Anti-angiogenic Activity Evaluation of Secondary Metabolites from *Calycolpus moritzianus* Leaves

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Angiogenesis is a crucial step in many pathological conditions like cancer, inflammation and metastasis formation; on these basis the search for antiangiogenic agents has widened. In order to identify new compounds able to interfere in the Vascular Endothelial Growth Factor Receptor-1 (VEGFR-1, also known as Flt-1) recognition by VEGFs family members, we screened *Calycolpus moritzianus* (O. Berg) Burret leaves extracts by a competitive ELISA-based assay. MeOH and $CHCl_3$ extracts and several their fractions demonstrated to be able to prevent VEGF or PlGF interaction with Flt-1, with an inhibition about 50% at concentration of 100 μg/mL. Phytochemical and pharmacological investigation of the active fractions led to the isolation of flavonoids, and terpenes.

Keywords: *Calycolpus moritzianus*, Angiogenesis, VEGFR1/Flt-1, VEGF and PlGF, bioassay-oriented study.

In the last decade the inhibition of angiogenesis and vascular targeting has been the focus of new treatment strategies against the cancer. Among the long list of growth factors involved in the angiogenic process, VEGF-A has been considered for years the most important mediator of tumor angiogenesis [1]. Consequently, several strategies have been developed to inhibit the release of this growth factor, or to interfere in its interaction with receptors, VEGF receptor 1 (Flt-1) and VEGF receptor 2 (Flk-1 in mouse, KDR in human) [2]. Recent data support the concept that tumor infiltration by bone marrow-derived myeloid cells confers resistance to current antiangiogenic drugs targeting primary VEGF-A and its receptors (VEGF(R)s) [3]. For this reason, novel targets out of VEGF-A have been studied to diversify antiangiogenic treatments and to overcome resistance [4]. Genetic and pharmacological studies have identified Flt-1 and Placental Growth Factor (PlGF) as possible therapeutic targets for anticancer therapy [5]. Furthermore, has been proven that a combination of lower amount of VEGF(R)s inhibitors and compounds able to block PlGF showed equal antitumor efficacy compared to the standard dose of VEGF(R)s [5].

These findings suggested that molecules able to inhibit the activity of both PlGF and VEGF-A driven angiogenesis may be an opportunity for patients with cancer who may suffer excessive or prohibitive adverse effects from VEGF(R)s inhibitors.
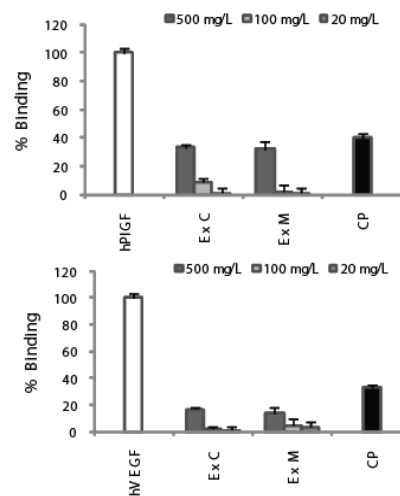

Figure 1: Inhibitory effect of *C. moritzianus* leaves extracts were assayed on PlGF/Flt-1 (A) and VEGF/Flt-1 interaction (B). The extracts were used at 500-100-20 μg/mL. As control a specific inhibiting peptide was used (CP). The white bar refers to ELISA experiment carried out without inhibitors. Each experiment was performed three times and average values ± SD were reported.

Accordingly to these data, the research of new natural compounds which may inhibit both PlGF and VEGF-A activity, has been the target of the present study. We carried out a screening of *Calycolpus moritzianus* (O. Berg) Burret leaves extracts by a competitive ELISA-based assay [6]. We aimed to identify natural molecules as inhibitors of PlGF and VEGF-A recognition by Flt-1.
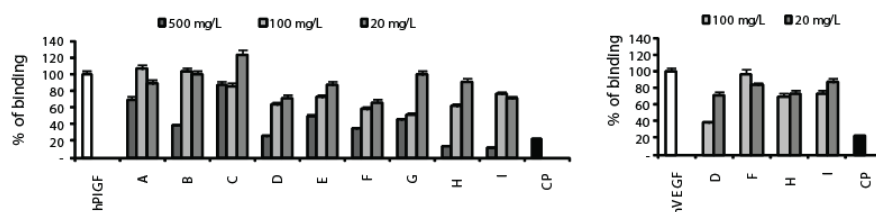
Figure 2: Inhibitory properties of plant fractions from MeOH extract were assayed on PlGF/Flt-1 interaction (A) and VEGF/Flt-1 interaction (B). The fractions were tested at 500-100-20 µg/mL. As control a specific inhibiting peptide was used (CP). The white bar refers to ELISA experiment carried out without inhibitors. Each experiment was performed three times and average values ± SD were reported.

Figure 3: Inhibitory activity of compounds isolated from active methanolic fractions assayed at concentration of 100 µg/ml on PlGF/Flt-1 (A) and VEGF/Flt-1 (B). As control a specific inhibiting peptide was used (CP). The white bar refers to ELISA experiment carried out without inhibitors. Each experiment was performed three times and average values ± SD were reported.
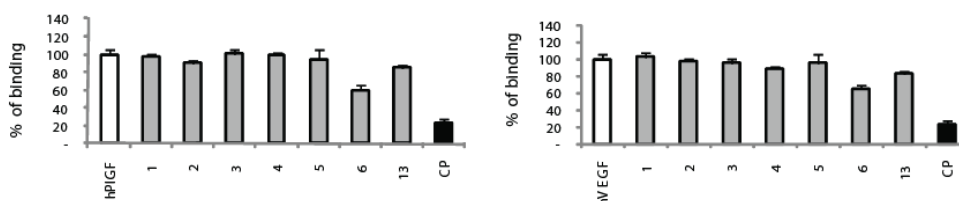
Several extracts from *C. moritzianus* leaves have been tested at the doses of 0.5, 0.1, and 0.02 mg/mL.

Methanol and chloroform residues exhibited a good activity in the inhibition on both PlGF/Flt-1 and VEGF-A/Flt-1 interaction, with a binding reduction higher than 60% at 20 µg/mL (Figure 1).Therefore these extracts were submitted to a bioassay-oriented fractionation. *C. moritzianus* MeOH extract was fractioned by sephadex column chromatography giving 9 fractions (A-I), while CHCl₃ extract was separated using silica gel column chromatography giving 7 fractions (AA-GG). The effect of the obtained fractions was tested on both PlGF/Flt-1 and VEGF-A/Flt-1, leading to the results reported in Figure 2-4.

C. moritzianus MeOH extract fractions were assayed in dose-dependent experiments at concentration ranging between 500 and 20 µg/mL on PlGF/Flt-1; the active frs. D-F and H were then assayed on VEGF-A/Flt-1 at concentration of 100 and 20 µg/mL. Among the MeOH fractions, Fr. H revealed the highest dose-dependent activity for PlGF/Flt-1 inhibition, provoking a reduction of its Flt-1 binding to 20% at 500 µg/mL and to 60% at 100 µg/mL, while at dose 100 µg/mL a 25% reduction of VEGF-A/Flt-1 interaction was observed.

Also D, E, F, I fractions exhibited a moderate inhibition for PlGF/Flt-1 complex, even if only Fr. D revealed to be able to inhibit also hVEGF-A / Flt-1 complex, leading to a binding reduction of 60% at 100 µg/mL (Figure 2). The active fractions were studied in order to identify the compounds responsible for this inhibitory activity.

Chromatographic and spectroscopic analyses of active Frs. D-F indicated the presence of flavonoidic derivatives

as main components, which were identified as quercetin 3-*O*-β-D-glucopyranoside **1** [7], kampferol-3-*O*-β-D-glucopyranoside **2** [7], quercetin-7-*O*-β-D-glucopyranoside **3** [8], kaempferol-3-*O*-β-D-rhamnopyranoside **4** [9], quercetin-3-*O*-β-D-rhamopyranoside **5** [9], and quercetin **6** [10].

The main compound identified in Fr. H was quercetin (85% w/w abundance). All isolated compounds were identified by means of 1D- and 2D-NMR spectroscopy, ESI-MS analysis, and by comparison of their data with those reported in the literature.

Among the pure compounds assayed, only quercetin showed a moderate activity (60% inhibition of PlGF/Flt-1 interaction at 100 µg/mL), compounds **1**, **3**, **5** its glycosides were inactive (Figure 3). These data suggest that the glycosylation at C-3 and C-7 of quercetin core is fatal for the activity. To better investigate a structure-activity relationship we tested also kaempferol aglycon of compounds **2**, **4**. Kaempferol was inactive in our test. The structures of quercetin and kaempferol are very similar except for the substituent at C-3'; the different activity observed for these compounds indicated that the presence of OH group at C-3' influence the resultant activity.

Fractions obtained from *C. moritzianus* CHCl₃ extract were assayed on PlGF/Flt-1 and VEGF-A/Flt-1 interaction by a competitive ELISA screening at concentration of 100 and 20 µg/mL.

Data in Figure 4 showed that the most active fraction was Fr. AA which revealed a moderate activity for both the growth factors causing a reduction of their Flt-1 binding to 40% for PlGF and 60% for VEGF at dose 100 µg/mL.
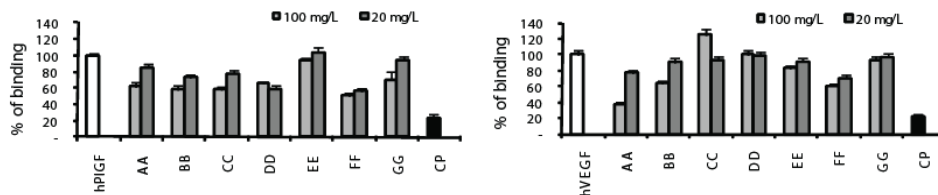
Figure 4: Inhibitory properties of chloroformic extract fractions were assayed on PlGF/Flt-1 interaction (A) and VEGF/Flt-1 interaction (B). The fractions were used at 100-20 µg/mL. As control a specific inhibiting peptide was used (CP). The white bar refers to ELISA experiment carried out without inhibitors. Each experiment was performed three times and average values ± SD were reported.

Figure 5: Inhibitory activity of compounds **7-12** assayed at concentration of 100-20 µg/mL on PlGF/Flt-1 (A) and VEGF/Flt-1 (B). As control a specific inhibiting peptide was used (CP). The white bar refers to ELISA experiment carried out without inhibitors. Each experiment was performed three times and average values ± SD were reported.
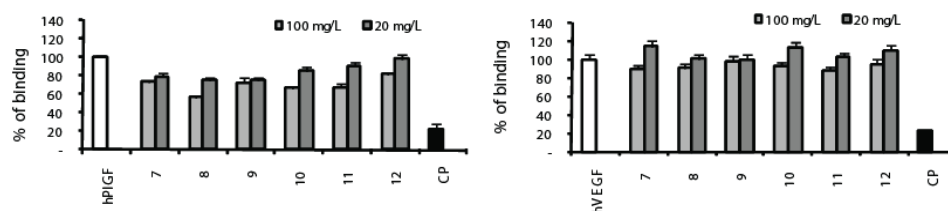
Nonetheless BB showed a moderate inhibition for both PlGF / Flt-1 and VEGF / Flt-1 interaction with a binding reduction of about 40% at dose 100 µg/mL.

Chromatographic separation of AA and BB fractions allowed to obtain the pure components: rosifoliol **7** [11], platanic acid **8** [12], oleanolic acid **9** [13], (-)-4,10-di-epi-5β,11-dihydroxyeudesmane **10** [14], 4,5-dioxoseco-γ-eudesmol **11** [15], and ursolic acid **12** [13], all identified by means of 1D- and 2D-NMR spectroscopy, ESI-MS analysis, and a comparison of their data with those reported in the literature.

The isolated pure compounds were tested in dose dependence manner on both PlGF/Flt-1 and hVEGF/Flt-1 systems (Figure 5). Only compound **8** was moderately able to inhibit PlGF/Flt-1 recognition; anyway the inhibition activity showed by this compound cannot explain by itself the activity of the original fraction .

On the basis of our results, we could hypothesize that the inhibition activity of PlGF and VEGF interaction with Flt-1 receptor by the *C. moritzianus* CHCl₃ extracts and fractions may be due to the presence of a combination of compounds acting synergistically or as vehicles enhancing the biological activity. However, we cannot rule out that the activity of the extracts and fractions could be due to a very minor compound not isolated.

Experimental

General experimental procedures: The instrumentation used in this work is described in our previous paper [13].

Plant material: The leaves of *C. moritzianus* were collected in Venezuela in 2008 and identified by Ing. Juan Carmona of Herbarium (MERF), Facultad de Farmacia y Bioanalisis - Universidad de Los Andes, Merida; where a voucher specimen n.761 is deposited.

Extraction and isolation: The air-dried powdered leaves of *C. moritzianus* (890 g) were defatted with *n*-hexane and extracted successively by exhaustive maceration (3 x 1 L, for 48 h) with CHCl₃, CHCl₃-MeOH (9:1), and MeOH. The MeOH extract (5 g) was chromatographed over a sephadex LH-20 column (100 x 5 cm) with MeOH as the eluent. A total of 110 fractions were collected (15 mL each) and combined according to TLC analysis [silica 60 F₂₅₄ gel-coated glass sheets with *n*-BuOH-AcOH-H₂O (60:15:25) and CHCl₃-MeOH-H₂O (40:9:1)] to give nine pooled fractions (A-I). Fraction D (106 mg) was purified by RP-HPLC with a C₁₈ µ-Bondapak column (30 cm x 7.8 mm, flow rate 2 mL/min) using MeOH-H₂O (35:65) to obtain compound **1** (4.0 mg, t_R = 20 min), and **2** (15.0 mg, t_R = 26 min).

Fraction E (95 mg) was purified by RP-HPLC with a C₁₈ µ-Bondapak column (30 cm x 7.8 mm, flow rate 2 mL/min) using MeOH-H₂O (35:65) to obtain compound **2** (5.0 mg, t_R = 26 min). Fractions F (90 mg) was separately purified by RP-HPLC using MeOH-H₂O (2:3) to give compounds **3** (16 mg, t_R = 10 min), **4** (6 mg, t_R = 16 min), and **5** (2 mg, t_R = 20 min). Fraction H (20 mg) was identified as quercetin.

The CHCl₃ extract (5.0 g) was submitted to silica gel flash column chromatography eluting with CHCl₃ followed by increasing concentrations of MeOH (between 1% and 70%). The following volumes of solvents were used: 4.2 L of CHCl₃, 1 L of CHCl₃-MeOH (99:1), 4.3 L of CHCl₃-MeOH (49:1), 1 L of CHCl₃-MeOH (95:5), 0.5 L of CHCl₃-MeOH (9:1), 0.5 L of CHCl₃-MeOH (1:1), 0.5 L of CHCl₃-MeOH (3:7), and 0.3 L of MeOH. Fractions of 30 mL were collected and analyzed by TLC on silica 60 F₂₅₄ gel-coated glass sheets eluting with CHCl₃ or mixtures CHCl₃-MeOH, 99:1, 49:1, 95:5, 9:1, 4:1, and grouped into seven fractions (AA-GG). Fraction AA (95 mg) was subjected to RP-HPLC on a C₁₈ µ-Bondapak column

(30 cm x 7.8 mm, flow rate 2.0 mL/min) with MeOH-H$_2$O (73:27) to yield compounds **7** (4 mg, t_R = 10 min), **8** (10 mg, t_R = 16 min) and **9** (2 mg, t_R = 34 min). Fractions BB (70 mg) and was purified by RP-HPLC with MeOH-H$_2$O (37:13) to give compounds **10** (3.5 mg, t_R = 16 min), **11** (8 mg, t_R = 4 min), **12** (1.5 mg, t_R = 26 min).

ELISA-based assays: The ELISA based assay for plant extract, fractions and pure compounds screening was performed as described elsewhere [6].

Plant extracts, fractions and compounds **1-13** dissolved in DMSO (Sigma) were properly diluted and added to the wells pre-mixed with ligand. For dose-dependent experiments, concentration ranging between 20 and 500 µg/mL were used.

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