Peltogynoids and 2-Phenoxychromones from *Peltophorum pterocarpum* and Evaluation of Their Estrogenic Activity

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Abstract

Phytochemical investigation of the dichloromethane extract of the leaves of *Peltophorum pterocarpum*, a tropical ornamental tree, led to the isolation of twelve compounds (**1**–**12**). One new derivative of peltogynoid ophioglonin (**1**) and a new 2-phenoxychromone (**2**) with its 3′-O-*β*-D-glucoside derivative (**3**) are described here for the first time. In addition, nine flavonoid derivatives, including peltogynoid ophioglonin (**4**), were isolated for the first time from this plant. The structures were determined by spectroscopic and chemical methods. Evaluation of the estrogenic activities of **1, 2**, and **4** using different model cell systems revealed that **4** was estrogenic and that **2** was largely inactive. Interestingly, **1** was unable to stimulate the proliferation of breast and endometrial cancer cells but exhibited substantial estrogen receptor *α*-mediated activation of gene expression. This observation indicates that **1** can be further evaluated for its cancer chemopreventive potential.

Introduction

"Yellow flame tree" is the name given to a beautiful Indonesian ornamental tree that is widespread in tropical regions. *Peltophorum pterocarpum* (DC.) Backer ex K. Heyne is the most frequent name encountered for this species which has also been referred to as *Peltophorum ferrugineum* (Decne.) Benth., *Peltophorum inerme* (Roxb.) Náves, or *Peltophorum roxburghii* Degener [1, 2].

The genus *Peltophorum* is included in the large family of Fabaceae and includes less than 10 species that are mostly distributed in tropical areas. Plants of this genus, often used as ornamental shade trees, represent a rich source of tannins and are used as a traditional remedy by tribes from Asia and Africa. These plants have not been investigated in detail from a phytochemical viewpoint [3]. Some studies on the fruits, flowers, and bark of *P. pterocarpum* have evidenced the presence of tannins (bergenin), triterpenes (lupeol), leucocyanidins, as well as several flavonoids and their related glycoside derivatives (quercetin, rhamnetin, melanoxetin, propelargonidin, and meratin) [2, 4–7].

This paper describes the isolation, characterisation, and biological evaluation of one new peltogynoid (**1**) and two new 2-phenoxychromones (**2, 3**) from the leaves of *P. pterocarpum.*

Material and Methods

General experimental procedures

UV spectra were measured on a Perkin-Elmer Lambda 20 spectrophotometer, and IR spectra were obtained on a Perkin-Elmer 1600 FTIR instrument. UV shift experiments were performed according to the protocol published by Markham [8]. EIMS spectra were obtained on a Finnigan-MAT/TSQ-700 triple stage quadrupole instrument (EIMS: 70 eV; positive-ion mode). HRESIMS data were obtained on a Micromass-LCT Premier time-of-flight (TOF) mass spectrometer (Waters) with an electrospray interface. ^{1}H and ^{13}C NMR were recorded on a Varian Inova 500 spectrometer (499.87 and 125.70 MHz, respectively) in DMSO-d_6 and $CDCl_3$ with TMS as an internal standard. Complete assignment was performed using 2D experiments gradient COSY, gradient HSQC, gradient HMBC, and NOESY. Analytical HPLC was carried out on a HP 1100 system equipped with a photodiode array detector (Agilent Technologies). MPLC separation was performed using a Buchi 681 pump equipped with a Knauer UV detector

and Lichroprep C18 as the stationary phase (15–25 µm, 460 × 70 mm i.d.; Merck). Semi-preparative HPLC was carried out with a Shimadzu LC-8A pump equipped with a Knauer UV detector using a µBondapak® C18 column (10 µm, 100 × 25 mm i.d.; Waters).

Plant material

The leaves of *Peltophorum pterocarpum* (Fabaceae) were collected in Surabaya, Indonesia, in September 1994. A voucher specimen was deposited at the School of Pharmaceutical Sciences, Phytochemistry and Bioactive Natural Products Research Unit, University of Geneva, Geneva, Switzerland (no. 94112) and identified by Dr. J.P. Mogea from the Herbarium Bogoriense (Bogor, Indonesia).

Extraction and isolation

The dried leaves (200 g) were ground in liquid nitrogen, successively extracted with solvents of increasing polarity (CH_2Cl_2 and MeOH) and concentrated under vacuum to give 3 g of CH_2Cl_2 extract and 36 g of MeOH extract. The CH_2Cl_2 (3 g) extract was first fractionated using MPLC with Lichroprep C18 as the stationary phase (15–25 µm, 460 × 70 mm i.d.; Merck) with a linear gradient of MeOH and H_2O from 10% to 100% MeOH and a flow rate of 4 mL/min over 3 days, which yielded 23 fractions. All of the fractions were analysed by HPLC and fractions 13 to17 were selected for further purification. The final fractionation steps were performed using semi-preparative HPLC on a µBondapak® C_{18} column (10 µm, 100 × 25 mm i.d.; Waters) using MeOH-H_2O-0.05% TFA as eluents for isocratic elution with a flow rate of 10 mL/min. Fraction 13 (54.5 mg) yielded **5** (2.3 mg) (MeOH 35%, flow rate 10 mL/min, UV 210 nm); fraction 14 (59.4 mg) yielded **7** (2.2 mg), **4** (4.1 mg), and **3** (3.2 mg), and fraction 15 (121.9 mg) yielded **6** (2.9 mg), **8** (2.2 mg), **9** (2.3 mg), and **10** (5.7 mg) (MeOH 40% isocratic conditions); fraction 16 (61.7 mg) yielded **11** (1.2 mg) (MeOH 50% isocratic conditions); fraction 17 (175.0 mg) yielded **2** (2.6 mg) and **12** (49.1 mg), and finally fraction 18 (131.0 mg) yielded **1** (7.0 mg) and **12** (12.4 mg) (MeOH 55% isocratic conditions).

LC-DAD-MS analysis

LC-DAD-MS data were obtained with an Agilent HPLC 1100 series system consisting of an autosampler, high-pressure mixing pump, and DAD detector connected to a Finnigan MAT LCQ ion trap mass spectrometer equipped with a Finnigan atmospheric pressure chemical ionization (APCI) interface. The following conditions were used: HPLC conditions: symmetry shield C-18 column (5 µm, 250 × 2.1 mm i.d.; Waters), solvent system: A-MeCN with 0.25% acetic acid, B–H_2O with 0.25% acetic acid; gradient mode: 5 to 100% of A in 65 min; flow rate: 1 mL/min; injection volume 10 µL; and sample concentration 10 mg/mL in MeCN. The UV traces were recorded at 210 and 254 nm, and UV-DAD spectra were recorded between 190 and 600 nm (2 nm steps). APCI-MS conditions: capillary voltage 30 V, capillary temperature 200 °C, source voltage 4.5 kV, and source current 80 µA, with nitrogen as the sheath gas in positive ion mode. Spectra (180–1200 mu) were recorded every 3 s.

7-Methoxy ophioglonin (**1**): UV (MeOH) λ_{max} (log ε) 259 (4.91), 383 (4.68) nm; UV (MeOH+NaOMe) λ_{max} 266, 426 nm; UV (MeOH + NaOAc) λ_{max} 265, 422 nm; UV (MeOH + NaOAc + H_3BO_3) λ_{max} 263, 400 nm; UV (MeOH + $AlCl_3$) λ_{max} 275, 451 nm; UV (MeOH + $AlCl_3$ + HCl) λ_{max} 264, 423 nm; IR ν_{max} (KBr) 3225 (-OH), 1660 (C=C), 1622 (C=O), 1590, 1502, 1448, 1350, 1288, 1207, 1151 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 3.86 (3H, s, OCH_3), 5.20 (2H, s, CH_2-7′), 6.35 (1H, d, J = 1.7 Hz, H-6), 6.69 (1H, d, J = 1.7 Hz, H-8), 6.92 (1H, d, J = 8.1 Hz, H-3′), 7.22 (1H, d, J = 8.1 Hz, H-2′), 12.78 (1H, s, OH-5); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 55.9 (CH_3,OCH_3), 62.9 (CH_2, C-7′), 92.3 (CH, C-8), 97.6 (CH, C-6), 105.3 (C, C-4a), 114.1 (CH, C-2′), 114.7 (CH, C-3′), 115.2 (C, C-1′), 119.8 (C, C-6′), 133.0 (C, C-3), 140.6 (C, C-5′), 149.2 (C, C-2), 149.7 (C, C-4′), 155.6 (C, C-8a), 161.1 (C, C-5), 164.6 (C, C-7), 174.0 (C, C-4); APCI-MS *m/z* 329 $[M + H]^+$; EI-MS *m/z* (rel. int.) 328 $[M]^+$ (95), 300 $[M-28]^+$ (100), 285 $[M-43]^+$ (27); HRESI-MS *m/z* 329.0660 $[M + H]^+$ (calcd. for $C_{17}H_{13}O_7$, 329.0655).

5-Hydroxy-7-methoxy-2-(3,4-dihydroxy)phenoxychromone (**2**): UV (MeOH) λ_{max} (log ε) 286 (4.30), 300 (sh) nm; UV (MeOH + NaOMe) λ_{max} 283, 340 (sh) nm; UV (MeOH + $AlCl_3$) λ_{max} 299, 350 (sh) nm; UV (MeOH + $AlCl_3$ + HCl) λ_{max} 298, 351 (sh) nm; IR ν_{max} (KBr) 3427 (-OH), 1671 (C=C), 1611 (C=O), 1501, 1443, 1384, 1361, 1206, 1207, 1144 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 3.85 (3H, s,OCH_3), 5.13 (1H, s, H-3), 6.40 (1H, d, J = 1.8 Hz, H-6), 6.63 (1H, dd, J = 8.6, 2.7 Hz, H-6′), 6.63 (1H, d, J = 1.8 Hz, H-8), 6.73 (1H, d, J = 2.7 Hz, H-2′), 6.83 (1H, d, J = 8.6 Hz, H-5′), 12.84 (1H, s, OH-5); ^{13}C NMR (DMSO-d_6,125 MHz) δ 56.0 (CH_3, OCH_3), 87.1 (C-3), 92.7 (CH, C-8), 98.2 (CH, C-6), 102.9 (C, C-4a), 108.2 (CH, C-2′), 110.8 (CH, C-6′), 115.8 (CH, C-5′), 143.0 (C, C-1′), 144.3 (C, C-4′), 146.3 (C, C-3′), 154.7 (C, C-8a), 161.1 (C, C-5), 164.8 (C, C-7), 168.0 (C, C-2), 183.1 (C, C-4); APCI-MS *m/z* 317 $[M + H]^+$; EI-MS *m/z* (rel. int.) 316 $[M]^+$ (100), 301 $[M-15]^+$ (17), 288 $[M-28]^+$ (14), 167 $[A_1 + H]^+$ (22); HRESI-MS *m/z* 317.0660 $[M + H]^+$ (calcd. for $C_{16}H_{13}O_7$, 317.0655).

5-Hydroxy-7-methoxy-2-(3′-O-β-glucopyranosyl,4′-hydroxy)phenoxychromone (**3**): UV (MeOH) λ_{max}(log ε) 282 (4.35), 305 (sh) nm; UV (MeOH + $AlCl_3$) λ_{max} 300, 347 (sh) nm; UV (MeOH + $AlCl_3$ + HCl) λ_{max} 300, 347 (sh) nm; IR ν_{max} (KBr) 3428 (-OH), 1658 (C=C), 1620 (C=O), 1498, 1437, 1224, 1026, 954 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 3.16 (1H, t, J = 8.9 Hz, H-4″), 3.27 (1H, m, H-3″), 3.30 (1H, m, H-2″), 3.33 (1H, m, H-5″), 3.44 (1H, m, H-6″ a), 3.66 (1H, dd, J = 11.7, 2.1 Hz, H-6″b), 3.85 (3H, s, OCH_3), 4.80 (1H, d, J = 7.2 Hz, H-1″), 5.17 (1H, s, H-3), 6.40 (1H, d, J = 2.2 Hz, H-6), 6.64 (1H, d, J = 2.2 Hz, H-8), 6.89 (1H, dd, J = 8.7, 2.6 Hz, H-6′), 6.92 (1H, d, J = 8.7 Hz, H-5′), 7.15 (1H, d, J = 2.6 Hz, H-2′), 12.84 (1H, s, OH-5); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 56.1 (CH_3, OCH_3), 60.6 (CH, C-6″), 69.7 (CH, C-4″), 73.2 (CH, C-2″), 75.8 (CH, C-3″), 77.0 (CH, C-5″), 87.3 (CH, C-3), 92.7 (CH, C-8), 98.2 (CH, C-6), 101.5 (CH, C-1″), 102.9 (C, C-4a), 109.3 (CH, C-2′), 114.4 (CH, C-6′), 116.2 (CH, C-5′), 142.8 (C, C-1′), 145.5 (C, C-4′), 145.7 (C, C-3′), 154.7 (C, C-8a), 161.1 (C, C-5), 164.5 (C, C-7), 168.0 (C, C-2), 182.9 (C, C-4); APCI-MS *m/z* 479 $[M + H]^+$, 317 $[(M + H) - Glc]^+$; EI-MS *m/z* (rel. int.) 316 $[M]^+$ (100), 301 $[M-15]^+$ (37), 167 $[A_1 + H]^+$ (21); HRESI-MS *m/z* 479.1158 $[M + H]^+$ (calcd. for $C_{22}H_{23}O_{12}$, 479.1190).

Preparation of acetyl derivatives (1a and 2a)

1 or **2** (2 mg) was dissolved in pyridine (0.2 mL); an excess of acetic anhydride (3 mL) was added, and the mixture stirred for 24 h at room temperature. The mixture was washed with a 1% $NaHCO_3$ solution and evaporated *in vacuo* to yield **1a** (2.10 mg, 76%) or **2a** (2.17 mg, 77.5%).

Compound (**1a**): ^1H NMR ($CDCl_3$, 500 MHz) δ 2.32 (3H, s, OAc), 2.35 (3H, s, OAc), 2.44 (3H, s, OAc), 3.92 (3H, s, OMe), 5.12 (2H, s, H-7′), 6.62 (1H, d, J = 2.3 Hz, H-6), 6.86 (1H, d, J = 2.3 Hz, H-8), 7.30 (1H, d, J = 8.4 Hz, H-3′), 7.70 (1H, d, J = 8.4 Hz, H-2′); APCI-MS *m/z* 455 $[M + H]^+$, 413 $[M + H - 42]^+$, 371 $[M + H-42-42]^+$, 329 $[M + H-42-42-42]^+$.

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Compound (**2a**): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.30 (3H, s, OAc), 2.31 (3H, s, OAc), 2.40 (3H, s, OAc), 3.89 (3H, s, OMe), 5.44 (1H, s, H-3), 6.61 (1H, d, J = 2.5 Hz, H-6), 6.77 (1H, d, J = 2.5 Hz, H-8), 7.07 (1H, dd, J = 8.6, 2.7 Hz, H-6′), 7.09 (1H, d, J = 2.6 Hz, H-2′), 7.26 (1H, d, J = 8.6 Hz, H-5′); APCI-MS *m/z* 443 $[M + H]^+$, 401 $[M + H-42]^+$, 359 $[M + H-42-42]^+$, 317 $[M + H-42-42-42]^+$.

Enzymatic hydrolysis

Hydrolysis was performed on **3** to determine the nature of its hexose. The reaction was carried out on 0.5 mg of product, once with 4 mg (28 units) of β-glucosidase from *Escherichia coli* (Sigma-Aldrich Chemical Co.) and once with 4 mg (20 units) of β-galactosidase from almonds (Sigma-Aldrich Chemical Co.), dissolved in 5 mL of acetate buffer pH 5 and incubated under agitation at 37 °C for 48 h. Hydrolysis occurred only with the glucosidase. The hydrolysis products were then compared on a TLC plate with the original glycoside **3** and the aglycone **2**. TLC eluent: $CHCl_3$-MeOH-H_2O (65 : 35 : 5). TLC support: Merck silica gel 60 F254 aluminium.

Human cell lines

MCF-7 cells (purchased from ATCC) and Ishikawa cells (purchased from ECACC) were cultured as previously described [10]. MCF-7 : D5 L cells, a clone of MCF-7 cells stably transfected with reporter plasmid pERE-βGlo-Luc, and HEK : ERβ cells, a clone of HEK-293 cells stably co-transfected with an estrogen receptor β (ERβ)-expression plasmid and reporter plasmid pERE-tk-Luc, were generated and cultured as previously described [9, 10].

ER binding assay

The relative binding affinity (RBA) values of **1, 2**, and **4** for isolated recombinant human estrogen receptor (ER) α and β were assessed as previously described [10]. The purity of compounds **1, 2**, and **4** were estimated to be > 95% by HPLC-UV and ^{1}H NMR. Briefly, 17β-estradiol (estradiol; 98% purity; Sigma-Aldrich) and the test compounds were dissolved in DMSO to form 10 mM stock solutions. Serial 1 : 3 dilutions of the stock solutions in DMSO were also prepared. Subsequently, 1 : 1000 dilutions of the solutions in DMSO were prepared in the assay buffer (contains 0.1% DMSO) and were tested for their ability to inhibit the binding of 1 nM ES2 (a fluorescein-labelled estrogen from Invitrogen) to the isolated human ERα or ERβ (Invitrogen) by 50% (IC_{50}), as assessed using a Beacon 2000 fluorescence polarization system (Invitrogen). The RBAα and RBAβ values (mean ± SEM of at least three independent experiments) were calculated by [(IC_{50} estradiol/IC_{50} test compound) × 100]. The RBAα and RBAβ values of estradiol were set equal to 100.

Cell proliferation assay

The effect of estradiol and compounds **1, 2**, and **4** on the proliferation of MCF-7 cells was determined as previously described [10]. Briefly, cells were plated in 96-well flat-bottomed microplates at a density of 8000 cells/well in phenol-red-free culture medium supplemented with 5% DCC-FBS. The following day, serial 1 : 1000 dilutions of estradiol or the test compounds, prepared as described above, were added and replenished every 48 h. Cell growth was assayed 6 days later using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Cells exposed only to 0.1% DMSO served as the control. The effect of estradiol and compounds **1, 2**, and **4** on the proliferation of Ishikawa cells was determined by using the same approach, with the exception that cell growth was assessed 3 days after plating. The proliferation of MCF-7 and Ishikawa cells was not affected by DMSO at concentrations ≤ 0.3%

Luciferase gene expression assay

The induction of estrogen response element (ERE)-dependent luciferase gene expression by estradiol and compounds **1, 2**, and **4** was assessed using MCF-7 : D5 L and HEK : ERβ cell lines as previously described [9, 10]. Briefly, the cells were plated in 96-well, flat-bottomed microplates at a density of 10 000 cells/well in phenol-red-free DMEM supplemented with 5% DCC-FBS, and 72 hours later the cells were treated for 16 h with serial 1 : 1000 dilutions of estradiol or the test compounds prepared in fresh medium as described above. Compound stimulation of gene expression was antagonised using fulvestrant (ICI 182, 780; > 99% purity; Tocris Bioscience) at 10 µM. Following the treatment, luciferase activity was assayed using the commercial Steady-Glo Luciferase Assay System (Promega). Cells exposed only to DMSO served as control. Cell viability and assay sensitivity were not affected by DMSO at concentrations ≤ 0.3%.

Alkaline phosphatase expression assay

Induction of alkaline phosphatase (AlkP) expression in Ishikawa cells by estradiol and compounds **1, 2**, and **4** was assessed as previously described [10]. Briefly, the cells were plated in 96-well flat-bottomed microculture plates at a density of 12 000 cells per well in phenol-red-free medium supplemented with 5% DCC-FBS. The following day, the cells were treated for 72 h with serial 1 : 1000 dilutions of estradiol or the test compounds in fresh medium (prepared as described above), prior to assaying the cell extracts for AlkP activity. Cells exposed only to 0.1% DMSO served as controls. Cell viability and assay sensitivity were not affected by DMSO at concentrations ≤ 0.3%.

Statistics

The significance of the induction of cell proliferation and luciferase and AlkP expressions by estradiol and the test compounds was determined using Student's t-test.

Results and Discussion

In our on-going search for new estrogenic compounds, we have investigated the leaves of *P. pterocarpum* based on chemotaxonomic considerations. The test compounds were selected for biological evaluation based on the structural features known to be associated with estrogen-like activity. To obtain more information on the chemical composition of the dichloromethane extract and further identify the constituents of *P. pterocarpum*, LC-DAD-APCI-MS analysis was performed. The DAD-UV spectra revealed the presence of numerous phenolic compounds and were characteristic of flavanones, dihydroflavonols, flavones, or flavonols (**Fig. 1**) [8]. Based on the molecular weights deduced from the LC-APCI-MS profiling of the extract, the majority of these polyphenols were present as free aglycones with the exception of **3**, which exhibited an $[M + H]^+$ at *m/z* 479 and a fragment at *m/z* 317 $[M-162 + H]^+$, suggesting the presence of a hexose residue [8]. These UV and MS data however were not sufficient for an unambiguous identification of the polyphenols. To fully characterise these compounds and assess their biological activities, the extract was first fractionated using reversed phase MPLC and finally purified by semi-preparative HPLC. This purification resulted in the isolation of 12 pure compounds (**1**–**12**) (**Fig. 2**).

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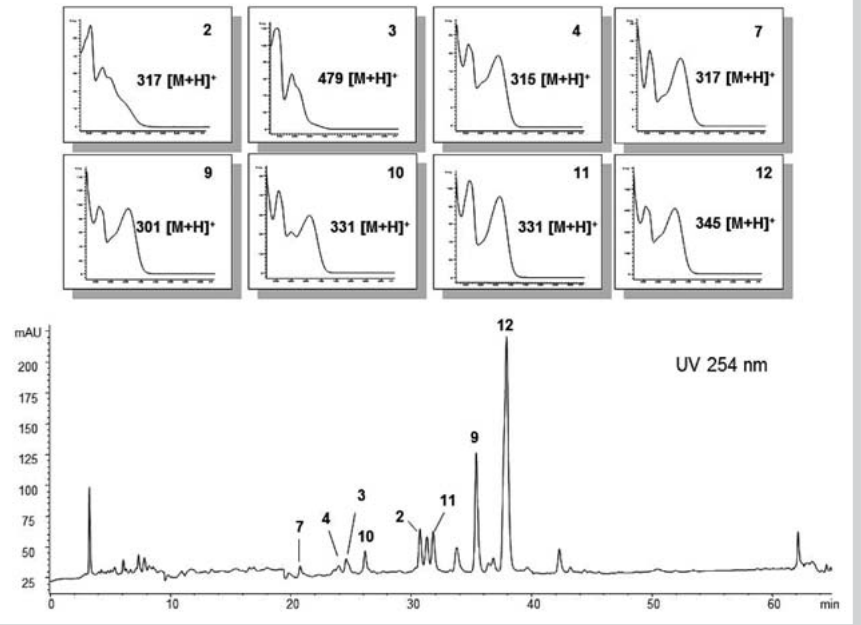

Fig. 1 LC-DAD-APCI-MS analysis of the CH_2Cl_2 extract of *P. pterocarpum*.

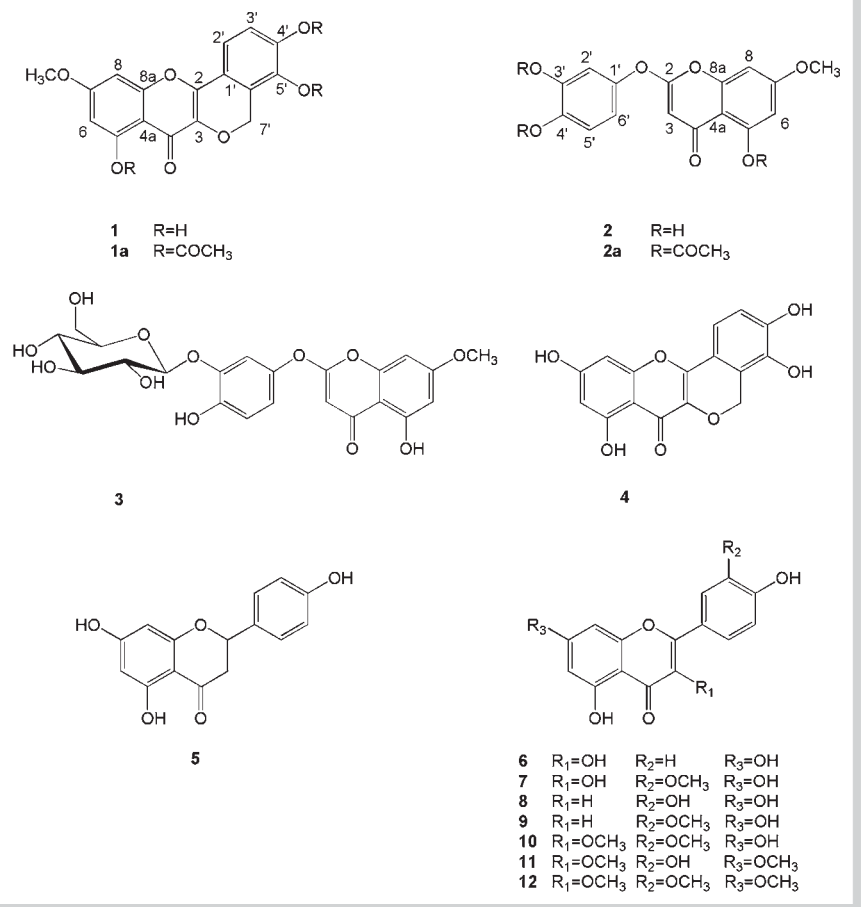

Fig. 2 Chemical structures of the compounds isolated from the leaves of *Peltophorum pterocarpum*.

Compound **1** was isolated as a brownish-yellow amorphous powder and presented an IR spectrum with absorptions at 1660 (C=C) and 1622 cm^{-1} (C=O) and UV maxima at 259 and 383 nm, suggesting the presence of a flavone nucleus [8]. The high-resolution ESI-MS displayed a $[M + H]^+$ ion at *m/z* 329.0660, in agreement with the molecular formula $C_{17}H_{13}O_7$ (calcd. for $C_{17}H_{13}O_7$, 329.0655).

The ^1H NMR spectrum of **1** revealed a pair of *meta*-coupled protons at δ_H 6.35 and δ_H 6.69 (J = 1.7 Hz), which were attributed to the protons H-6 and H-8 in ring A and a second aromatic system consisting of a pair of *ortho*-coupled protons at δ_H 7.22 and δ_H

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6.92 (J = 8.1 Hz), which were attributed to H-2′ and H-3′ in ring B. Additionally, a singlet at δ_H 12.78 was observed corresponding to a hydrogen-bonded hydroxyl group at C-5. A singlet at δ_H 5.20 (2H, s, CH_2-7′) suggested a pyran ring [11–13]. The HMBC experiment confirmed these hypotheses as the methylene protons (δ_H 5.20) were coupled to C-3 (δ_C 133.0), C-1′ (δ_C 115.2), C-5′ (δ_C 140.6), and C-6′ (δ_C 119.8). Moreover, the ^{1}H NMR spectrum of **1** indicated a methoxyl group at δ_H 3.86 (3H, *s*), which was confirmed by the methoxyl carbon signal at δ_C 55.9 in the ^{13}C NMR spectrum. The position of this methoxyl in C-7 was assigned by long-range HMBC correlations of its protons with C-7 (δ_C 164.6). NOESY experiments further confirmed this relationship with correlations between the methoxyl group and H-6 and H-8. To confirm the structure, acetylation of **1** was undertaken and yielded the acetylated derivative **1a**. The APCI-MS spectrum of **1a** showed an ion at *m/z* 455 $[M + H]^+$, corresponding to the addition of three acetyl groups, which was confirmed by ^{1}H NMR (three singlets at δ_H 2.32, 2.35, and 2.44). Based on these results and the comparison with **1a, 1** was identified as a new peltogynoid and named 7-methoxy-ophioglonin.

Compound **2** was obtained as a fine brown powder and exhibited a UV spectrum with a maximum at 286 nm and a shoulder at 300 nm [8]. The high-resolution ESI-MS analysis presented a $[M + H]^+$ ion at *m/z* 317.0660, suggesting a molecular formula of $C_{16}H_{12}O_7$ (calcd. for $C_{16}H_{13}O_7$, 317.0655). The EI-MS analysis exhibited an $[M]^+$ at *m/z* 316 and two fragments at *m/z* 301 $[M-15]^+$ and 167 $[A + 1]^+$ [14]. The first fragment suggested the loss of a methyl group, and the second at *m/z* 167, was attributed to a typical retro-Diels-Alder fragment and indicated hydroxyl and methoxyl groups on ring A [14]. The ^{1}H NMR spectrum of **2** exhibited two signals at δ_H 6.40 and 6.63, attributed to the protons at H-6 and H-8, respectively. It also displayed an AMX system at δ_H 6.63, 6.83, and 6.73, for the aromatic protons H-6′, H-5′, and H-2′, respectively. This observation was confirmed by COSY and HMBC correlations. Ring B was linked to the C ring via an oxygen atom, because no correlations were observed between the protons at H-2′ and H-6′ and C-2 in the HMBC experiment [15]. The chemical shift of C-2 at δ_C 168.0 corresponded well with published values for this type of compound and reinforced the structural assignment. The NOESY correlations between H-2′, H-6′, and H-3 (δ_H 5.13) confirmed the position of the aromatic protons in ring C. The ^{1}H NMR spectrum also showed a methoxyl group at δ_H 3.85 (3H, *s*), correlated with δ_C 164.8 (C-7) in HMBC. Acetylation of **2** yielded the acetylated derivative **2a**. The APCI-MS spectrum of **2a** showed a $[M + H]^+$ ion at *m/z* 443 indicating the addition of three acetyl groups, and this was confirmed by ^{1}H NMR (δ_H 2.30, 2.31, and 2.40). Based on the spectral evidences, this new compound was characterized as 5-hydroxy-7-methoxy-2-(3′,4′-dihydroxy)-phenoxychromone and was named pterophenoxychromone.

Compound **3** was isolated as a brownish amorphous powder. Its UV spectrum was very similar to that of **2,** with a maximum at 282 nm and a shoulder at 305 nm. The high-resolution ESI-MS analysis revealed an $[M + H]^+$ at *m/z* 479.1158, suggesting $C_{22}H_{22}O_{12}$ as the molecular formula (calcd. for $C_{22}H_{23}O_{12}$, 479.1190). An ion at *m/z* 317.0619, revealed the presence of a hexose moiety $[M + H-162]^+$. In the ^{1}H-NMR as for **2**, a singlet was observed at δ_H 5.16 ppm which is characteristic for a 2-phenoxychromone [14]. All the ^{1}H- and ^{13}C NMR signals and 2D experiments correlations revealed the same substitution pattern as for **2**. The other signals were attributed to the sugar moiety. The anomeric proton H-1″ signal at δ_H 4.80 exhibited a coupling constant of J = 7.2 Hz characteristic of a *β*-hexose. An HMBC correlation between H-1″ and δ_c 145.7 indicated that the hexose was linked at C-3′. To identify the sugar moiety, two different enzymatic hydrolyses were undertaken using *β*-D-glucosidase and *β*-D-galactosidase [16], and the products were analysed by TLC. The enzymatic hydrolysis only occurred with the *β*-D-glucosidase and confirmed the presence of a *β*-D-glucose. Based on these data, compound **3** was identified as new natural product characterised as 5-hydroxy-7-methoxy-2-(3′-O-*β*-D-glucopyranosyl,4′-hydroxy)-phenoxychromone and was named pterophenoxychromone-3′-O-glucoside.

Nine other known flavonoids were isolated and identified from the leaves of *P. pterocarpum*, including the peltogynoid ophioglonin (**4**) [11], the flavanone naringenin (**5**) [17], the flavonols kaempferol (**6**) [18] and isorhamnetin (**7**) [2], and the flavones luteolin (**8**) [18], chrysoeriol (**9**) [18], 3,3′-dimethylquercetin (**10**) [19], 3,7-dimethylquercetin (**11**) [19], and pachypodol (**12**) [20] (**Fig. 2**).

The phytochemical investigation led to the identification of five flavonols, two flavones, and one flavanone. These three classes of compounds are characteristic for the *Peltophorum* genus and have already been reported to display estrogen-like activities [21]. However, limited information was available for the estrogenic activity of peltogynoids and phenoxychromones. Because these compounds possess structural features that are distinct from those of flavonoids, they were further investigated to determine their estrogenic potential. Compounds **1, 2**, and **4** were selected for this experiment, while **3** was excluded because it was the glycosidic form of **2**.

The steroidal hormone 17*β*-estradiol (E_2) controls cell growth, differentiation, survival, and homeostasis through two ER subtypes, ER*α* and ER*β*. The hormone is known to change the conformation of ER in a manner that allows a receptor homodimer or heterodimer to bind to an **e**strogen **r**esponse **e**lement (ERE) in the promoter region of estrogen target genes and regulate transcription by recruiting other transcription factors (cofactors) [22]. The estrogenic activity of a specific ER ligand is therefore largely dependent on the structure of the promoter and the cofactor complement of the cell as well as the ER subtype(s) involved [22]. ER*α* and ER*β* are known to bind a diversity of relatively flat compounds that happen to possess two hydroxyl groups with an O-O distance in the range of 9.7–12.3 Å, which allows them to mimic the 3- and 17*β*-OH groups of estradiol [10, 23]. For compounds **1, 2**, and **4**, the O-O distances between one or more pairs of distant OH groups were estimated to lie in this range, indicating that these compounds may display ER binding activity. The relative binding affinity (RBA) of **1, 2**, and **4** for ER*α* and ER*β* compared to estradiol was determined by a fluorescence polarization assay using isolated recombinant receptor subtypes [10]. The RBA values of **1, 2**, and **4** for ER*α* (RBA*α*) were found to be 0.51 ± 0.10, 0.41 ± 0.04, and 0.17 ± 0.03, respectively, whereas the corresponding RBA*β* values were 0.22 ± 0.02, 0.29 ± 0.07, and 0.27 ± 0.04, suggesting that all three compounds bind ER*α* and ER*β* with similar affinity and a lack of selectivity. Compound **1**, with two pairs of distant OH groups (5/4′ and 5/5′, with a distance of 10.3 and 9.0 Å, respectively), likely binds to ER through the 5/4′ pair, which has a distance within the optimal range for binding to the receptor (9.7–12.3 Å). Compound **2**, with two pairs of distant OH groups (5/3′ and 5/4′, with a distance of 9.5 and 10.1 Å, respectively), may bind to ER predominantly through the 5/4′ pair. Compound **4**, with three pairs of distant OH groups with a distance within the optimal range (5/4′, 7/3′, and 7/4′ with a dis-

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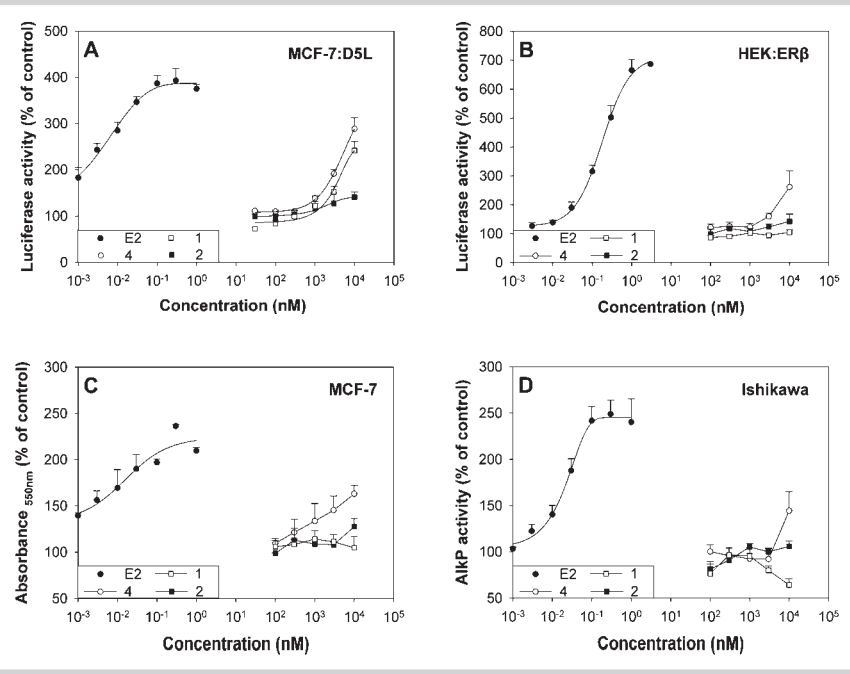

Fig. 3 Estrogenic activities of compounds **1, 2**, and **4** compared to that of estradiol (E_2). **A, B** Dose-response curves of the induction of luciferase expression in MCF-7 : D5 L cells (**A**) and in HEK : ER*β* cells (**B**) by estradiol and the test compounds. **C, D** Induction of the proliferative response of MCF-7 cells (**C**) and the alkaline phosphatase (AlkP) response of Ishikawa cells (**D**) caused by estradiol and the test compounds. Data, expressed as the % of the response of cells treated only with vehicle (0.1 %DMSO), are presented as the mean ± SEM of triplicate measurements in three independent experiments.

tance of 10.3, 10.5, and 10.7 Å, respectively), presumably could bind to ER through any one of the three pairs.

The ability of **1, 2**, and **4** to induce ERE-dependent gene expression through ER*α* was assessed using MCF-7 : D5 L cells, a clone of MCF-7 human breast cancer cells that was generated by stably transfecting the cells with a reporter plasmid comprising an ERE upstream of a minimal promoter (of the globin gene) and the cDNA of luciferase [10]. In these cells, estradiol displayed a ~ 4-fold induction of luciferase expression at concentrations ≥ 0.1 nM (**Fig. 3A**), in accordance with previous findings [9, 10]. Treatment of MCF-7 : D5 L cells with increasing concentrations of **1, 2**, and **4** induced luciferase expression in a manner that was strictly ERα-dependent, as assessed using the ER destabiliser fulvestrant (**Fig. 3A** and data not shown). At 10 µM concentration, **2** was weakly effective but **1** and **4** displayed high activity. The ability of **1, 2**, and **4** to induce ERE-dependent gene expression through ER*β* was assessed using HEK : ER*β* cells, a clone of the ER-less HEK-293 human embryonic kidney cells that was generated by stably transfecting the cells with an ER*β* expression vector as well as a reporter plasmid comprising an ERE, a minimal promoter (of thymidine kinase), and the cDNA of luciferase [10]. In these cells, estradiol displayed a ~ 7-fold induction of luciferase expression at concentrations ≥ 1 nM (**Fig. 3B**), in accordance with previous findings [10]. Treatment of HEK : ER*β* cells with increasing concentrations of the three compounds revealed that while **4** displayed a non-significant ($p \geq 0.05$; t-test) induction of luciferase expression, **1** and **2** were totally ineffective (**Fig. 3B**). Thus, it appears that **1** and **4** are essentially active only through ERα, and **2** through neither form of ER. Because **1, 2**, and **4** bind to ERα and ER*β* with similar affinities, a lack of induction efficacy through ERα and/or ER*β* may be taken to indicate a ligand-induced receptor conformation unable to recruit cofactors and thus activate ERE-dependent transcription [22]. Because the ligand binding pocket of ER*β* is considerably smaller than ERα [23], relatively bulky ER ligands may act as agonists through the latter receptor but as antagonists through the former by stabilising a conformation that allows coactivator recruitment to ERα but not to ER*β* [24].

MCF-7 cells are known to cease proliferating following transfer to estrogen-free media supplemented with foetal bovine serum (FBS) treated with dextran coated charcoal (DCC-FBS) to remove endogenous estrogens. Estradiol is known to stimulate the proliferation of these cells primarily through ERα [25]. **Fig. 3C** shows that **4** stimulated the proliferation of MCF-7 cells significantly ($p \leq 0.05$; t-test), whereas both **1** and **2** were ineffective up to 10 µM. Taken together, the data of **Figs. 3A** and **C** suggest that **1** changes the conformation of ERα so that it can induce ERE-dependent gene expression but not cell proliferation. Notably, we have previously reported that some deoxybenzoins can exhibit a similar degree of dissociation between their gene expression and cell proliferation efficacies [10]. Interestingly, the biased behaviour of **1** with breast cancer cells is nicely complemented by its inability to significantly stimulate ($p \geq 0.05$; t-test) the induction of alkaline phosphatase (AlkP) activity in Ishikawa human endometrial adenocarcinoma cells (**Fig. 3D**). Induction of AlkP by estrogens and phytoestrogens is reportedly a measure of their estrogenic activity [25]. The maximum AlkP activity observed with estradiol at concentrations ≥ 0.1 nM, was drastically reduced in the presence of **1** at 10 µM, while this was not the case in the presence of **2** and **4** (**Fig. 4**), suggesting that unlike the latter, **1** strongly antagonises estradiol induction of AlkP expression in Ishikawa cells. The lower AlkP induction efficacy of estradiol in the presence of **1** was not due to a change in the number of viable cells, as determined using MTT (data not shown), in accordance with previous findings with many but not all of the phytoestrogens at this concentration [26–29]. The effects of estradiol and **1, 2**, and **4** were fully suppressed by the ER destabiliser fulvestrant (**Fig. 4**), suggesting that they were strictly mediated by ER. In light of these findings one may propose that **1** constitutes a safe cancer chemopreventive agent because it cannot stimulate the proliferation of breast and endometrial cancer cells, but it can display ERα-mediated activation of gene expression. Notably, ERα-specific agonists such as **1** were found to be as efficacious as estradiol in preventing ovariectomy-induced loss of bone mineral density [30].

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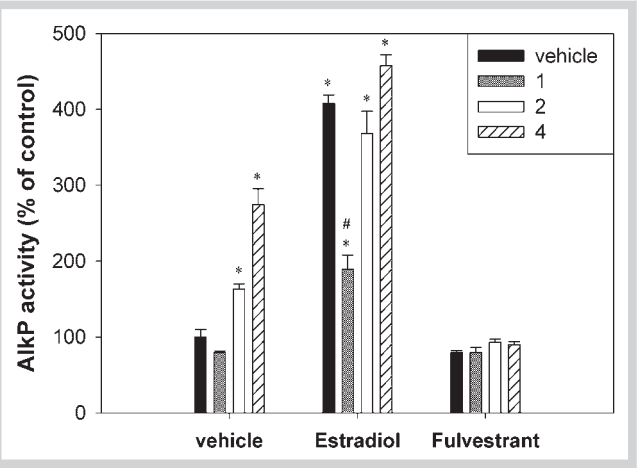

Fig. 4 Effect of compound **1, 2**, or **4** (10 µM) or the vehicle (0.2% DMSO) on the alkaline phosphatase (AlkP) expression of Ishikawa cells growing in the presence of estradiol (0.1 nM), fulvestrant (1 nM), or the vehicle. Data (% of the vehicle) are the mean ± SEM. * $p < 0.05$ vs. vehicle, # $p < 0.05$ vs. effect of estradiol or fulvestrant in the presence of the vehicle.

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Conflict of Interest

None. The authors certify that there is no conflict of interest with any organization, financial or other, regarding the material discussed in the manuscript.

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