.

2.8. Ex vivo angiogenesis assay

The *ex vivo* angiogenesis assay was performed according to the method slightly modified with the use of ECM gel® instead of collagen gel [21]. Briefly, male Swiss mice (6–8-week old) were sacrificed by CO_2 asphyxia. Thoracic aorta was removed and washed with cell medium to avoid contamination with blood. It was then turned inside out and cut into short segments of about 1 mm. Briefly, 150 μL of ECM gel® substrate diluted with FBS-free medium (1:1 dilution) was added into a four-well plate and allowed to solidify for 1 h at 37 °C. Each aortic segment was placed in the center of a well of four-well culture plate and covered with 50 μL pure ECM gel®. The solution was allowed to gel at 37 °C for 1 h, and then overlaid with 1 mL medium containing 10% FBS. Sample solution or vehicle was then added. Plates were incubated for 4 days in a fully humidified system of 5% CO_2 at 37 °C. Tube formation was examined by phase-contrast microscopy (MOTIC AE21) after 4 days. To determine pro- or anti-angiogenic properties of tested compounds, aortic rings were treated with tested compounds or the combination of VEGF and tested compounds, as described above.

2.9. Adhesion assay

Evaluation of adherent cells was performed using crystal violet staining [22]. For adhesion experiments, 5×10^3 cells per well were seeded into 96-well plates and stimulated by tested compounds. After 24 h of incubation, the plate was shacked for 15 s. The supernatant with non-adherent cells was removed by three washes with washing buffer (0.1% bovine serum albumin in medium without serum; Sigma–Aldrich). Attached cells were fixed with 4% of paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) for 15 min at room temperature. Cells were rinsed two times with washing buffer, stained with crystal violet (Sigma–Aldrich) (1 mg/mL in 2% of ethanol) for 10 min at room temperature protected from light and extensively washed with distilled water. Then, SDS 2% was added and incubates for 30 min at room temperature. Absorbance was then evaluated using a microplate reader at 550 nm (Sinergy HT Biotek).

2.10. Proliferation assay

Effects of compounds on proliferation on HUVECs were analyzed by using CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). Briefly, 5×10^3 cells per well were seeded into 96-well plates and allowed to attach overnight and then cells were treated with compounds for 24 h. After growth medium removal, dye-binding solution was added into each microplate well and cells were incubated at 37 °C for 30 min. The fluorescence levels were read on a fluorescent microplate reader (Synergy HT, Biotek) with filters for 485 nm excitation and 530 nm emission.

2.11. Migration assay

The Transwell cell culture chambers (Corning, Cambridge, MA) were used for cell migration assay. Enriched-medium with

Table 1
pD_2 values and maximal relaxation (E_{max}) obtained with isocalolongic acid (IA), 2-deprenylrheediaxanthone (DRX), griffipavixanthone, mammea A/AA cycloF, caloxanthone C and calothwaitesixanthone in pre-contracted mouse thoracic aorta. Values are means ± SEM from four independent experiments for each group. +*E* and −*E* indicate, respectively, with and without a functional endothelium.

	pD_2		E_{max} (% relaxation)	
	+*E*	−*E*	+*E*	−*E*
Isocalolongic acid (IA)	5.15* ± 0.12	4.80 ± 0.11	99.9 ± 0.1	99.1 ± 0.9
Griffipavixanthone	4.98 ± 0.11	4.93 ± 0.08	49.1 ± 14.1	35.0 ± 5.3
Mammea A/AA cycloF	4.83 ± 0.12	4.70 ± 0.12	58.6 ± 15.5	47.0 ± 18.3
2-Deprenylrheediaxanthone (DRX)	5.22 ± 0.08	4.98 ± 0.20	99.3 ± 0.7	98.5 ± 0.7
Caloxanthone C	4.76 ± 0.15	4.81 ± 0.12	54.8 ± 16.9	41.1 ± 20.6
Calothwaitesixanthone	4.83 ± 0.11	4.89 ± 0.09	66.1 ± 11.1	60.8 ± 14.2

* $P < 0.05$ is the statistical differences *versus* −*E*.

20% FBS was added into the lower chamber. 2×10^4 HUVEC cells were added onto the upper compartment (8 μm pore size) in 250 μL of starvation medium containing 0.5% FBS with IA (0.25 μM, 8 μM, 25 μM), DRX (0.25 μM, 8 μM), vehicle or VEGF (20 ng/mL). After 24 h of incubation at 37 °C, the non-migrated cells were removed from the upper surface of the membrane by wiping with a cotton swab. The membrane was then fixed with 4% paraformaldehyde for 15 min and then stained with crystal violet solution for 10 min at room temperature protected from light and extensively washed with distilled water. Then, SDS 2% was added and incubated for 30 min at room temperature. Absorbance was then evaluated using a microplate reader at 550 nm (Sinergy HT Biotek).

2.12. Western blot

After treatment, cells were homogenized and lysed. Proteins (20 μg) were separated on 10% SDS-polyacrylamide gel electrophoresis (Invitrogen). Blots were probed with E-selectin, ICAM-1, MMP-2, PECAM-1 and VEGF antibodies (Santa Cruz Biotechnology). Monoclonal anti-β-actin antibody (Sigma–Aldrich) was used to visualize protein gel loading. The membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ). The protein–antibody complexes were detected by enhanced chemiluminescence plus (Amersham Biosciences).

2.13. Quantitative real-time reverse transcription-polymerase chain reaction analysis

HUVEC cells were grown for 24 h in the presence or absence of tested compounds (8 μM). Cells were detached using trypsin-EDTA and after two subsequent steps of centrifugation at 500 × *g* for 10 min, the pellet containing cells were frozen in liquids N_2 and used to investigate the expression of messenger RNA (mRNA) for E-selectin, ICAM-1, IL-6, IL-1β, MT-MMP1, MMP2, PECAM-1, TNFα, VEGF-A, and VCAM-1 transcripts by real-time reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR analyses were carried out by the Service Commun de Cytométrie et d'Analyses Nucléotidiques from Angers University on Chromo 4™ (Bio-Rad, Hercules, CA) using the SYBR Green PCR Master Mix (Invitrogen). The PCR reaction consisted of 7.5 μL of SYBR Green PCR Master Mix, 10 nM of forward and reverse primers and 2.0 μL of 1:20-diluted template cDNA in a total volume of 20 μL. Cycling was performed using these conditions for 10 min at 95 °C, followed by 40 rounds of 15 s at 95 °C and 1 min at 60 °C. To verify that the used primer pair produced only a single product, a dissociation protocol was added after thermocycling, determining dissociation of the PCR products from 65 °C to 95 °C. We selected the GAPDH gene as an endogenous control. Finally, the quantification of mRNA was performed according to the ΔC_t method.

2.14. Data analysis

Relaxation was measured as the reduction of the contraction (tension in mN) induced by tested compounds and expressed as mean ± SEM, where *n* is the number of preparations from different animals used, which is at least 4 for all experiments. Sensitivities to drugs were expressed as pD_2 values, where $pD_2 = -\log EC_{50}$, EC_{50} being the drug concentration required to give a half-maximal relaxant response. Graph Pad Prism software was used to fit sigmoidal curves and to determine the E_{max} and EC_{50} values from the concentration-response curves. Results were compared by two-way ANOVA followed by Bonferroni post-hoc test, as appropriate.

For cellular assays, data are represented as mean ± SEM, *n* represents the number of experiments repeated at least in triplicate. Statistical analyses were performed by Mann–Whitney *U*-tests (non-parametric).

All tests were two-tailed and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of polyphenolic compounds from Clusiaceae plants on the sustained contraction induced by U46619 in endothelium intact and denuded aorta

In aortic rings with functional endothelium, all the polyphenolic compounds (2.5 nM to 25 μM) induced a concentration-dependent relaxation of the sustained contraction induced by U46619 (30 nM). The mean pD_2 and E_{max} values are given in Table 1. In rings without functional endothelium, the concentration–response relaxation curves of IA and DRX but not the other molecules were shifted to the right (Fig. 2A and B).

In intact blood vessels, the EC_{50} values for IA and DRX were 7.1 ± 2.3 μM ($n = 4$) and 6.0 ± 1.3 μM ($n = 6$), respectively. But, in rings without functional endothelium, the EC_{50} values were increased (15.8 ± 4.4 μM ($n = 4$, $^*P < 0.05$) for IA and 10.4 ± 6.5 μM ($n = 6$) for DRX). Even though EC_{50} values for DRX in the presence and in the absence of intact endothelium were not significantly different, the concentration–response curves of DRX with or without endothelium showed a significant difference by the two-way ANOVA test. These results suggest that the two compounds were more potent to induce relaxation when the endothelium is intact. They also demonstrate that IA and DRX induced relaxation in an endothelium-dependent and -independent manner.

3.2. IA and DRX induced NO release on endothelial cells

It is well known that the most potent endothelial-derived relaxant factor is NO. The ability of the two compounds in producing NO in endothelial cells was thus assessed by using NO spin trapping and electron paramagnetic resonance studies.

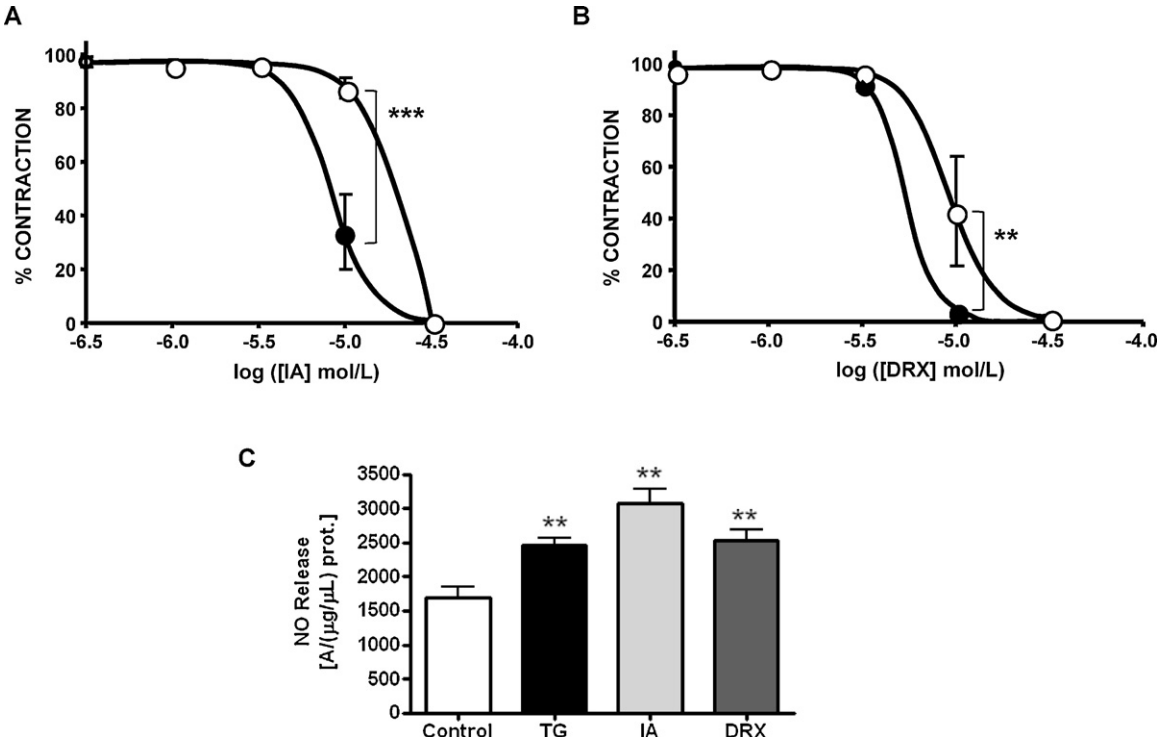


Fig. 2. Concentration–response curves for isocalolongic acid (A) and 2-deprenylrhhediaxanthone (B) in U46619 pre-contracted mouse thoracic aortic rings with (black circles) or without functional endothelium (open circles). Values are means ± SEM, $n = 4$ for IA and $n = 6$ for DRX. $^{**}P < 0.01$, $^{***}P < 0.001$ are the statistical differences between curves with or without functional endothelium. (C) Quantification of the amplitude of the NO-Fe(DETC)$_2$ complex signal in EaHy.926 endothelial cells revealed a significant increase of NO production in cells treated with IA (25 µM) and DRX (25 µM). Values are expressed as units/µg/µL of protein in the samples. Thapsigargin (TG) 10 µM was used as positive control. Results are means ± SEM from six independent experiments. $^{**}P < 0.01$ *versus* control.

Treatment of EaHy.926 cells with IA or DRX (25 µM) significantly increased NO production (Fig. 2C). Thapsigargin (10 µM) was used as positive control. These data showed that both compounds, IA and DRX, were able to induce NO release from endothelial cells.

3.3. IA promoted but DRX reduced in vitro angiogenesis

Sprouting angiogenesis includes successive phases leading to the formation of a new vessel. These steps need the migration and proliferation of endothelial cells behind the migration front, and the organization of endothelial cells into capillary-like structures [23]. These multistep processes can be reproduced with *in vitro* assays.

These cellular experiments were performed after 24 h of treatment with either IA or DRX. The cytotoxicity on HUVECs of IA and DRX were previously evaluated by MTT assay to avoid interfering with their own effect on proliferation, migration, adhesion and tube formation. After 48 h, the tested concentrations of IA (0.25, 8 and 25 µM) and DRX (0.25 and 8 µM) were not cytotoxic (Fig. 3). But, DRX at 25 µM decreased the cell viability on HUVECs up to 65%. Thus, this concentration was not tested on angiogenesis assays.

VEGF is an endothelial cell-specific mitogen and an angiogenesis inducer released by a variety of tumor cells and is known to play an important role in several pathological events such as tumor angiogenesis [24]. VEGF was thus used as a chemoattractant or an angiogenic factor in all *in vitro* assays. In the absence of VEGF, cultured HUVECs on the ECM gel® normally formed tube-like structures but the capillary network formation upon VEGF treatment resulted in elongated and robust tube-like structures (Fig. 4A). The ability of endothelial cells to form tubular structures was assessed by calculating the length of tubes with an inverted

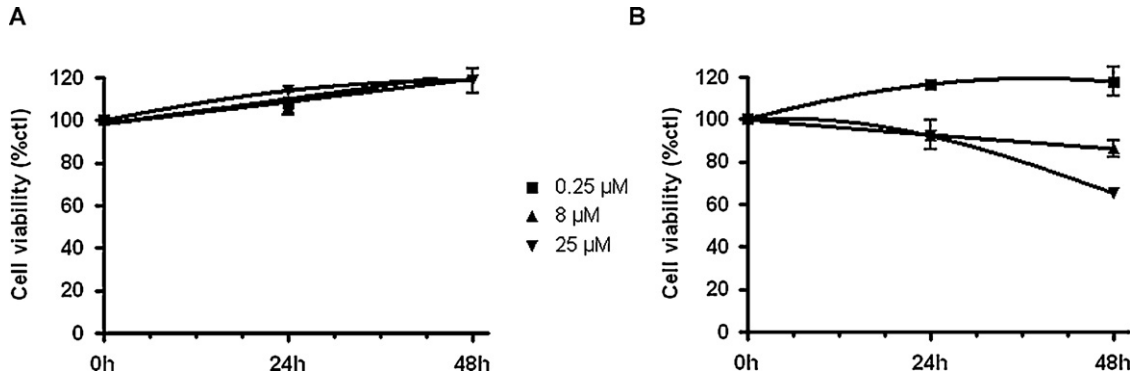


Fig. 3. Effect of isocalolongic acid (A) and 2-deprenylrheediaxanthone (B) on HUVEC viability (MTT assay). (A) Isocalolongic acid did not affect cell viability at 0.25 µM, 8 µM and 25 µM after 24 h and 48 h stimulation. (B) 2-Deprenylrheediaxanthone was not cytotoxic at 0.25 µM and 8 µM, except for a 48 h stimulation at 25 µM.

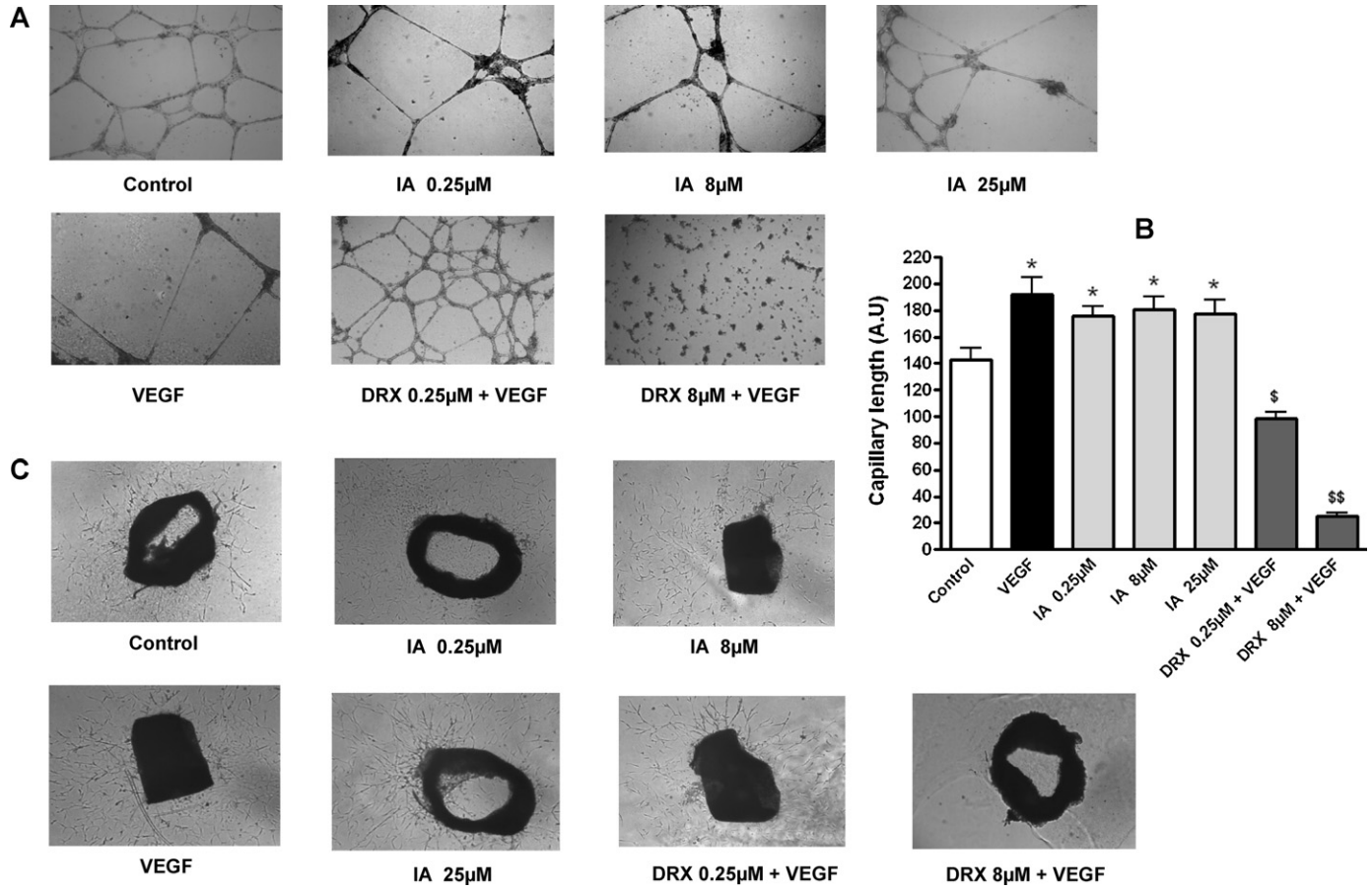


Fig. 4. Pro-angiogenic properties of isocalolongic acid (IA) and anti-angiogenic properties of 2-deprenylrheediaxanthone (DRX) on *in vitro* HUVEC tube formation and mouse aortic ring *ex vivo* angiogenesis. (A) HUVEC cells were cultured in medium supplemented with 10% FBS and treated with IA (0.25 μM, 8 μM, 25 μM) and DRX (0.25 μM, 8 μM) + VEGF (20 ng/mL) for 24 h. IA treatment enhanced capillary-like structure formation while DRX prevented the VEGF-induced increase in capillary-like tube formation in a concentration-dependent manner. VEGF (20 ng/mL) was used as positive control. (B) Capillary length was used to quantify *in vitro* angiogenesis. These results clearly showed an opposite effect of IA and DRX on angiogenesis. Results are means ± SEM from four independent experiments. $^{*}P < 0.05$ *versus* control, $^{\$}P < 0.05$ *versus* VEGF. (C) *Ex vivo* mouse aortic ring were cultured in medium supplemented with 10% FBS and treated with IA (0.25 μM, 8 μM, 25 μM) and DRX (0.25 μM, 8 μM) + VEGF (20 ng/mL) for 96 h. This *ex vivo* angiogenesis assay confirmed the *in vitro* results. IA stimulated capillary-like structure formation while DRX treatment blocked VEGF-induced tube formation. VEGF (20 ng/mL) was used as positive control.

photomicroscope. The length of tubes has been previously reported to characterize the angiogenic effect of several compounds including plant-derived polyphenols [25–28]. As shown in Fig. 4A and B, IA treatment enhanced capillary-like structure formation at all concentrations tested in an identical manner than VEGF whereas DRX (0.25 and 8 μM) efficiently reduced VEGF-induced tube formation in a concentration-dependent manner. These results suggested that IA possessed pro-angiogenic properties while DRX inhibited VEGF-induced *in vitro* angiogenesis.

3.4. Effect on ex vivo angiogenesis

The impact of both compounds IA and DRX was also assessed on a mouse aortic ring (*ex vivo*) angiogenesis model. This assay was widely used to evaluate pro- or anti-angiogenic agents in a complex system in which endothelial cells, fibroblasts, pericytes and smooth muscle cells are involved [29,30]. In the absence of VEGF, fibroblastic fusiform cells migrated from the ends of the aortic rings after 2 days and spread in the ECM gel®. Microvessels appeared after 4 days and elongated (Fig. 4C). As positive control, VEGF increased the formation of microvessels with a more disseminated area. Interestingly, the IA treatment enhanced *ex vivo* microvessel formation at all tested concentrations, which confirmed the ability of IA to promote angiogenesis. On the contrary, a reduced vessel area was observed when a combination of DRX and VEGF was used as stimulus (Fig. 4C). On the basis of this *ex vivo* model, the pro-angiogenic effect of IA and the anti-angiogenic property of DRX were confirmed.

3.5. IA decreased (at high concentration) endothelial cell adhesion but increased cell proliferation while DRX reduced endothelial cell adhesion, proliferation and migration in a concentration-dependent manner

After 24 h treatments, only high concentration (25 μM) of IA had a weak effect on the adhesion of HUVEC cells, as determined with the adhesion assay using crystal violet (Fig. 5A). On the other hand, DRX significantly decreased the adhesion of HUVEC cells in a concentration-dependent manner. VEGF (20 ng/mL) increased cell adhesion under similar experimental conditions.

Because endothelial cell proliferation represents a critical step in angiogenesis, the effect of both compounds on this cellular process was investigated. We highlighted proliferative properties for compound IA at 0.25 μM and 8 μM. Indeed, these concentrations were able to increase significantly HUVEC proliferation (Fig. 5B) while IA at 25 μM exhibited no effect. In contrast, compound DRX reduced HUVEC proliferation at 8 μM. As expected, VEGF treatment induced an increase of endothelial cell proliferation.

Migration of endothelial cells, which allows cells to disseminate from the pre-existing vessel to form new vessels, contributes to

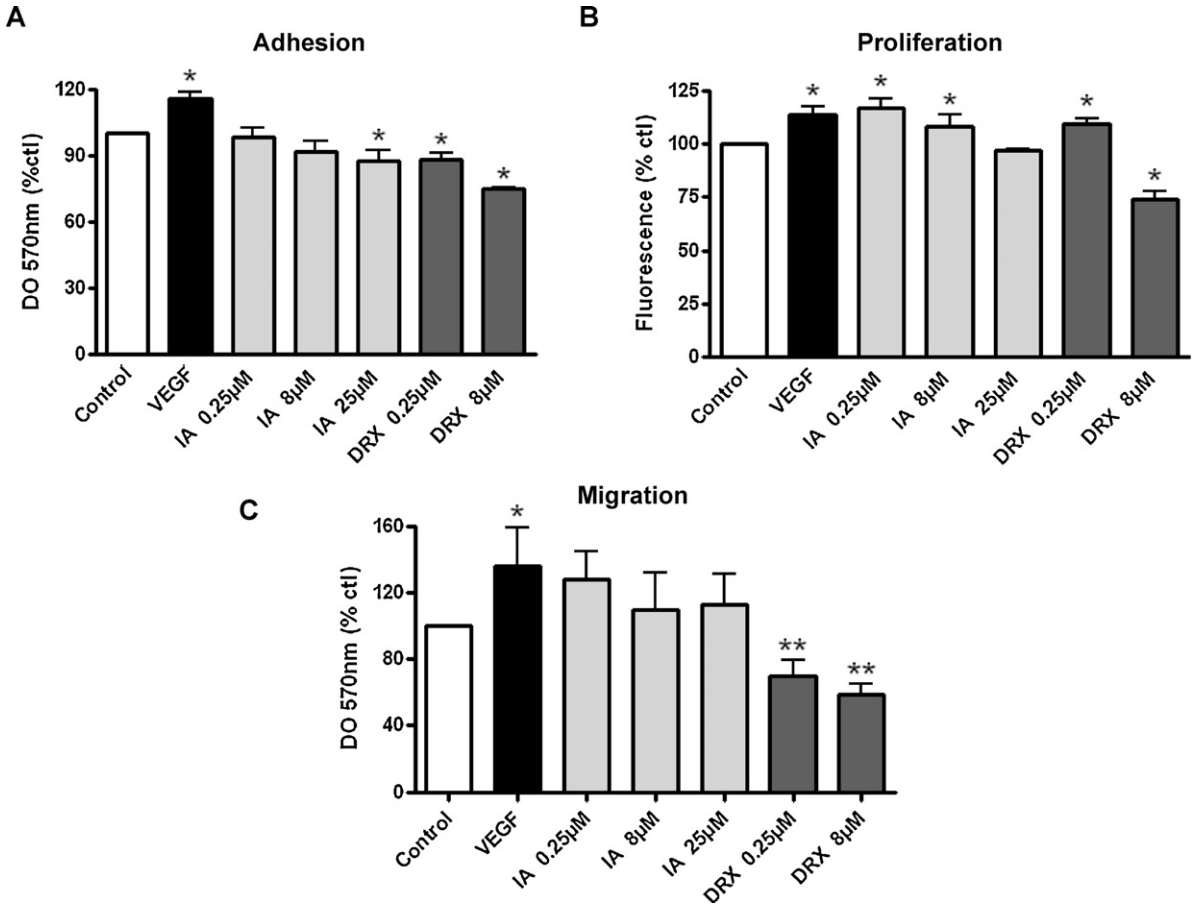


Fig. 5. Properties of isocalolongic acid (IA) and 2-deprenylrheediaxanthone (DRX) in endothelial cellular processes implicated on angiogenesis: endothelial cell adhesion (A), proliferation (B) and migration (C). (A) Effect of IA and DRX on adhesion of HUVECs. IA had only a slight effect at 25 μM on HUVEC adhesion while DRX decreased it in a concentration-dependent manner. VEGF (20 ng/mL) was used as positive control. Results are means ± SEM from four independent triplicate experiments. $^{*}P < 0.05$ *versus* control. (B) Effect of IA and DRX on proliferation of HUVECs. IA favored cell proliferation while DRX decreased it in a concentration-dependent manner. VEGF (20 ng/mL) was used as positive control. Results are means ± SEM from four independent triplicate experiments. $^{*}P < 0.05$ *versus* control. (C) Effect of IA and DRX on migration of HUVECs. IA had only a tendency at 0.25, 8 and 25 μM to increase HUVEC migration while DRX strongly inhibited this process at 0.25 and 8 μM. VEGF (20 ng/mL) was used as positive control. Results are means ± SEM from six independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* control.

angiogenesis. The effect of both compounds on endothelial cell migration using a Transwell *in vitro* migration assay was assessed. IA did not significantly modify endothelial cell migration at all concentrations tested (Fig. 5C). On the other hand, compound DRX (0.25 μM and 8 μM) decreased endothelial cell migration. As positive control, VEGF was able to increase endothelial cell migration.

Table 2
Effects of isocalolongic acid (IA) and 2-deprenylrheediaxanthone (DRX) on different mRNA expressions on HUVECs. Values are expressed as ratio of mRNA expression from HUVECs treated with IA (8 μM) or DRX (8 μM) for 24 h *versus* control group. Results are means ± SEM from four independent triplicate experiments.

mRNA	Ratio (IA *versus* CTL)	Ratio (DRX *versus* CTL)
E-selectin	0.97	0.94
ICAM-1	1.46*	1.07
IL-6	1.27	0.81
IL-1β	ND	ND
MT-MMP1	1.04	1.19
MMP2	1.22	0.93
PECAM-1	1.04	0.95
TNFα	ND	ND
VCAM-1	1.17	1.38
VEGF A	1.17	0.94

ND: not detected.
* $P < 0.05$ *versus* control.

3.6. The effect of DRX on the adhesion of endothelial cells is associated with reduced ICAM-1 expression

To determine the molecular changes modulating endothelial cell adhesion, the expression and activation of ICAM-1, VCAM-1, PECAM-1 and E-selectin were assessed by real-time RT-PCR and Western blotting. Only an increase in ICAM-1 mRNA was observed in cells treated with IA (8 μM) whereas no effect on mRNA level was noted after DRX stimulation at 8 μM (Table 2). Western blot analysis also revealed that IA (8 μM) had no effect whereas DRX (8 μM) decreased ICAM-1 expression (Fig. 6A). IA or DRX did not modify VCAM-1, PECAM-1, E-selectin expression on endothelial cells (Table 2). Also, no changes in expression of IL-1β, IL-6, MT-MMP1 and MMP2 were observed (Table 2). In contrast, although VEGF mRNA was not modified by IA or DRX treatment, Western blot analysis revealed a significant increase of VEGF-A protein expression by IA but not DRX treatment (Fig. 6B).

4. Discussion

The present study provides evidence that polyphenolic compounds such as xanthones, coumarins and chromanones exhibited vasorelaxant effects on isolated mouse aorta. However, only IA and DRX were able to induce a vasorelaxation that is partially dependent on the presence of functional endothelium and to

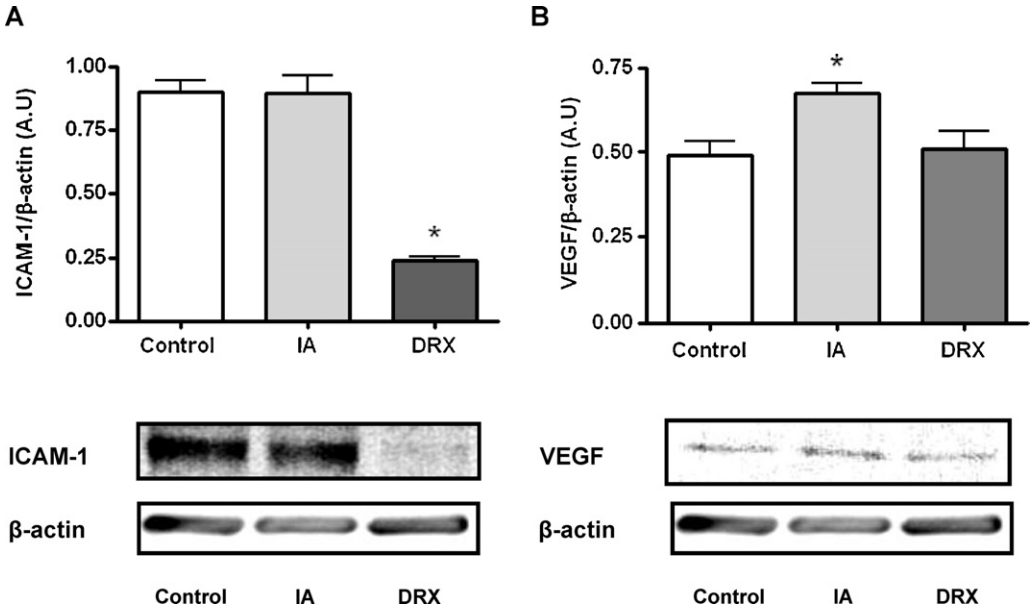


Fig. 6. (A) Western blot shows VEGF protein expression after treatment with IA and DRX at 8 μM for 24 h. Ratio between VEGF and β-actin expressions. IA increased and DRX had no effect on VEGF expression after 24 h treatment. (B) Western blot showed ICAM-1 protein expression after treatment with IA and DRX at 8 μM for 24 h. Ratio between ICAM-1 and β-actin expressions. DRX inhibited and IA had no effect on ICAM-1 expression after 24 h treatment. Results are means ± SEM from four independent experiments. $^{*}P < 0.05$ *versus* control.

enhance endothelial production of NO. With regard to angiogenesis, IA belonging to chromanones class displayed pro-angiogenic properties whereas DRX exerted anti-angiogenic ones. Thus, this study highlights a part of diverse biological activities induced by unrecognized polyphenolic compound on the endothelium.

Plants of family Clusiaceae (formerly Guttiferae) contain xanthones and chromanones. Among Clusiaceae plants, very few extracts have been assessed for their vasorelaxant effects on isolated blood vessel. *Calophyllum brasiliense* is used against inflammation and for the treatment of ulcers. This plant, from which acid chromanones were isolated, has been reported to induce relaxation of guinea pig ileum and rat duodenum [31]. But, no study describes the vasorelaxant effect of acid chromanones. The closest chemical structures of IA are coumarins, even if the position of the ketone function is angular for the last ones. Indeed, the coumarin praeruptorin A, isolated from the roots of *Peucedanum praeruptorum* Dunn and used in Chinese medicine for the treatment of hypertension, exerts endothelium-dependent relaxation on isolated rat aorta (EC_{50} = 35.4 ± 3.6 μM) via NO pathway [32]. Moreover, an extract of *Cedrelopsis grevei*, rich in coumarins, also shows the capacity to enhance endothelium-dependent vasorelaxation [10]. In comparison with praeruptorin A, IA (EC_{50} = 7.1 ± 2.3 μM) exhibits a more potent vasorelaxant property.

Several xanthones have also been evaluated on isolated blood vessels and have been shown to induce an endothelium-independent relaxation by acting directly on smooth muscle cells. In this case, the suggested mechanism of xanthone-induced vasorelaxation might involve the increase of intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) content and blockade of Ca^{2+} channels [33]. Only one study reports that HM-1 xanthone induces endothelium-independent and -dependent relaxation [34]. Vascular tone is regulated by endothelium through an equilibrium between the synthesis and release of vasorelaxing factors (NO and prostacyclin) and vasoconstricting factors such as endothelin and angiotensin II [35]. In this study, the removal of the endothelium from aortic rings led to a loss of DRX efficiency, suggesting that an endothelium-dependent mechanism was involved. This mechanism was confirmed by the capacity of this xanthone to increase endothelial NO production. The vasorelaxant effect of DRX (EC_{50} = 6.0 ± 1.3 μM) was comparable to that reported for HM-1 (EC_{50} = 1.67 ± 0.27 μM) in rat thoracic aorta.

In both cases, the endothelium-dependent relaxation induced by IA and DRX implicates, in part, NO pathway. Indeed, the short stimulation (30 min) of endothelial cells by IA and DRX induced an increase in NO production. However, a long stimulation (24 h) of endothelial cells by IA and DRX did not show increased phosphorylation of eNOS at the activator site, since they were not modified under the experimental conditions used in the present work (not shown). Thus, one has to take into account that the phosphorylation of eNOS at the activator site might occur earlier (at 15–30 min stimulation) to lead to an increase of NO production and does not last after 24 h. Moreover, IA and DRX might negatively regulate caveolin-1 expression that is implicated for the production of bioactive NO [36]. Recently, in human endothelial cells, we reported that the production of NO can be increased without changes in eNOS expression and its phosphorylation, but the decrease on caveolin-1 expression and the increase on its phosphorylation [37]. Such a mechanism might occur for IA. Another possibility is that the increase of NO upon IA and DRX stimulation results from the antioxidant properties of the two compounds leading to an increased NO bioavailability. We cannot distinguish among these possibilities. Thus, the complete mechanism through the activation of eNOS and Ca^{2+} pathway remains to be elucidated.

In adults, angiogenesis occurs sporadically during inflammation, wound healing and bone repair and represents an essential step in tumor expansion. It also takes part in other pathological conditions such as diabetic retinopathy, rheumatoid arthritis and ischemic diseases. The induction of angiogenesis is characterized by an angiogenic switch, i.e. an imbalance between pro-angiogenic factors, such as VEGF, fibroblast growth factors (FGF-1, FGF-2), platelet-derived growth factor (PDGF-B and PDGF-C) and anti-angiogenic factor production, such as thrombospondin-1 (TSP-1). The ability of endothelial cells to form capillary tubes is a specialized function of this cell type resulting from a finely tuned balance between cell migration, proliferation and adhesion [38]. All the concentrations tested for IA and DRX did not induce any cytotoxicity.

Interestingly, IA had pro-angiogenic properties as shown by its ability to induce formation of capillary-like structures in HUVECs. This result was confirmed by the increase of endothelial cell proliferation and the upregulation of VEGF expression. Among stimulator molecules of angiogenesis, VEGF appears to have a central role in the angiogenic process. It is the target of many pro-angiogenic factors, but it also regulates molecules that are implicated in the proliferation and migration of endothelial cells [39]. An upregulation of VEGF expression, regulated in part by NO, leads to an increase of vascular permeability. Enhancing the release of VEGF in vascular tissue is a promising approach to treat cardiovascular diseases such as ischemia and stroke events. In this context, IA might be a candidate to treat these diseases. One of the interesting data reported here was that the efficiency of IA to promote angiogenesis was greater (0.25 μM) than that needed to induce endothelium-dependent relaxation (EC_{50} = 7.1 μM). Further investigations are necessary to elucidate both mechanisms. Nevertheless, we underscore the potent pro-angiogenic property of IA.

On the other hand, angiogenesis is critical for tumor development [13] and neovascularization is known as a prerequisite to the rapid expansion of tumor cells associated with formation of macroscopic tumors [40]. Blockade of angiogenesis is therefore an important approach for cancer treatment and prevention. In the present study, DRX displayed anti-angiogenic properties as it strongly reduced capillary-like structures formation induced by VEGF in HUVECs. This compound belongs to the xanthones class, more particularly furanoxanthones. However, only few studies reported the effect of xanthones on angiogenesis. 2,6-Di(2,3-epoxypropoxy)xanthone (EPOX) has been shown to inhibit endothelial cell proliferation and angiogenesis. EPOX acts in part through selective induction of endothelial cell apoptosis to promote active vessel regression [41]. In response to EPOX treatment, the ERK inactivation that results in the dephosphorylation and down-regulation of Mcl-1 might participate in the inhibition of angiogenesis. The inhibition of capillary-like structure occurs at 0.1 μM for EPOX, which is comparable to the result obtained with DRX treatment at 0.25 μM. Indeed, DRX greatly reduced capillary formation and all the processes of angiogenesis including proliferation, migration and adhesion, when endothelial cells were activated by VEGF. The main data related to xanthones class in the literature are their activity as cytotoxic and antiproliferative agents. The most potent xanthones belong to furanoxanthones class such as psorospermin and its chlorohydrin derivatives. This xanthone isolated from *Psorospermum febrifugum* (Hypericaceae) exhibits significant cytotoxicity against drug resistant leukemia and a variety of human solid tumor cell lines [42–44]. More recently, two novel dihydrofuranoxanthones, namely psoroxanthin and psoroxanthin chlorohydrin, have been isolated from *Psorospermum molluscum* (Hypericaceae) and have been found to be selectively active against bovine endothelial ABAE cell line, with IC_{50} values of 0.102 and 0.004 μM, respectively, with regard to cytotoxicity [45]. However, the effect of such compounds on angiogenesis has not been assessed yet.

ICAM-1, a potent member of the immunoglobulin superfamily, is present on resting endothelial cells. *In vitro* and *in vivo* angiogenic activity of soluble ICAM-1 have been observed [46]. Besides, Kevil et al. [47] reported the involvement of ICAM-1 in migration by using aortic endothelial cells from ICAM-1-deficient mice. In line with these reports, we found that DRX treatment decreased the formation of capillary-like structures induced by VEGF in HUVECs. This effect was associated with the inhibition of endothelial cell migration and adhesion via down-regulation of ICAM-1 expression. Our results are in accordance with those obtained with a synthesized xanthone which is able to down-regulate adhesion molecules of endothelial cells such as ICAM-1 [48]. This mechanism might participate to the anti-angiogenic property of DRX.

Our data highlight a novel biological function for xanthone derivatives and acid chromanones. Nevertheless, further studies are required to address whether DRX inhibits tumor angiogenesis and tumor growth in mice and whether IA acid is able to restore blood flow and vascular density *in vivo* after ischemic event. Thus, IA might be a compound with potential therapeutic relevance to be used as a pro-angiogenic factor able to stimulate collateral blood vessel formation in the ischemic heart.

Altogether, these data allow identifying isolated molecules from Clusiaceae plants with potential activity in modulating endothelium-dependent relaxation involving NO release. Interestingly, they underscore paradoxical effects of the two compounds on *in vitro* cellular angiogenic processes, IA being pro-angiogenic and DRX anti-angiogenic.

Acknowledgments

We thank Angers Loire Métropole for granting a PhD scholarship to A. Lavaud. We sincerely thank Dr. MC Martinez for careful reading of this manuscript and C. Guillet from Service de Cytométrie et d'Analyses Nucléotidiques from Institut Fédératif de Recherche 132 (Université d'Angers) for her assistance in quantitative PCR quantification.

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