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Antiangiogenic Activity and Cytotoxicity of Triterpenoids and Homoisoflavonoids from *Massonia pustulata* and *Massonia bifolia.*

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Abstract:

The Hyacinthaceae family (*sensu* APGII) with approximately 900 species in around 70 genera, plays a significant role in traditional medicine in Africa as well as across Europe and the Middle and Far East. The dichloromethane extract of the bulbs of *Massonia pustulata* (Hyacinthaceae *sensu* APGII) yielded two known homoisoflavonoids, (*R*)-5-hydroxy-3-(4-hydroxybenzyl)-7-methoxy-4-chromanone **1** and 5-hydroxy-3-(4-hydroxybenzyl)-7-methoxy-4-chromone **2** and four spirocyclic nortriterpenoids, eucosterol **3**, 28-hydroxyeucosterol **4** and two previously unreported triterpenoid derivatives, (*17S,23S*)-17α,23-epoxy-3β,22β,29-trihydroxylanost-8-en-27,23-olide **5** and (*17S, 23S*)-17α,23-epoxy-28,29-dihydroxylanost-8-en-3-on-27,23-olide **6**. Compounds **1**, **2**, **3**, and **5** were assessed for cytotoxicity against CaCo-2 cells using a neutral red uptake assay. Compounds **1**, **2** and **5** reduced cell viability by 70% at concentrations of 30, 100 and 100 µM respectively. *Massonia bifolia* yielded three known homoisoflavonoids, (*R*)-(4'-hydroxy)-5-hydroxy-7-methoxy-4-chromanone **1**, (*R*)-(4'-hydroxy)-5,7-dihydroxy-4-chromanone **7** and (*R*)-(3'-hydroxy-4'-methoxy)-5,7-dihydroxy-4-chromanone **9**, two previously unreported homoisoflavonoids, (*E*)-3-benzylidene-(3',4'-dihydroxy)-5-hydroxy-7-methoxy-4-chromanone **8** and (*R*)-(3',4'-dihydroxy)-5-hydroxy-7-methoxy-4-chromanone **10,** and a spirocyclic nortriterpenoid, 15-deoxoeucosterol **11**. Compounds **1**, **1Ac**, **7**, **8**, **9** and **10** were screened for antiangiogenic activity against human retinal microvascular endothelial cells. Some compounds showed dose-dependent antiproliferative activity and blocked endothelial tube formation, suggestive of antiangiogenic activity.

Keywords: *Massonia species*, Hyacinthaceae, homoisoflavonoids, spirocyclic nortriterpenoids, cytotoxicity, angiogenesis

Introduction

The genus *Massonia* Houttuyn (Hyacinthaceae, subfamily Hyacinthoideae, tribe Massonieae) [1] is found in the dry areas of South Africa and southwestern Namibia. The genus was named after a Scottish student gardener at Kew, Francis Masson, who described the genus while collecting seeds in the Cape in 1772. The genus is not known to be used by traditional healers in the region. *Massonia pustulata*, first

described in 1791 by Nikolaus von Jacquin, flowers in winter and a dry summer dormancy is required. *Massonia bifolia* (syn. *Whiteheadia bifolia*) was originally described by von Jacquin in 1791 as *Eucomis bifolia*, however, the plant was 'rediscovered' as *Melanthium massoniifolium* in 1804 and as *Whiteheadia latifolia* in 1865. It was soon after renamed as *Whiteheadia bifolia* due to similarities between the collected specimen and that described by Jaquin. It remained the only species in the *Whiteheadia* genus until 2004 when it was transferred to *Massonia* by Manning et al. due to the results of its DNA sequence analysis [2]. This analysis was prompted by the discovery of a second species, *W. etesionamibensis*, which was tentatively placed in the *Whiteheadia* genus. However, the DNA analysis indicated that this species fell between the genera *Whiteheadia* and *Massonia*, suggesting it was an evolutionary stepping stone from *Massonia* to *Whiteheadia*. As such, *Massonia* was given a broader scope to include *Whiteheadia* rather than to create a second, new single-species genus for *W. etesionamibensis* [3]. The phytochemistry of the genus *Massonia* has not been investigated previously.

The subfamily Hyacinthoideae (*sensu* APGII) is characterised by the presence of homoisoflavonoids and spirocyclic nortritepenoids, usually of the lanostane type [4]. Genera from this family are widely used by traditional healers [4] and homoisoflavonoids isolated from this subfamily have shown anti-inflammatory activity [5-7] and activity against colon (HT-29) and breast cancer (MDA-MB-435) [8,9] cells. Spirocyclic nortriterpenoids have shown activity against HeLa cells [10] and HSC-2 oral squamous carcinoma cells [11-13]. Homoisoflavonoids **1** and **2** and spirocyclic nortriterpenoids **3**, **4**, **5** and **6** were isolated from the dichloromethane extract of the bulbs of *Massonia pustulata* and were assessed for anti-proliferative activity against CaCo-2 cells. Homoisoflavonoids **1, 7, 8, 9** and **10** and the spirocyclic nortriterpenoid **11** were isolated from the ethanol extract of the bulbs of *Massonia bifolia*. Compound **1** was acetylated to aid in separation, forming compound **1Ac**. Since some homoisoflavonoids have been shown to have antiangiogenic activity [14- 17], the ability of compounds **1**, **1Ac**, **7**, **8**, **9** and **10** to block in vitro angiogenesis of human retinal microvascular endothelial cells (HRECs) was assessed. Structures are provided in Fig. 1.

Results and Discussion

(*R*)-5-Hydroxy-3-(4-hydroxybenzyl)-7-methoxy-4-chromanone **1** and 5-hydroxy-3-(4-hydroxybenzyl)-7-methoxy-4-chromone **2** have been isolated previously from *Lachenalia rubida* Jacq. [18]. This species, like *Massonia pustulata,* is from the subfamily Hyacinthoideae and endemic to the dry areas of South Africa and Namibia. (*R*)-5-Hydroxy-3-(4-hydroxybenzyl)-7-methoxy-4-chromanone **1** has also been isolated from *Ledebouria graminifolia* Bak. (Jessop) [19]. Eucosterol **3** was first isolated from *Eucomis autumnalis* [20] and later from *Eucomis bicolor* [21], (17*S*, 23*S*)-23,17-epoxy-3β,28,29-trihydroxy-27-norlanost-8-en-24-one **4** has been isolated previously from *Eucomis zambesiaca* [21], (*R*)-(4'-hydroxy)-5,7-dihydroxy-4-chromanone **7** has been extracted from *Scilla scilloides* [22] , *Leopoldia* (*Muscari*) *comosa* [23] and several *Ledebouria* species [7] and (*R*)-(3'-hydroxy-4'-methoxy)-5,7-dihydroxy-4-chromanone **9** has been extracted from *Scilla nervosa* [9]. 15-Deoxoeucosterol **11** has been isolated from several plant species including *Scilla scilloides* [24] and *Pseudoprospero firmifolium* [25] .

Compound **5** was isolated as an amorphous white powder. HR-ESIMS indicated a formula of $C_{30}H_{46}O_6$ with [M^++1] at *m/z* = 503.33666 ($C_{30}H_{46}O_6$+H requires 503.33726). The FTIR spectrum showed absorption peaks at 3391 cm^{-1} and 1758 cm^{-1} due to hydroxyl and carbonyl stretches respectively. NMR spectra indicated a lanosterol-type triterpenoid, with a spirocyclic γ-lactone side chain, typical of the subfamily Hyacinthoideae. The spectroscopic data obtained was very similar to that obtained for the co-isolated eucosterol **3**. As with eucosterol, an 8,9-double bond (δ_c 134.7 and 134.6 respectively), a 3β-hydroxyl group (δ_c 80.7 and δ_H 3.50 dd, *J* = 6.0Hz and 12.2 Hz, $W_{1/2}$ = 20.3 Hz) [21] and an oxymethylene group at C-29 (δ_c 64.3 and δ_H 4.27, δ_H 3.36) were noted. Four tertiary methyl groups were present at δ_H 0.94 (3H-18), δ_H 0.96 (3H-19), δ_H 1.25 (3H-28) and δ_H 2.24 (3H-30), together with two secondary methyl group resonances at δ_H 1.31, (3H-21) and δ_H 1.39, (3H-26). The presence of the C-17 carbon resonance at δ_c 99.4 together with C-23 fully substituted carbon resonance at δ_c 115.5 indicated a spirocyclic ring system as shown in Fig 2. [26]. An oxymethine proton resonance ascribed to H-22 was noted (δ_H 3.99), and the corresponding carbon resonance (δ_c 84.0) showed correlations in the HMBC spectrum

with the H-21 (δ_H 1.31) resonance. Further correlations were seen between the H-22 (δ_H 3.99) and C-17 (δ_c 99.4) resonances, the 3H-26 (δ_H 1.39) and C-27 (δ_c 178.8) and C-24 (δ_c 42.0) resonances and the H-24 (δ_H 2.45) and C-27 (δ_c 178.8) and C-23 (δ_c 115.5) resonances. A correlation was seen between the 3H-21 (δ_H 1.31) and H-22 (δ_H 3.99) resonances in the NOESY spectrum, indicating the hydroxyl group at C-22 was β. The compound was identified as the previously unreported (*17S,23S*)-17α,23-epoxy-3β,22β,29-trihydroxylanost-8-en-27,23-olide.

Compound **6** was isolated as an amorphous white powder. The FTIR spectrum showed absorption peaks at 3353 cm^{-1} and 3250 cm^{-1} indicative of the presence of hydroxyl groups as well as absorption peaks at 1772 cm^{-1} and 1723 cm^{-1} indicative of carbonyl groups. The molecular ion was not seen in the LCMS. The NMR data for compound **6** was similar to that obtained for compound **5,** but differed in showing the presence of a keto group at C-3 (δ_c 213.6), an oxymethylene group at C-28 (δ_c 63.4) and the absence of the hydroxyl group at C-22 (δ_c 32.3 instead of δ_c 84.0). Correlations were seen in the HMBC spectrum between the H-24α (δ_H 2.00) and H-24β (δ_H 2.72) and C-23 (δ_c 113.6) resonances, and between 3H-26 (δ_H 1.28) and C-25 (δ_c 35.8) resonances. Correlations were also noted in the HMBC spectrum between the 3H-30 (δ_H 1.05) and C-8 (δ_c 133.5) resonances and between the 3-H-19 (δ_H 0.98) and C-9 (δ_c 135.4) resonances. Various glycoside derivatives of compound **6** have been isolated from *Scilla peruviana* [10] but the aglycone, (*17S,23S*)-17α,23-epoxy-28,29-dihydroxylanost-8-en-3-on-27,23-olide, has not been reported previously. Key correlations seen in the HMBC spectra of compounds **5** and **6** are shown in **Fig. 2.**

HR-ESIMS of compound **9** indicated a $[M + H]^+$ ion at *m/z* 317.1020 corresponding to a molecular formula of $C_{17}H_{16}O_5$ for the compound. The ^1H NMR spectrum indicated this compound was a 3-benzyl-4-chromanone type homoisoflavonoid due to characteristic proton peaks and coupling patterns, including the two H-2 proton resonances at δ_H 4.30 (dd, *J*=11.3, 4.4 Hz) and δ_H 4.14 (dd, *J*=11.3, 7.4 Hz), the H-3 resonance at δ_H 2.84 (m) and the two H-9 resonances at δ_H 3.08 (dd, *J*=14.4, 5.3 Hz) and δ_H 1.62 (dd, *J*=14.4, 10.1 Hz). Also seen in the proton spectrum was a singlet peak at δ_H 3.83 integrating to 3H, indicating that a single methoxy group was present.

The C-4 carbonyl carbon resonance was present at δ_C 200.1 and a H-bonded OH group proton resonance was seen at δ_H 12.10, confirming the placement of a hydroxy group at C-5. Two *meta*-coupled proton resonances were present (δ_H 6.05, *J* = 2.3 Hz, H-6 and 6.02 *J* = 2.3 Hz, H-8). Both resonances showed a correlation with the single methoxy group proton resonance in the NOESY spectrum, so a methoxy group was placed at C-7.

The B ring was 1,3,4-trisubstituted as shown by the typical splitting patterns of H-2' (δ_H 6.70, d, *J*=2.1 Hz), H-5' (δ_H 6.73, d, *J* = 8.0 Hz) and H-6' (δ_H 6.57, dd, *J* = 8.0, 2.1 Hz) in the ^{1}H NMR spectrum. Hydroxy groups were placed at the remaining C- 3' and C-4' positions. CD analysis *confirmed* the configuration at C-3 as *R* due to the negative Cotton effect seen at 290 nm [27] and the compound was identified as the previously unreported 3*R*-(3',4'-dihydroxybenzyl)-5-hydroxy-7-methoxy-4-chromanone.

Compound **10** was isolated as a brown oil. HR-ESIMS indicated a protonated molecular ion at *m/z* 315.0863 indicating a molecular formula of $C_{17}H_{14}O_6$ for the compound. This compound was identified as the *E*-3-benzylidene homoisoflavonoid analogue of compound **9,** *E*-3-(3',4'-dihydroxybenzylidene)-5-hydroxy-7-methoxy-4-chromanone**.** The two equivalent H-2 resonances occurred at δ_H 5.39 (*J*=1.8 Hz) and the H-9 resonance occurred at δ_H 7.72 (br s). The *E* configuration of the double bond was confirmed by the characteristic H-9 proton shift. For the *E*-configuration, H-9 occurs in the δ_H 7.58-7.79 region while for the *Z*-configuration, the H-9 resonance occurs at about δ_H 6.86 [28].

Compounds **1**, **2**, **3** and **5** were assessed for activity against CaCo-2 cells using a neutral red uptake assay [29, 30] (Figure 3). Healthy cells take up neutral red into lysosomes whereas dead or damaged cells do not. The amount of neutral red recovered from cells at the end of the assay (as determined by absorbance) is related to the number of viable cells after exposure to a test compound. Cell viability was reduced below 70% by compound **1** at 30 µM and for compounds **2** and **5** at 100 µM. Compound **3** had no effect on cell viability. Compound **2** at 100 µM ($p<0.05$) was of similar activity (22% viability) to actinomycin D (10 µg/mL or 7.96 µM, purchased from Sigma-Aldrich cat no: A1410, 98% purity), a known cytotoxic agent, but at 13x concentration (Figure 3) Overall, the reported activity was moderate for these compounds.

Compounds **1, 1Ac** and **7-10** were tested for their effect on proliferation of Human Retinal Microvascular Endothelial Cell (HRECs) (Figure 4). GI_{50} values for the compounds, the concentration at which the proliferation of cells is reduced by 50% relative to the DMSO control, were calculated and compounds that only reduced cell proliferation at the highest concentration tested (relative to DMSO control) were reported as having a GI_{50} > 100 µM. Compound **1** was reported to have the most potent GI_{50} value of 24.8 µM, compounds **7** and **8** were found to have GI_{50} values of 35.8 µM and 93.2 µM respectively. Compounds **1Ac**, **9** and **10** had negligible effect on cell proliferation.

We then tested these compounds for anti-angiogenic activity in an *in vitro* Matrigel tube formation assay. This assay models the endothelial cells' ability to form vascular structures. HRECs overall tubule formation decreased significantly and in a dose-dependent manner when compared to DMSO (Figure 5). Compounds **1**, **7** and **8** again proved most potent of the compounds to block vascular tube formation by HRECs.

The majority of (synthetic) antiangiogenic homoisoflavonoids described previously have trisubstituted A-rings [16], thus the finding that compound **1**, disubstituted on this ring, had reasonably potent activity is novel. Taken together with previous SAR studies on synthetic homoisoflavonoids, these findings help identify homoisoflavonoids that could be pursued towards therapies for neovascular eye diseases such as wet age-related macular degeneration.

Materials and Methods

General experimental procedures

Optical rotations were measured at room temperature on a JASCO-P-1020 polarimeter and IR spectra were recorded using a Perkin-Elmer (2000 FTIR) spectrophotometer using KBr windows. ECD spectra were measured on an Applied Photophysics Chirascan CD spectrometer using a 1 mm cell and CH_3CN as the solvent. ^{1}H, ^{13}C and 2D NMR spectra were recorded on a Bruker AVANCE III NMR spectrometer, operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C, using standard experiments from the Bruker pulse programs library. Chemical shifts are reported in ppm (δ) referencing the solvent signal ($CDCl_3$) as internal standard respect to TMS (0

ppm), and coupling constants (*J*) are measured in Hz. HR-ESIMS was performed on a Bruker MicroToF Mass Spectrometer, using an Agilent 1100 HPLC to introduce samples. Gravity column chromatography was performed using silica gel (Merck Art. 9385) packed 1 or 4 cm diameter columns. TLC was performed on aluminium precoated silica gel plates (Merck 9385) visualised using anisaldehyde spray reagent. Mass spectra of compounds **9** and **10** were recorded on a Thermo Q-Exactive Orbitrap Mass Spectrometer using direct HESI injection.

Plant material

Massonia pustulata Jacq. and *Massonia bifolia* Jacq. bulbs were obtained from the collection of the Royal Horticultural Society, Wisley Gardens, Wisley, Surrey, UK (W20150204A WSY and W20150850 WSY respectively).

Extraction and isolation of compounds from *M. pustulata* and *M. bifolia*

The bulbs of *Massonia pustulata* (672.1 g) were chopped and extracted by shaking successively for 24 hours in dichloromethane (CH_2Cl_2) and then methanol (MeOH) to yield 8.35 g of CH_2Cl_2 extract and 29.67 g of MeOH extract. The dichloromethane extract was fractionated over silica gel starting with EtOAc:CH_2Cl_2 (1:9) and increasing the concentration of EtOAc. 110 fractions of 20 mL each were collected. Further fractionation over Sephadex LH20 (1:1, CH_2Cl_2:MeOH) yielded compounds **1**-**6** (20 mg, 8.9 mg, 50 mg, 15 mg, 4.6 mg and 28 mg respectively).

Bulbs of *Massonia bifolia* (263.0 g) were chopped and extracted by shaking for 2x24 hours in ethanol (EtOH) yielding a combined 5.13 g of extract. The extract was fractionated over silica gel, starting with CH_2Cl_2 as solvent and gradually increasing the polarity by addition of use of EtOAc. 95 fractions of 20 mL each were collected. Further fractionation over Sephadex LH20 (1:1, CH_2Cl_2:MeOH) yielded compounds **1** and **7-11** (19 mg, 9.8 mg, 2.3 mg, 3.2 mg,1.4 mg and 6.9 mg respectively). Structures of compounds **1**-**11** are shown in **Fig. 2**.

(*17S,23S*)-17α,23-epoxy-3β,22β,29-trihydroxylanost-8-en-27,23-olide (**5):** Amorphous white solid (4.6 mg, 75% pure; from NMR); $[\alpha]_D^{23.6}$ +44.4, ($CHCl_3$, *c* = 2.7 mg/mL); IR ν_{max} cm^{-1}: 3391, 2934, 1758, 1455, 1375, 1034, 736; ^{1}H- and ^{13}C-NMR

(500 MHz, $CDCl_3$) see **Table 1**. HR-ESI-MS: *m/z* 503.33667 for $[M^+ + H]^+$ (calcd. for $C_{30}H_{47}O_6$, 503.33726)

(*17S, 23S*)-17α,23-epoxy-28,29-dihydroxylanost-8-en-3-on-27,23-olide (**6**): Amorphous white solid (28 mg, 70% pure from NMR); $[\alpha]_D^{23.6}$ +3.08 ($CHCl_3$, C = 20.3 mg/mL); IR ν_{max} cm^{-1}: 3353, 3250, 2937, 1772, 1723, 1456, 1371, 1046, 736; ^{1}H- and ^{13}C-NMR (500 MHz, $CDCl_3$) see **Table 1**. HR-ESI-MS: M^+ not detected.

3*R*-(3',4'-dihydroxybenzyl)-5-hydroxy-7-methoxy-4-chromanone **(9)**: cream/brown oil (3.2 mg); $[\alpha]_D^{20.2}$ = +67.5 (MeOH, C = 1.6 mg/mL); CD (CH_3CN) 256 nm (Δε + 0.25), 286 nm (Δε – 9.19), 313 nm (Δε + 1.05); IR ν_{max} cm^{-1}: 3349, 2949, 2925, 2848, 1639, 1449, 1014; ^{1}H- and ^{13}C-NMR (500 MHz, MeOD) see **Table 2.** HESIMS *m/z* 317.1020 $[M + H]^+$, (calcd. for $C_{17}H_{17}O_6$, 317.1025).

E-3-(3',4'-dihydroxybenzylidene)-5-hydroxy-7-methoxy-4-chromanone **(10)**: brown oil (1.4 mg); IR ν_{max} cm^{-1}: Insufficient quantity of compound; ^{1}H- and ^{13}C-NMR (500 MHz, MeOD). See **Table 2**. HESIMS *m/z* 315.0863 $[M + H]^+$, (calcd. for $C_{17}H_{15}O_6$, 315.0868)

Pharmacological Assays

Neutral red assay

CaCo-2 human colon carcinoma cells (CACO-2 (ECACC 86010202)) from Public Health England, UK, were cultured in EMEM (Eagle's Minimum Essential Medium, ATCC/LGC Standards) supplemented with 10% FBS, 2 mM glutamine, 1% non-essential amino acids, 100 U/mL penicillin/100 U/mL streptomycin, and maintained in 5% CO_2/95% air in an incubator at 37°C. Cells were plated in 96 well plates at $2x10^4$ cells/well for 48 hours. Cells were then treated for 48 hours with one of the following, in 4 replicates: culture medium alone (untreated control); 0.1% DMSO (vehicle control; maximum DMSO exposed to cells); actinomycin D 10 µg/mL (positive control); test substances at 1, 3, 10, 30 and 100 µM (diluted from a 100 mM stock in DMSO). Cells were washed with PBS, and neutral red (25 µg/mL in culture medium, diluted from a stock of 2.5 mg/mL in ultrapure water) was added for 3 hours, cells were washed with PBS, and neutral red extracted in 50% ethanol/water with 1% acetic acid. Absorbance was read at 540 nm. Data are reported from 5 independent experiments (different cell passages). The mean absorbance of wells without cells (blank) was subtracted from

all other readings. Plates were accepted for analysis given adequate neutral red loading in vehicle treated cells (>0.18 absorbance units) and positive control activity (<60% viability); Z factor values were in the range 0.68 to 0.88. Replicates were averaged to give one treatment value per plate. All plate treatment values were divided by the *mean* value of vehicle-treated cells on the same plate, so data was normalised as percentage of maximum cell viability. The plate treatment values were averaged to produce group means of n=5. Analysis of multiple means was performed by one way ANOVA with Dunnett's multiple comparison test against the untreated cells (negative control) value, using GraphPad Prism 6.

HREC proliferation assay

Endothelial Growth Medium (EGM-2) was prepared by mixing the contents of an EGM-2 "Bullet Kit" (Cat. no. CC-4176) with Endothelial Basal Medium (EBM) (Lonza). The EGM-2 "Bullet Kit" contains hydrocortisone, human fibroblast growth factor (hFGF), VEGF, R3-insulin like growth factor (R3-IGF-1), ascorbic acid, human epidermal growth factor (hEGF), gentamycin and heparin along with 2% foetal bovine serum (FBS). Human Retinal Endothelial Cells (HRECs) and proprietary Attachment Factor were purchased from Cell Systems (Kirkland, WA, USA). HRECs were used for cell proliferation studies between passages 5 and 7.

The base of a tissue culture flask (10 cm^2) was washed with 1 mL of attachment factors and aspirated. Cryopreserved cells were removed from liquid nitrogen storage and defrosted just prior to use. Cells were transferred into EGM-2 media (5 mL) which was then centrifuged (2 mins, 270x*g*). The liquid above the cell pellet was aspirated off and the cell pellet was resuspended in 10mL EGM-2 and transferred into the flask. The tube was then incubated overnight (37°C, 5% CO_2) until cells reached confluence. Medium was aspirated and the cells washed with PBS (3 mL). PBS was aspirated and 500 µL TrypLE (Life Technologies) added and coated over the surface of the cells for a maximum of 30 seconds. Trypsin was then aspirated and EGM-2 medium (2 mL) was added to the tube to wash cells from the surface. The resulting cell slurry was transferred to a Falcon tube (15 mL) and diluted with sufficient EGM-2 to seed approximately 2,500 cells per well.

The required 2,500 cells/well in 100 uL medium were incubated in the centre 48 wells of a 96-well clear bottom black plates for 24 hours, with the surrounding 48 wells containing 100 µL deionized, sterilised water. Standard 1:10 dilutions of compounds in DMSO ranged from 100 mM to 100 nM such that seven concentrations of each compound were tested and the eighth and final well for testing containing DMSO as a control. Cells were treated with 1 µL of different concentrations of each test compound and control, resulting in a final in-well concentration range of 1 nM to 1 mM with a final DMSO concentration of 1% in all wells. All compounds were tested in triplicate, and our previously-reported synthetic, antiangiogenic homoisoflavonoid SH-11037 [16] was included as a positive control. Treated cells were incubated for a further 48 hour period.

At the end of this incubation period, AlamarBlue reagent (11.1 µL) was added and after 4 hours of incubation, fluorescence readings were taken with excitation and emission wavelengths of 560 nm and 590 nm respectively. Data were analyzed and dose response curves generated using GraphPad Prism software (v. 6.0).

In vitro Angiogenesis Assay

A Matrigel based tube formation assay was performed as previously described [31]. Briefly, 50 µL Matrigel was allowed to solidify in a 96 well black, clear bottom plate at 37°C for 20 minutes. HRECs were added to the solid Matrigel at 15,000 cells/well in 100 µL EGM-2 and dosed with appropriate concentrations of compound with 1 µL DMSO/well. Tube formation was observed every 2 hours by brightfield microscopy and images were taken after 8 hours of tube formation. Six images per treatment were analyzed with AngiogenesisAnalyzer plugin for ImageJ [32], and HREC total tubule length for treated cells was normalized to DMSO. Statistical analysis was completed using GraphPad Prism.

Supporting information

Photographs of plant material, spectra for compounds **5, 6, 8** and **10** and graphs showing effects of compounds on HREC cell proliferation are available as Supporting Information.

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Conflict of Interest

The authors declare there is no conflict of interest.

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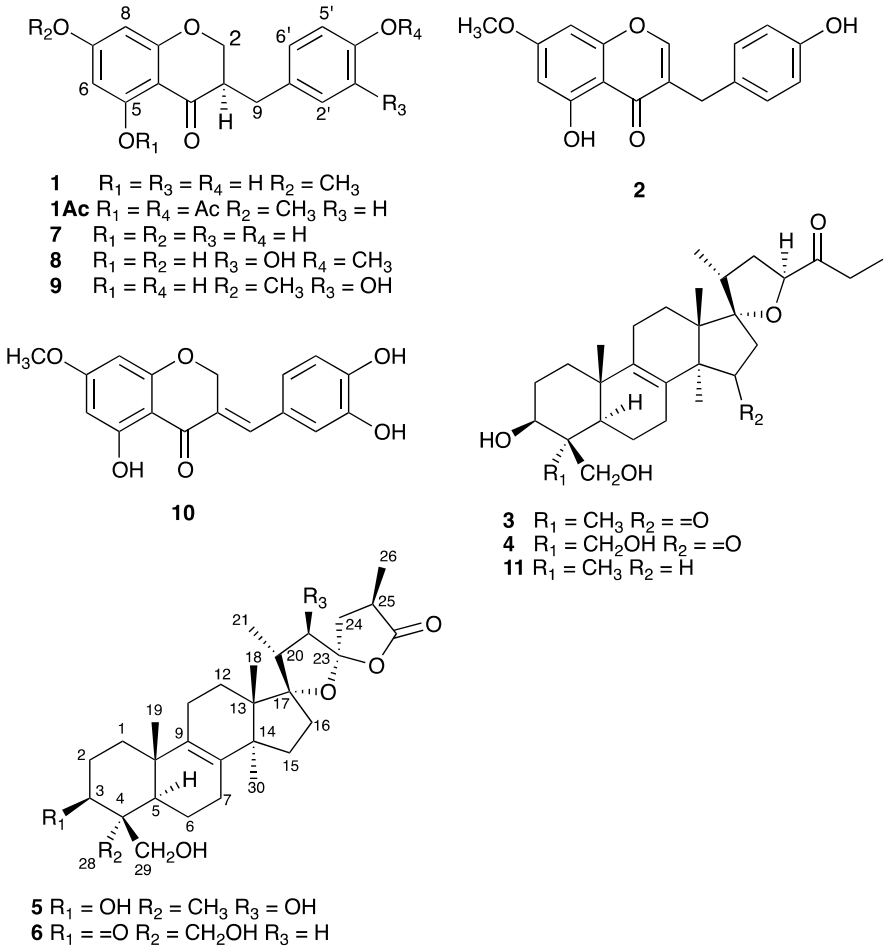

Fig. 1 Structures of compounds **1** – **11**

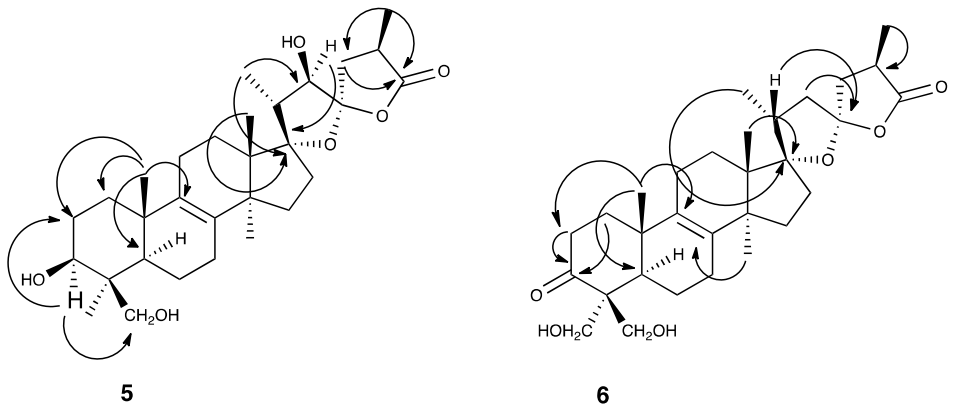

Fig. 2 Key correlations seen in the HMBC spectra for compounds **5** and **6**.

Table 1 ^{1}H and ^{13}C NMR data for Compounds **5** and **6** ($CDCl_3$).

Compound 5			**Compound 6**	
No.	**δC**	**δH (*J* in Hz)**	**δC**	**δH (*J* in Hz)**
1	35.3	1.21 m, 1.80 m	34.8	1.25 m, 1.79 m
2	28.3	1.30 m	35.8	1.75, 1.25
3	80.7	3.50 dd (6.0, 12.2)	213.6	
4	43.5		48.3	
5	51.2	1.20 m	45.3	1.80 m
6	18.6	1.50 m, 1.80 m	19.5	1.5 m, 1.8 m
7	26.4	1.90 m	26.3	2.05 m
8	134.7		133.5	
9	134.6		135.4	
10	37.4		36.9	
11	20.6	1.99 m, 2.13 m	20.5	1.94, 2.05
12	24.9	2.23 m, 1.55 m	24.7	1.5, 2.0 m
13	49.6		46.7	
14	50.6		50.6	
15	32.0	α 1.35 m β 1.75 m	31.3	α 1.38 m β 1.68 m
16	37.7	α 1.85 dd (5.6, 13.4) β 2.80 dd (9.1, 13.4)	36.9	α 1.80 m β 2.50 m
17	99.4		98.3	
18	19.6	0.94 s	18.6	0.90 s
19	19.8	0.96 s	19.5	0.98 s
20	50.3	2.30 m	43.7	2.25 m
21	15.5	1.31 d (6.8)	18.6	1.05 d (6.8)
22	84.0	3.99 bs ($W_{1/2}$ = 5.4)	32.3	α 1.80 m β 2.55 m
23	115.5		113.6	
24	42.0	α 2.45 dd (4.5, 7.8), β 2.10 dd (7.8, 8.0)	45.2	α 2.00 m, β 2.72 m
25	35.5	2.70 m	35.8	2.97 m
26	16.5	1.39 d (7.4)	15.1	1.28 d (7.0)
27	178.8		179.6	
28	22.2	1.25 s	63.4	α 4.10 d (12.5) β 3.80 d (12.5)
29	64.3	α 3.36 d (11.2) β 4.27 d (11.2)	70.7	α 3.70 d (14.0) β 4.40 d (14.0)
30	26.0	2.24 s	24.7	1.05 s

Table 2 ^{1}H and ^{13}C NMR data for Compounds **9** and **10** (CD_3OD).

	Compound **9**		Compound **10**	
No.	**^{13}C**	**^{1}H (*J* in Hz)**	**^{13}C**	**^{1}H (*J* in Hz)**
2	70.4	α 4.14 (dd, 11.3, 7.4) β 4.30 (dd, 11.3, 4.4)	68.9	5.39 (d, 1.79)
3	48.3	2.84 m	127.4	
4	200.1		187.2	
4a	103.8		105.0	
5	165.2		169.6	
6	95.9	6.05 (d, 2.3)	95.7	6.08 (d, 2.3)
7	169.6		169.5	
8	94.7	6.02 (d, 2.3)	94.7	6.02 (d, 2.3)
8a	164.8		163.9	
9	33.3	α 3.08 (dd, 14.4, 5.3) β 2.62 (dd, 14.4, 10.1)	139.8	7.72 s
1’	131.1		127.0	
2’	117.3	6.70 (d, 2.1)	118.5	6.86 (d, 2.1)
3’	146.7		150.3	
4’	145.2		163.8	
5’	116.6	6.73 (d, 8.0)	116.9	6.87 (d, 8.1)
6’	121.6	6.57 (dd, 8.0, 2.1)	125.1	6.81 (dd, 8.2, 2.1)
5-OH (in $CDCl_3$)		12.10 s		
7-OCH_3	56.4	3.83 s	56.1	3.84 s

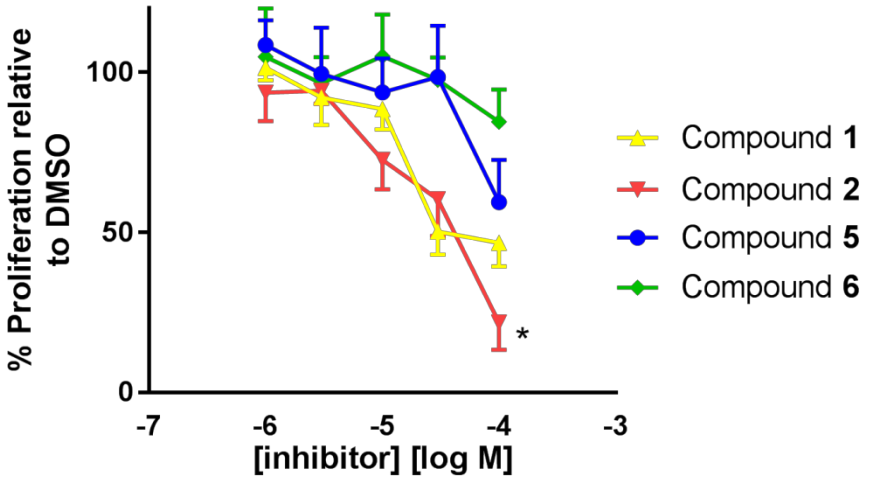

Fig 3. Effect of test compounds on Caco-2 proliferation after 48 hours as measured by neutral red uptake. Mean values are shown with SEM. N=5 independent experiments. Effect of positive control (Actinomycin D) was 23% +/- 8% (not shown). * p<0.05 compared to DMSO control.

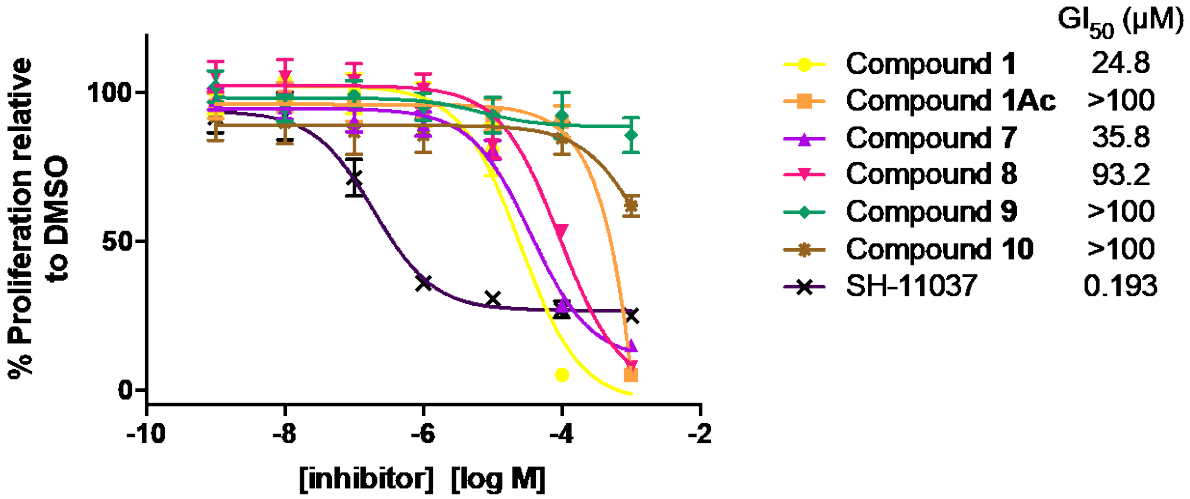

Fig. 4 Effect of compounds on HREC cell proliferation, normalised to DMSO control. Mean values are shown with SEM, n=3 wells, representative data from at least triplicate experiments. Synthetic homoisoflavonoid SH-11037 was positive control.

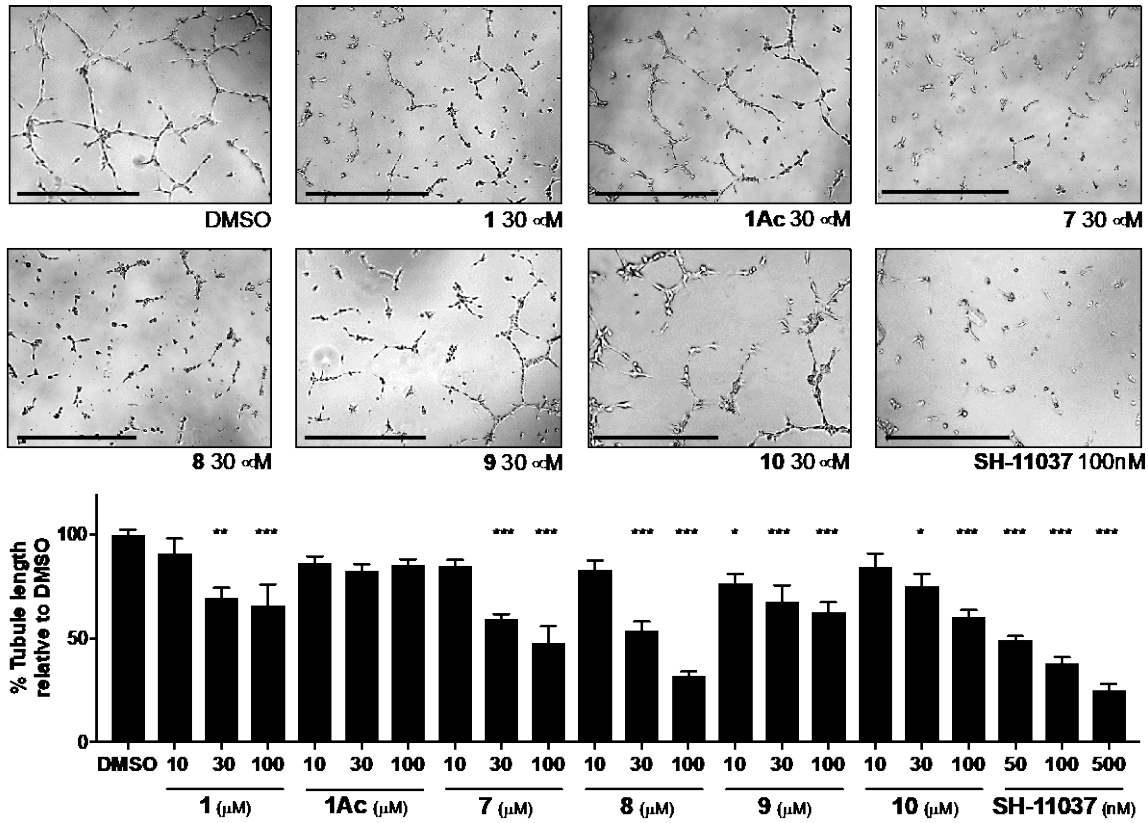

Fig. 5 Tube formation ability of HRECs treated with compounds at 3 concentrations (10 µM, 30 µM, and 100 µM) was observed in Matrigel over 8 hours and images were taken using brightfield microscopy. Tubule length was determined using ImageJ. Synthetic homoisoflavonoid SH-11037 was positive control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to DMSO, ANOVA with Dunnett's *post hoc* tests. Mean values are shown with SEM, n=6, representative data from at least triplicate experiments. Images above are tubes formed by HRECs in the presence of 30 µM of each compound above, or 100 nM SH-11037 positive control, or DMSO control. Scale bars = 500 µm.