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Isolation and structural characterization of glycosides from an anti-angiogenic extract of *Monnina obtusifolia* H.B.K.

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ABSTRACT

Angiogenesis, the growth of new blood vessels from the pre-existing vasculature is of physiological and pathological importance. Substantial data over the last decade has implicated uncontrolled angiogenesis with various pathological states. Vascular endothelial growth factors (VEGFs) play a critical role in its regulation, and have become one of the most interesting anti-angiogenesis targets. We have investigated the anti-angiogenic potential of plant extracts in a preliminary ELISA screening. The *n*-BuOH extract obtained from the leaves of *Monnina obtusifolia* (Polygalaceae) demonstrated an inhibition of VEGF-A or Placental Growth Factor interaction with Flt-1 (VEGF receptor 1), with an inhibition over 50% in particular for VEGF-A/Flt-1 interaction at a concentration of 500 μg/mL. Successively fractionation of the bioactive *n*-BuOH extracts of *M. obtusifolia* aerial parts led to the isolation of six new compounds, 1-*O*-(4-hydroxy-2-methylene-butanoic acid)-6-*O*-β-D-(4-hydroxy-2-methylene-butanoyl)-glucopyranose (**1**), 1-*O*-(isopentenyl)-6-*O*-β-D-(4-hydroxy-2-methylene-butanoyl)-glucopyranose (**2**), 1-*O*-(4-hydroxy-2-methylene-butanoic acid)-6-*O*-β-D-(isovaleroyl)-glucopyranose (**3**), 1-*O*-(3-methylbut-3enyl)-6-*O*-β-D-(isovaleroyl)-glucopyranose (**4**), two new sucrose esters, 3,4-*O*-β-D-di-feruloyl-fructofuranosyl-6-*O*-α-D-(*p*-coumaroyl)-glucopyranoside (**5**), and 3,4-*O*-β-D-di-feruloyl-fructofuranosyl-6-*O*-α-D-(caffeoyl)-glucopyranoside (**6**), together with known flavonoids. Their structures were established on the basis of detailed spectral analysis. Since none of the isolated compounds showed a relevant inhibition of VEGFs, the biological activity observed for the butanolic extract might be due to the presence of a combination of compounds acting synergistically.

1. Introduction

Angiogenesis, the growth of new blood vessels from the pre-existing vasculature, is of physiological and pathological importance. Substantial data over the last decade has implicated uncontrolled angiogenesis with various pathological states [1]. Vascular endothelial growth factors (VEGFs) and their membrane receptors (Flt-1 and Flk-1, corresponding to VEGF receptor 1 and VEGF receptor 2) play a critical role in its regulation, and have become an attractive anti-angiogenesis target [2,3]. Therefore, there is an increasing need of molecules able to modulate the activation of VEGFs for therapeutic approaches. In an effort to identify new VEGFs/Flt-1 inhibitors, we have recently developed a competitive ELISA-based screening and use this method to test several plant extracts [4]. The ELISA screening showed a 50% inhibition of VEGF-A interaction with Flt-1, for *Monnina obtusifolia* *n*-BuOH extract at 500 μg/mL. Therefore this extract was submitted to a bioassay-guided fractionation [4].

M. obtusifolia (Polygalaceae) is mostly distributed in Ecuador and its herbs are used as a remedy of heat-clearing and detoxicating, removing food retention, promoting blood flow and expelling phlegm to arrest coughing in the folk medicine traditions [5]. Previous phytochemical investigations on Polygalaceae have reported that the main chemical

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constituents are saponins, xanthones and oligosaccharide esters [6–8].

In the present investigation on *M. obtusifolia* *n*-BuOH extract, we describe the isolation and structural characterization by spectroscopic and spectrometric analyses of six new compounds, four alkyl glysosides (**1**–**4**) and two new sucrose esters (**5**–**6**), together with known flavonoids. All compounds were also evaluated for their anti-angiogenic activity.

2. Experimental procedures

2.1. General experimental procedures

Optical rotations were measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1-dm microcell. All the 2D NMR spectra were acquired in CD_3OD in the phase-sensitive mode with the transmitter set at the solvent resonance and TPPI (time proportional phase increment) used to achieve frequency discrimination in the ω_1 dimension. The standard pulse sequence and phase cycling were used for DQF-COSY, TOCSY, HSQC, HMBC, NOESY and ROESY experiments. The NMR data were processed on a Silicon Graphic Indigo2 Workstation using UXNMR software. Column chromatographies were performed over silica gel (63–200 μm, Merck, Darmstadt, Germany); electrospray ionization mass spectra were acquired in positive or in negative ion mode on a LC-Q Advantage Termoquest spectrometer, equipped with Xcalibur software (Thermo-Fisher, Waltham, USA). Mass calibrations of the spectrometer were performed using quercetin (molecular mass 302) or amentoflavone (molecular mass 538) as standards. HPLC separations were conducted on a Waters 590 system equipped with a Waters R401 refractive index detector, and with a Waters μ-Bondapak C_{18} column (Waters, Milford, MA). TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck, Darmstadt, Germany); compounds were detected by $Ce(SO_4)_2/H_2SO_4$ (Sigma-Aldrich, Milano, Italy) solution.

2.2. Plant material

The aerial parts of *M. obtusifolia* were collected in November 2006 at San Andrés, Chimborazo, in Ecuador. A voucher specimen (voucher n° 231) was deposited at the Herbario of Escuela Superior Politécnica de Chimborazo, ESPOCH, Riobamba, Ecuador.

2.3. Extraction and isolation

The air-dried powdered aerial parts of *M. obtusifolia* (200 g) were extracted in sequence with MeOH (15 g). The MeOH extract was defatted with *n*-hexane and extracted successively with $CHCl_3$, and *n*-BuOH. The extracts were concentrated under reduced pressure to afford 1.8 and 4.0 g of dried residues, respectively. A portion of the *n*-BuOH extract (3 g) was chromatographed over a Sephadex LH-20 column (100 cm × 5 cm) with MeOH as the eluent. A total of 85 fractions was collected (10 mL each). These were combined according to TLC analysis [silica 60 F_{254} gel-coated glass sheets with *n*-BuOH–AcOH–H_2O (60:15:25) and $CHCl_3$–MeOH–H_2O (40:9:1)] to give 9 pooled fractions (A–I). Fraction B (75 mg) was purified by RP-HPLC using MeOH–H_2O (25:75) to give compounds **1** (10 mg, t_R = 12 min), **4** (6 mg, t_R = 20 min), and 1-*O*-(3′-methylbut-3enyl)-β-D-glucopyranoside (7 mg, t_R = 7 min). Fraction C (60 mg) was purified by RP-HPLC using MeOH–H_2O (25:75) to give compounds **1** (4 mg, t_R = 12 min) and **2** (8 mg, t_R = 16 min). Fraction D (70 mg) was purified by RP-HPLC using MeOH–H_2O (3:7) to give compounds **3** (9 mg, t_R = 10 min), **4** (3 mg, t_R = 15 min), and 1-*O*-(3′-methylbut-3enyl)-β-D-glucopyranoside (5 mg, t_R = 8.5 min). Fraction E (110 mg) was purified by RP-HPLC using MeOH–H_2O (2:3) to give compounds quercetin 3-*O*-β-D-galactopyranosyl-(1-6)-glucopyranoside (15 mg, t_R = 14 min), quercetin 3-*O*-β-D-xylopyranosyl-(1-2)-galactopyranoside (5 mg, t_R = 15 min), and quercetin 3-*O*-β-D-apiofuraranosyl-(1-2)-galactopyranoside (11 mg, t_R = 10 min). Fraction F (40 mg) was purified by RP-HPLC using MeOH–H_2O (1:1) to give compound **5** (2 mg, t_R = 24 min). Fraction H (95 mg) was purified by RP-HPLC using MeOH–H_2O (1:1) to give compounds **5** (6 mg, t_R = 24 min), and **6** (7 mg, t_R = 22.5 min). Fraction I yielded quercetin 3-*O*-β-D-glucopyranoside (25.0 mg).

1-*O*-(4-hydroxy-2-methylene-butanoic acid)-6-*O*-β-D-(4-hydroxy-2-methylene-butanoyl)-glucopyranose (**1**, Fig. 1): colourless needles (MeOH); $[\alpha]_D$: −28 (*c* 0.1, MeOH); ESI-MS: negative ion mode *m/z* 375 $[M-H]^-$; ^1H NMR ^{13}C NMR see Table 1.

1-*O*-(isopentenyl)-6-*O*-β-D-(4-hydroxy-2-methylene-butanoyl)-glucopyranose (2, Fig. 1): colourless needles (MeOH); $[\alpha]_D$: −34.0 (*c* 0.1, MeOH); ESI-MS in negative ion mode (*m/z*): 345 $[M-H]^-$, ^1H NMR ^{13}C NMR see Table 1.

1-*O*-(4-hydroxy-2-methylene-butanoic acid)-6-*O*-β-D-(isovaleroyl)-glucopyranose (3, Fig. 1): colourless needles (MeOH); $[\alpha]_D$: −25.5 (*c* 0.1, MeOH); ESI-MS in negative ion mode (*m/z*): 361 $[M-H]^-$; ^1H NMR ^{13}C NMR see Table 1.

1-*O*-(3-methylbut-3enyl)-6-*O*-β-D-(isovaleroyl)-glucopyranose (4, Fig. 1): colourless needles (MeOH), $[\alpha]_D$: −18.3 (*c* 0.1, MeOH). ESI-MS in negative ion mode (*m/z*): 331 $[M-H]^-$; ^1H NMR δ_H 4.36 (1H, d, *J* = 7.5, H-1); 3.19 (1H, br t, *J* = 8.5, H-2); 3.36 (1H, t, *J* = 9.0, H-3); 3.31 (1H, t, *J* = 9.0, H-4); 3.55 (1H, m, H-5); 4.28 (1H, dd *J* = 12.0, 2.5, H-6a) 4.00 (1H, dd *J* = 12.0, 5.0, H-6b); 3.99 (1H, dd *J* = 16.5, 8.0, H-1a′); 3.75 (1H, m, H-1b′); 2.37 (2H, m, H-2′); 4.80 (1H, br s, H-4a′); 4.77 (1H, br s, H-4b′); 1.78 (1H, br s, H-5′); 2.26 (1H, m, H-2a″); 2.50 (1H, m, H-2b″); 250 (1H, m, H-3″); 1.03 (6H, d, *J* = 6.5, H-4″, H-.5″), ^{13}C NMR see Table 2.

1-*O*-(3-methylbut-3enyl)-6-*O*-β-D-(isovaleroyl)-glucopyranose (5, Fig. 1): amorphous powder (MeOH); $[\alpha]_D$: −48.8 (*c* 0.1, MeOH); ESI-MS in negative ion mode (*m/z*): 839 $[M-H]^-$; ^1H NMR ^{13}C NMR see Table 2.

3,4-*O*-β-D-di-feruloyl-fructofuranosyl-6-*O*-α-D-(caffeoyl)-glucopyranoside (6, Fig. 1): amorphous powder (MeOH); $[\alpha]_D$: −47.0 (*c* 0.1, MeOH); ESI-MS in negative ion mode (*m/z*): 855 $[M-H]^-$; ^1H NMR ^{13}C NMR see Table 2.

2.4. ELISA-based assays

The ELISA-based assay for the screening of plant extracts, fractions and pure compounds was performed coating on a 96-well plate a recombinant form of Flt-1 (VEGFR-1-Fc chimera R&D Systems) at 0.5 μg/mL, 100 μL/well, 16 h at

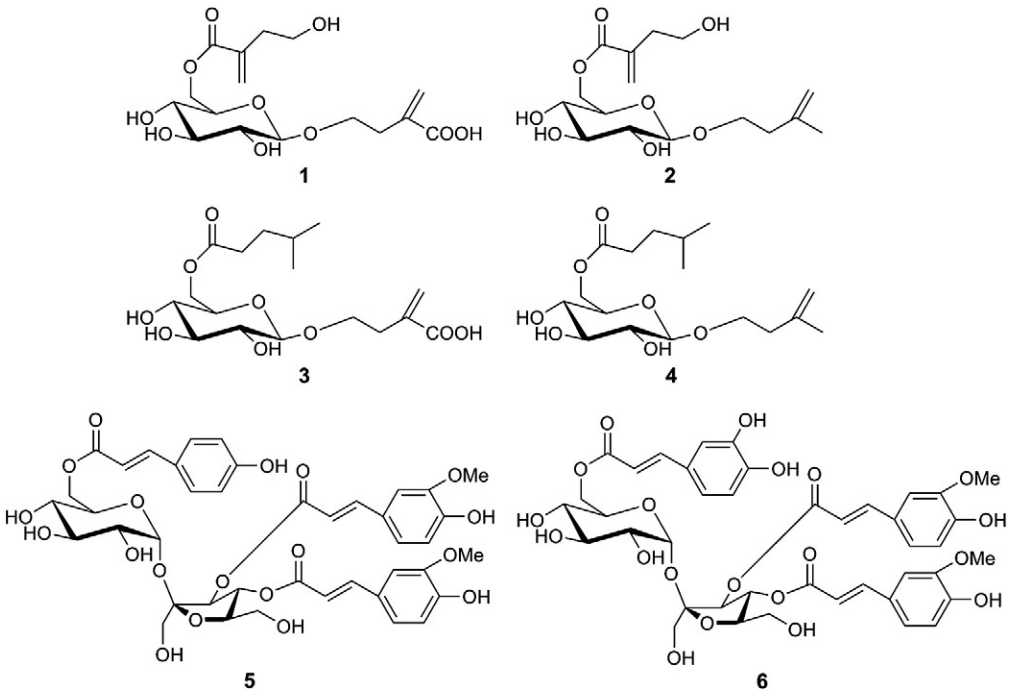

Fig. 1. Structure of compounds 1–6.

room temperature. Wells were washed five times with PBS containing 0.004% Tween-20 (PBT) and the plate was then blocked for 3 h at RT with 1% bovine serum albumin (BSA) in PBS, 200 μL/well. Wells were washed as stated previously, and a recombinant form of human PlGF (R&D Systems, Minneapolis, MN) at 5 ng/mL or human VEGF-A (R&D Systems) at 10 ng/mL in PBS containing 0.1% BSA, 5 mM EDTA, 0.004% Tween 20 (PBET), 100 μL/well, was added and incubated for 1 h at 37 °C followed by 1 h at RT. Plant extracts, fractions and compounds dissolved in DMSO (Sigma-Aldrich) were properly diluted and added to the wells pre-mixed with ligand. Biotinylated anti-human PlGF or anti-human VEGF-A polyclonal antibody (R&D Systems), diluted in PBET at 300 ng/mL, 100 μL/well, was added to the wells and incubated for 1 h at 37 °C followed by 1 h at RT. A solution containing a preformed avidin and biotinylated HRP (horseradish peroxidase) macromolecular complex (Vectastain *elite* ABC kit, Vector LAB, Burlingame, CA) was added to each well and incubated for 1 h at RT. After the last wash, 100 μL of HRP substrate composed of 1 mg/mL of *ortho*-phenylenediamine in 50 mM citrate phosphate buffer pH 5, 0.006% of H_2O_2, was added and incubated for 40 min in the dark at RT. The reaction was blocked by adding 30 μL/well of 4 N H_2SO_4 and the absorbance was measured at 490 nm on a microplate reader (Biorad BenchMark, Milano, Italy).

Each point was done in triplicate and each experiment was repeated two times.

3. Results and discussion

The ELISA assay allowed detecting the interaction of 0.5–1.0 ng of soluble growth factors to 50 ng of immobilized recombinant Flt-1 receptor, and permitted to use high concentration of DMSO, up to 25%, without loss of binding activity. In a first screening we tested all extracts from *M. obtusifolia*. The obtained results showed an activity for *n*-BuOH extract (50% of inhibition at 500 μg/mL for VEGF-A/Flt-1 system). Therefore, this extract was submitted to a bioassay-guided fractionation. Fractionation and analysis of the *n*-BuOH extract led to isolation and structure elucidation of four new alkyl glycosides, 1-*O*-(4-hydroxy-2-methylene-butanoic acid)-6-*O*-β-D-(4-hydroxy-2-methylene-butanoyl)-glucopyranose (**1**), 1-*O*-(isopentenyl)-6-*O*-β-D-(4-hydroxy-2-methylene-butanoyl)-glucopyranose (**2**), 1-*O*-(4-hydroxy-2-methylene-butanoic acid)-6-*O*-β-D-(isovaleroyl)-glucopyranose (**3**), 1-*O*-(3-methylbut-3enyl)-6-*O*-β-D-(isovaleroyl)-glucopyranose (**4**), two new sucrose esters, 3,4-*O*-β-D-di-feruloyl-fructofuranosyl-6-*O*-α-D-(*p*-coumaroyl)-glucopyranoside (**5**), and 3,4-*O*-β-D-di-feruloyl-fructofuranosyl-6-*O*-α-D-(caffeoyl)-glucopyranoside (**6**), together with other known compounds, including quercetin 3-*O*-β-D-glucopyranoside [9], quercetin 3-*O*-β-D-galactopyranosyl-(1-6)-glucopyranoside [10], quercetin 3-*O*-β-D-xylopyranosyl-(1-2)-galactopyranoside [11], quercetin 3-*O*-β-D-apiofuraranosyl-(1-2)-galactopyranosid [12], and 1-*O*-(3′-methylbut-3enyl)-β-D-glucopyranoside [13]. Their structures were established on the basis of detailed spectral analysis.

Compound **1** was isolated as a colourless powder, and its molecular formula was determined as $C_{16}H_{24}O_{10}$ by means of ESI-MS ($[M-H]^-$ at *m/z* 375), ^{13}C, ^{13}C-DEPT NMR, and elemental analysis.

The ^1H NMR spectrum (Table 1) showed four terminal methylene protons at δ 5.72 (2H, br s), and 6.27 (2H, br s), oxymethylene protons at δ 3.72, 3.96 (both 1H-4′, m), and 3.68 (2H-4″, t), four methylene protons at δ 2.46 (2H-3′, t, $J=6.5$ Hz), and 2.52 (2H, t, $J=6.5$ Hz), attributed to the presence of two 4-hydroxy-2-methylen-butanoic acid units

Table 1
^{1}H and ^{13}C NMR data of compounds 1–4 (CD_3OD, 600 MHz) [a].

Position	1		2		3		4
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_C
1	4.38 d (7.5)	104.0	4.35	102.9	4.34 d (7.5)	103.1	102.9
2	3.19 br t (8.5)	73.8	3.19 br t (8.5)	74.0	3.20 br t (8.5)	74.0	74.0
3	3.38 t (9.0)	76.6	3.38 t (9.0)	77.0	3.35 t (9.0)	77.0	77.0
4	3.33 t (9.0)	70.8	3.33 t (9.0)	70.4	3.30 t (9.0)	70.6	70.4
5	3.52 m	74.2	3.52 m	74.6	3.56 m	74.8	74.6
6a	4.28 dd (12.0, 5.0)	62.5	4.27 dd (12.0, 5.0)	62.4	4.00 dd (12.0, 5.0)	63.2	62.6
6b	4.52 dd (12.0, 2.5)	–	4.49 dd (12.0, 2.5)	–	4.30 dd (12.0, 2.5)	–	–
1′a	–	170.1	4.01 dd (8.0, 16.5)	68.2	–	170.0	68.2
1′b			3.72 m	–	–	–	–
2′	–	138.0	2.35 m	37.0	–	139.5	34.2
3′	2.52 t (6.5)	34.2	.	142.4	2.44 t (6.5)	33.0	142.0
4′a	3.72 m	68.0	4.82 br s	109.8	3.75 m	68.2	109.6
4′b	3.96 m	–	4.77 br s	–	4.00 m	–	–
5′a	5.72 s	127.9	1.80 br s	21.8	5.73 s	126.0	22.0
5′b	6.27 s	–	–	–	6.28 s	–	–
1″	–	170.3	–	169.3	–	176.0	176.2
2″a	–	138.3	–	138.0	2.26 m	27.0	27.2
2″b	–	–	–	–	2.22 m	–	
3″	2.46 br t (6.5)	35.2	2.47 t (6.5)	34.8	2.48 m	45.2	45.0
4″	3.68 t (6.5)	61.0	3.67 t (6.5)	60.8	1.02 d (6.5)	22.8	22.6
5′a′	5.72 s	128.1	6.22 s	129.5	1.02 d (6.5)	22.8	22.6
5′b′	6.27 s	–	5.68 s	–	–		–

[a] Coupling pattern and coupling constants (J in Hertz) are in parentheses.

in **1** [1,14]. In addition the ^{1}H NMR spectrum showed an anomeric proton at δ 4.38 (1H, d, J = 7.5 Hz) and six proton between δ 3.19 and 4.52 attributed from 1D-TOCSY and COSY experiments to a β-D-glucopyranose unit, which was also supported by ^{13}C NMR signals (Table 1) [15]. All the ^{1}H and ^{13}C NMR signals of **1** were assigned using 1D-TOCSY, DQF-COSY, HSQC, and HMBC experiments. The configuration of the sugar unit was assigned after hydrolysis of **1** with 1 N HCl. The hydrolysate was trimethylsilylated, and GC retention times of sugar were compared with those of authentic sugar samples prepared in the same manner [15]. The lower field shifts of H_2-6 (δ 4.28, and 4.52) suggested the substitution pattern of one 4-hydroxy-2-methylene-butanoic acid unit. Unequivocal information could be obtained by 2D-NMR spectra; the HMBC experiment indicated connections between δ 4.38 (H-1) and 68.0 (C-4′); δ 4.52 (H-6) and 170.3 (C-1″) [16]. Thus, the structure of **1** was determined as 1-*O*-(4-hydroxy-2-methylene-butanoic acid)-6-*O*-β-D-(4-hydroxy-2-methylene-butanoyl)-glucopyranose.

Compound **2** showed a quasimolecular ion peak at *m/z* 345 $[M–H]^-$, in the negative ion mode (ESI-MS spectrum). The molecular formula was established to be $C_{16}H_{26}O_8$ by ESI-MS and NMR analyses. Data from NMR spectra suggested a glucoside structure (Table 1). The ^{13}C NMR spectrum of **2** showed 16 signals of which six could be assigned to a β-D-glucopyranose unit by comparison with the ^{13}C NMR data of compound **1**. The remaining 10 signals were assigned to a 4-hydroxy-2-methylenebutanoate and 3-methylbut-3-enyl groups (Table 1) [13,17]. The presence of an isopentenyl moiety in **2** was further supported by the ^{1}H NMR signals at δ 1.80 (3H-5′, s), 2.35 (2H-2′, m), 4.01 (1H-1′a, dd, J = 8.0, 16.5 Hz), 3.70 (1H-1′b, m), 4.77 and 4.82 (both 1H-4′, br s). The downfield resonance of the C-6 protons in 2 (δ 4.49 and 4.27) is due to an ester linkage at C-6, indicating that the 4-hydroxy-2-methylenebutanoate moiety is linked at this position, which is also in agreement with the ^{13}C NMR data (Table 1). From the aforementioned results the structure of **2** was determined to be 1-*O*-(isopentenyl)-6-*O*-β-D-(4-hydroxy-2-methylene-butanoyl)-glucopyranose. The molecular formula $C_{16}H_{26}O_9$ for compound **3** was determined by ESI-MS ($[M–H]^-$ at *m/z* 361), ^{13}C, ^{13}C-DEPT NMR analyses Its ^{1}H and ^{13}C NMR spectra (see Table 1) indicated that it was a hemiterpenoid glucoside [18].

Analysis of NMR data of 3 and their comparison with those of **1** showed that 3 differed from 1 only in hemiterpenoid esterified at C-6 of glucose unit. 1D-TOCSY and 2D-COSY experiments allowed identifying the spin system of isovaleroyl group at C-6 [19].

All the ^{1}H and ^{13}C NMR signals of **3** were assigned using COSY, 1D-TOCSY, HSQC, HMBC experiments. An unambiguous determination of the linkage sites was obtained from HMBC experiment, showing cross peak correlations between δ 4.34 (H-1) and 68.2 (C-1′), δ 4.00 and 4.30 (H_2-6) and 176.0 (C-1″). Therefore, the structure 1-*O*-(4-hydroxy-2-methylene-butanoic acid)-6-*O*-β-D-(isovaleroyl)-glucopyranose was assigned to compound **3**.

Compound **4** ($C_{16}H_{28}O_7$) showed a quasimolecular ion peak at *m/z* 331 $[M–H]^-$ in the negative ion mode ESI-MS spectrum. A comparison of the NMR spectral data of 4 (Table 1) with those of 3 showed that the 4-hydroxy-2-methylene-butanoyl moiety of **3** was replaced by one isopentenyl unit in **4** (Table 2) [13,17]. Therefore, the structure 1-*O*-(3-methylbut-3enyl)-6-*O*-β-D-(isovaleroyl)-glucopyranose was assigned to compound **4**.

Compound **5** exhibited a $[M–H]^-$ peak at *m/z* 839 in the negative ion ESI-MS spectrum and was formulated as $C_{41}H_{44}O_{19}$. The NMR spectra of compound **5** showed the presence of three substituted benzene rings conjugated with three *trans*-disubstituted double bonds. The substitution pattern at the three benzene rings and their conjugated α,β-

Table 2
^{1}H and ^{13}C NMR data of compounds 5, 6 (CD_3OD, 600 MHz) [a].

Position	5		6	
	δ_H	δ_C	δ_H	δ_C
1	3.98	65.8	4.00	66.0
2		105.0	–	105.0
3	5.85 d (8.0)	79.8	5.90 d (8.0)	79.9
4	5.70 t (8.0, 7.0)	75.2	5.68 t (8.0, 7.0)	75.3
5	4.21 m	84.6	4.21 m	84.6
6a	4.05 s	64.0	4.03 s	63.9
6b	3.96 s	–	3.96 s	–
1′	5.60 d (4.0)	92.9	5.57 d (4.0)	93.0
2′	3.58 dd (4.0, 9.0)	73.5	3.60 dd (4.0, 9.0)	73.6
3′	3.76 t (9.0)	74.5	3.80 t (9.0)	74.5
4′	3.40 m	72.1	3.42 m	72.0
5′	4.35 m	72.8	4.36 m	72.9
6′a	4.65 br d (12.0)	65.9	4.70 br d (12.0)	66.0
6′b	4.12 dd (12.0, 5.0)	–	4.15 dd (12.0, 5.0)	–
1″	–	127.3	–	127.5
2″	7.46 d (8.0)	131.3	7.03 d (1.5)	114.9
3″	6.78 d (8.0)	116.8	–	146.8
4″	.	161.2	–	149.6
5″	6.78 d (8.0)	116.8	6.77 d (8.0)	116.8
6″	7.46 d (8.0)	131.8	6.55 dd (8.0, 1.5)	123.1
7″	7.63 d (16.0)	146.9	7.55 d (16.0)	147.7
8″	6.43 d (16.0)	115.0	6.36 dd (16.0)	115.4
9″	–	169.3	–	169.2
1‴	–	127.1	–	127.0
2‴	7.24 d (1.5)	112.0	7.22 d (1.5)	112.1
3‴	–	149.3	–	149.5
4‴	–	150.0	–	150.2
5‴	6.79 d (8.2)	116.4	6.75 d (8.2)	116.5
6‴	7.11 dd (8.2, 1.5)	124.2	7.10 dd (8.2, 1.5)	124.2
7‴	7.70 d (16.0)	147.7	7.68 d (16.0)	147.8
8‴	6.40 d (16.0)	114.8	6.42 d (16.0)	114.9
9‴	–	168.3	–	168.4
–OMe	3.91	56.3	3.93	56.3
1⁗	–	127.3	–	127.2
2⁗	7.23 d (1.5)	112.2	7.24 d (1.5)	112.2
3⁗	–	149.3	–	149.5
4⁗	–	150.0	–	150.0
5⁗	6.80 d (8.2)	116.8	6.79 d (8.2)	117.0
6⁗	7.13 dd (8.2, 1.5)	123.9	7.12 dd (8.2, 1.5)	124.0
7⁗	7.67 d (16.0)	147.9	7.67 d (16.0)	147.9
8⁗	6.38 d (16.0)	115.0	6.32 d (16.0)	115.0
9⁗	–	169.0	–	169.1
–OMe	3.91	56.3	3.93	56.3

[a] Coupling pattern and coupling constants (J in Hertz) are in parentheses.

unsaturated carbonyl system were inferred from the signals at δ 7.24 (d, J = 1.5 Hz, H-2‴), 6.79 (d, J = 8.2 Hz, H-5‴), 7.11(dd, J = 8.2, 1.5 Hz, H-6‴), 7.70 (d, J = 16.0 Hz, H-7‴), 6.40 (d, J = 16.0 Hz, H-8‴), 7.23 (d, J = 1.5 Hz, H-2⁗), 6.80 (d, J = 8.2 Hz, H-5⁗), 7.13 (dd, J = 8.2, 1.5 Hz, H-6⁗), 7.67 (d, J = 16.0 Hz, H-7⁗), 6.38 (d, J = 16.0 Hz, H-8⁗), and 3.91 (6H, s, OCH_3)], and were assigned to the two feruloyl moieties [20,21], while signals at δ 7.46 (d, J = 8 Hz, H-2″, H-6″), 6.78 (d, J = 8 Hz, H-3″, H-5″), 7.63 (d, J = 16 Hz, H-7″), 6.43 (d, J = 16 Hz, H-8″) were assigned to a *p*-coumaroyl group [20]. The ^{13}C NMR spectrum (Table 2) of compound **5** showed signals for 41 carbons, resolved as 10 aromatic methine, 6 olefinic methine, 5 oxygenated quaternary aromatic, 3 quaternary aromatic, 3 α,β-unsaturated, 8 oxymethines, 3 oxymethylenes, 2 oxymethylen, and one oxygenated quaternary carbons. Among them, 12 signals were characteristic of a sucrose unit [22]. The assignments of all sugar protons were based on 1D-TOCSY and 2D-COSY experiments (Table 2). Cross peaks in both experiments displayed full coupling information, which helped with the assignments and allowed identification of proton pattern. Chemical shift and coupling constants were in good agreement with values reported for sucrose [23]. The signals of the C-3 and C-4 protons of fructose and the C-6′ proton of glucose resonated downfield about 1–1.5 ppm, as compared to unsubstituted sucrose [22], indicating the ester linkages at these position. The substitution of feruloyl and *p*-coumaroyl groups at the sucrose unit was deduced from the HMBC spectrum of compound **5**. Correlation peaks were observed between H_2-6′ protons (δ 4.05 and 3.96) and the α,β-unsaturated carbonyl carbon (δ 169.3) of the *p*-coumaroyl group. The carbonyl carbon at δ_C 169.3 also exhibited HMBC correlations with *trans*-olefinic protons, resonating at δ 7.63 and 6.43 (H-7″ and H-8″), respectively. These protons were further correlated with the aromatic carbon δ_C 127.3 (C-1‴) and δ_C 131.3 (2×CH, C-2′, C-6″). These correlations indicated that a *p*-coumaroyl residue was attached at the C-6′ position of the α-glucose. The HMBC correlation peaks confirmed the presence of two feruloyl residues esterified at C-3 and C-4 of fructose. Compound **5** was therefore identified as 3,4-*O*-β-D-di-feruloyl-fructofuranosyl-6-*O*-α-D-(*p*-coumaroyl)-glucopyranoside.

Compound **6** was assigned a molecular formula, $C_{41}H_{44}O_{20}$, as determined by ^{13}C, ^{13}C DEPT NMR, and ESI-MS. The ESI-MS spectrum of **6** showed an $[M–H]^-$ ion at m/z 855. In the MS^2 spectrum, prominent fragments at m/z 693 $[M–H-162]^-$, 517 $[M–H-(162+176)]^-$, and 341 $[M–H-(162+176+176)]^-$ were observed, due to consecutive losses of one caffeoyl and two feruloyl residues, respectively.

NMR spectral data of **6** in comparison to those of **5** showed that *p*-coumaroyl moiety of **5** was replaced by one caffeoyl unit in **6** (Table 2) [9,24]. The lower field shift of H-3 (δ 5.90) and C-3 (δ 79.9), H-4 (δ 5.68) and C-4 (δ 75.3), and H_2-6′ (δ 4.15, 4.70) and C-6′ (δ 66.0) of the sucrose unit suggested the substitution sites of the caffeoyl and feruloyl residues that were confirmed by the HMBC cross peak between δ 4.70 and 4.15 (H-6′) and 169.2 (C-9″), δ 7.55 (H-7″) and 169.2 (C-9″), and δ 7.55 (H-7″) and 127.5, 114.9 (C-1″, C-2″ respectively). Compound **6** was therefore identified as 3,4-*O*-β-D-di-feruloyl-fructofuranosyl-6-*O*-α-D-(caffeoyl)-glucopyranoside.

All the isolated compounds were evaluated for their anti-angiogenic activity, and all were inactive. On the basis of our results, we could hypothesize that the inhibition activity of VEGF-A interaction with Flt-1 receptor showed by *n*-BuOH extract 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.

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