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Bioactive Constituents of *Indigofera spicata*

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Abstract

Four new flavanones, designated as (+)-5-deacetylporpurin (**1**), (+)-5-methoxypurpurin (**2**), (2*S*)-2,3-dihydrothephroglabin (**3**), and (2*S*)-2,3-dihydrothephroapollin C (**4**), together with two known flavanones (**5** and **6**), three known rotenoids (**7–9**), and one known chalcone (**10**) were isolated from a chloroform-soluble partition of a methanol extract from the combined flowers, fruits, leaves, and twigs of *Indigofera spicata*, collected in Vietnam. The compounds were obtained by bioactivity-guided isolation using HT-29 human colon cancer, 697 human acute lymphoblastic leukemia, and Raji human Burkitt's lymphoma cell lines. The structures of **1–4** were established by extensive 1D- and 2D-NMR experiments and the absolute configurations were determined by the measurement of specific rotations and CD spectra. The cytotoxic activities of the isolated compounds were tested against the HT-29, 697, Raji and the CCD-112CoN human normal colon cells. Also, the quinone reductase induction activities of the isolates were determined using the Hepa 1c1c7 murine hepatoma cell line. In addition, *cis*-6a-12a-hydroxyrotenone (**7**) was evaluated in an in vivo hollow fiber bioassay using HT-29, MCF-7 human breast cancer, and MDA-MB-435 human melanoma cells.

Indigofera spicata Forssk. (synonyms: *I. endecaphylla* Jacq. ex Poir.; *I. hendecaphylla* Jacq.; *I. parkeri* Baker; *I. pusilla* Lam.), also known as “creeping or trailing indigo”, belongs to the plant family Fabaceae, subfamily Papilionoideae. This species, native to parts of East Africa, Madagascar, the Philippines, and Indonesia, is a ground-cover plant with butterfly-shaped flowers of varied colors, from red to orange to pink.¹ Species of the genus *Indigofera*, such as *I. tinctoria* L., *I. arrecta* Hochst. ex A. Rich., and *I. suffruticosa* Mill., have been cultivated widely to obtain indican, the source of the blue dye, indigo. However, *I. spicata* contains only low concentrations of indican, and is not grown commonly for this

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ASSOCIATED CONTENT

Supporting Information Available. ¹H-, ¹³C- and 2D-NMR spectra of compounds **1–4**, the in vivo hollow fiber assay results and protocol information for *cis*-(6a,12a)-hydroxyrotenone (**7**) are available free-of-charge via the Internet at <http://pubs.acs.org>.

purpose.¹ This species has been used for cover and erosion control in coffee, oil palm, rubber, sisal, and tea plantations.¹ Also, *I. spicata* was used formerly as forage for cattle, but it is no longer used for this purpose due to observed CNS toxicity to animals, which may lead to death.¹ The genus *Indigofera*, distributed worldwide, is the third largest genus in the family Fabaceae (legumes), comprising about 750 species.² Members of this genus have been studied widely resulting in the identification of alkaloids, flavonoids, and rotenoid-type compounds.^{3–8} Previous phytochemical studies on *I. spicata* are limited, but have revealed the presence of the toxic amino acids indospicine and canavanine (arginine inhibitors), and of a further toxic compound, 3-nitropropanoic acid, and a non-toxic galactomannan polysaccharide.^{9–13}

In a continuing effort to isolate anticancer compounds from various natural sources,^{14,15} a chloroform extract from a small-scale collection of the flowers, fruits, leaves, and twigs of *I. spicata*, collected in Vietnam, revealed an IC₅₀ value of 3.4 µg/mL when evaluated against the HT-29 human colon cancer cell line. Due to the cytotoxicity of the initial chloroform extract, a recollection of the combined flowers, fruits, leaves, and twigs of *I. spicata* was obtained, and the chloroform-soluble extract showed IC₅₀ values for HT-29, 697 human acute lymphoblastic leukemia, and Raji human Burkitt's lymphoma cells of 4.3, 2.2, and 5.0 µg/mL, respectively. Bioactivity-guided fractionation of this cytotoxic chloroform extract using these three cell lines was performed leading to the isolation of four new flavanones, (+)-5-deacetylpuirpurin (**1**), (+)-5-methoxypuirpurin (**2**), (2*S*)-2,3-dihydrotephroglabrin (**3**), and (2*S*)-2,3-dihydrotephroapollin C (**4**), together with two known flavanones consisting of (+)-puirpurin (**5**),^{16–18} (2*S*)-7-methoxy-8-(3-methoxy-3-methyl-but-1-enyl)flavanone (**6**),¹⁹ three known rotenoids inclusive of *cis*-(6a, 12a)-hydroxyrotenone (**7**),^{20,21} rotenone (**8**),^{21–23} and tephrosin (**9**),^{21, 24} in addition to a known chalcone, (+)-tephropuirpurin (**10**).²⁵ Also, a semi-synthetic compound, (+)-tephrosone (**11**),¹⁷ was produced from (+)-puirpurin (**5**) and tested biologically. Herein, the isolation and structure elucidation of the new compounds **1–4** and the biological activities of all the isolates and the semi-synthetic compound are reported.

RESULTS AND DISCUSSION

Compound **1** was obtained as a yellow amorphous solid. Its molecular formula was assigned as C₂₁H₂₀O₅ based on the [M + Na]⁺ ion peak at *m/z* 375.1197 (calcd 375.1208) in the HRESIMS. The ¹H NMR spectrum showed typical signals for a flavanone nucleus with resonances at ¹H 5.56 (1H, dd, *J* = 12.8 and 3.0 Hz, H-2_{ax}), 2.91 (1H, dd, *J* = 16.9 and 3.1 Hz, H-3_{eq}), and 3.05 (1H, dd, *J* = 16.9 and 12.9 Hz, H-3_{ax}).²⁶ The large *trans*-diaxial coupling constant (*J*_{2,3}) between H-2 and H-3_{ax} indicated that the C-2 aryl group is equatorial, as found for many natural flavanones.²⁷ Compound **1** gave a dextrorotatory specific rotation (+50.0), the same as reported for (+)-puirpurin (**5**).¹⁸ The relative configurations at H-2 and H-6 were assigned based on the chemical shifts observed at ¹H 6.51 and 3.95, respectively, and by comparison with values reported in the literature for (+)-puirpurin,¹⁷ which was also isolated in the present study as compound **5**. In addition, the coupling constant observed for H-2 was 6 Hz, the same as reported in the literature for **5**.¹⁷ However, an upfield shift of **1** at H-5 (¹H 4.25) was observed when compared with (+)-puirpurin (**5**), (¹H 5.46), consistent with the replacement of the acetoxy group in **5** with a hydroxy group in **1**. The ¹³C NMR spectrum of **1** showed 21 carbon signals, which were classified from the DEPT and HSQC spectra into two methyls, one methylene, eleven methines, and seven quaternary carbons. The complete ¹H- and ¹³C NMR assignments of **1** were made by a combination of ¹H, ¹³C, ¹³C DEPT, COSY, HSQC, HMBC, and NOESY experiments, and comparison with assignments previously reported for (+)-puirpurin (**5**).¹⁷ Compound **1** differed from **5** only in the replacement of the C-5 acetoxy group with a hydroxy group, and was assigned as (+)-5-deacetylpuirpurin.

Compound **2** was obtained as a white powder. Its molecular formula was assigned as $C_{24}H_{24}O_7$ based on the $[M + Na]^+$ ion peak at m/z 447.1439 (calcd 447.1420) in the HRESIMS. The 1H NMR spectrum showed typical signals for a flavanone nucleus with resonances at 1H 5.53 (1H, H-2_{ax}), 2.87 (1H, dd, J = 16.5 and 3.5 Hz, H-3_{eq}), and 2.97 (1H, dd, J = 16.4 and 12.5 Hz, H-3_{ax}).²⁶ The resonance at 1H 5.53 for H-2 was not observed as a doublet of doublets due to an overlapping signal with H-5. Nevertheless, the large coupling constant of 12.5 Hz observed at H-3_{ax} indicated that the C-2 aryl group is equatorial, as observed for compound **1**. Compound **2** gave a dextrorotatory specific rotation (+14.0), the same as **1** and that reported for (+)-purpurin (**5**).¹⁸ The relative configurations assigned for H-2, H-5 and H-6 were based on the proton chemical shifts observed at 1H 6.50 (d, J = 6.3 Hz), 1H 5.50 (d, J = 4.4 Hz), and 1H 3.96 (d, J = 6.6 Hz), respectively, and comparison to those reported for **5**.¹⁷ The singlet observed for H-6, the NOESY correlation of H-6 with the methoxy protons at position 5, and the HMBC correlation of the methoxy protons with C-5 supported the position of the methoxy group at C-5. The 1H - and ^{13}C NMR values of the dihydro-bisfuran moiety observed for compound **2** were very similar to those of **5**, and to analogous data of a flavone analogue of **2** with the trivial name of enantiomultijugin, previously isolated from *Tephrosia vicioides* Schtdl.²⁸ The ^{13}C NMR spectrum of **2** showed 24 carbon signals, which were classified from DEPT and HSQC data into four methyls, one methylene, ten methines, and nine quaternary carbons. Complete 1H - and ^{13}C NMR assignments of **2** were made by a combination of 1D- and 2D-NMR experiments, and comparison of the NMR values reported for **5** and enantiomultijugin.^{17,28} The only difference of compound **2** when compared to **5** was found to be a methoxy group substituted at position C-5. Thus, compound **2** was assigned as (+)-5-methoxypurpurin.

Compound **3** was obtained as a white powder. Its molecular formula was determined as $C_{22}H_{20}O_5$, based on the $[M + H]^+$ ion peak at m/z 365.1390 (calcd 365.1389) in the HRESIMS. The 1H NMR spectrum showed typical signals for a flavanone nucleus with resonances at 1H 5.48 (H-2), 2.88 (H-3_a), and 3.01 (H-3_b).²⁶ The NOESY correlation observed between H-6 and the protons of a methoxy group at C-7, together with the COSY correlation observed for H-5 and H-6, provided evidence for the location of an OCH₃ group at C-7. A flavone analogue of **3**, tephroglabrin, has been previously reported as an isolate from *Tephrosia apollinea* Klotzsch.²⁹ and *Tephrosia purpurea* (L.) Pers.³⁰ It can be proposed that substitution at C-8 in **3** is by a α , β -unsaturated ketone rather than a α -lactone arrangement, another five-membered ring substitution pattern at C-8 reported previously for some flavones, since the ^{13}C NMR values of the ring substituted at C-8 in compound **3** are closely comparable to values reported for tephroglabrin.^{29,30} The (*S*)-absolute configuration at C-2 in **3** was proposed from the levorotatory specific rotation and by CD spectroscopy, which indicated a positive Cotton Effect at 337 (1.72) nm for a $n \rightarrow \pi^*$ absorption band and the negative Cotton Effect at 303 (−2.21) nm for a $\pi \rightarrow \pi^*$ absorption band, typical of a 2*S* flavanone.²⁷ The ^{13}C NMR spectrum of **3** showed 22 carbon signals, which were classified from the DEPT and HSQC NMR spectra into three methyls, one methylene, nine methines, and nine quaternary carbons. The complete 1H - and ^{13}C NMR assignments of **3** were made by a combination of 1D- and 2D-NMR experiments. Since a flavone analogue of **3** has been assigned the trivial name, tephroglabrin,^{29,30} compound **3** was proposed as (2*S*)-2,3-dihydrotephroglabrin.

Compound **4** was obtained as a white powder. Its molecular formula was established as $C_{21}H_{22}O_4$, based on the $[M + Na]^+$ ion peak at m/z 361.1406 (calcd 361.1416) in its HRESIMS. In the 1H NMR spectrum, compound **4** showed typical signals for a flavanone with resonances at 1H 5.49 (H-2), 2.91 (H-3_a), and 3.05 (H-3_b).²⁶ The 1H NMR spectrum also showed doublets at 1H 5.88 (J = 12.5 Hz, H-8) and 6.00 (J = 12.5 Hz, H-7), which were assigned to two *cis*-oriented olefinic protons. The J value of 12.5 Hz is consistent with

a *cis*-arrangement of H-7 and H-8 as reported previously for a flavone analogue of **4**.²⁶ The NOESY correlation observed between H-6 and the methoxy protons together with the COSY correlation observed for H-5 and H-6 gave evidence for the location of the methoxy group at C-7. An (*S*)-absolute configuration at C-2 was evident from the levorotatory specific rotation and appearance of a similar CD curve as observed for **3** and for 2*S* flavanones.²⁷ The ¹³C NMR spectrum of **4** indicated the presence of 21 carbon signals, which were classified from DEPT and HSQC data into three methyls, one methylene, ten methines, and seven quaternary carbons. Complete ¹H- and ¹³C assignments of **4** were made by a combination of 1D- and 2D-NMR experiments, and the comparison of these values with those of a flavone analogue, tephroapollin C, and structurally related prenylated flavanones.^{31,26} Tephroapollin C was isolated previously from *Tephrosia apollinea*²⁶, and other prenylated flavanones similar to **4** have also been obtained from *Tephrosia leiocarpa* A. Gray.³¹ The new compound **4** was assigned as (2*S*)-2,3-dihydrotephroapollin C.

Compounds **3–11** were evaluated for their cytotoxicity against the HT-29 human colon cancer cell line using a sulforhodamine B (SRB) assay (Table 3). Also, the growth inhibition of compounds **1–11** was tested against 697 human acute lymphoblastic leukemia and Raji human Burkitt's lymphoma cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS) assay. Of the compounds tested, only the rotenoids were cytotoxic, with IC₅₀ values less than 10 μM. Numerous reports have documented the cytotoxicity of rotenoids for cancer cells.^{32–36} However, the cytotoxicity of the rotenoids isolated (**7–9**) has not been previously reported in HT-29, 697, or Raji cells, except for one report of the cytotoxicity induced by rotenone in iron-deprived sensitive Raji cells.³² The known rotenoids, *cis*-(6a, 12a)-hydroxyrotenone (**7**) and rotenone (**8**) exhibited potent activity against HT-29 and 697 cells with IC₅₀ values ranging from 0.1 to 0.7 μM, but were less potent for Raji cells (Table 3). Tephrosin (**9**) showed moderate to low growth inhibition in the 697 cells with IC₅₀ values ranging from 5.4 to 9.0 μM, and was inactive (IC₅₀ > 10 μM) against HT-29 and Raji cells (Table 3). The cytotoxicity of tephrosin (**9**) in 697 cells decreased from 5.4 μM at 48 h to 9.0 μM at 72 h (Table 3). Therefore, a propidium iodide (PI) experiment using flow cytometry was conducted simultaneously with the MTS assay to assess cell death and in this way confirm the results obtained for compound **9**. These experiments showed that tephrosin (**9**) has a low cytotoxicity at 10 μM (24% – 28% cell death, as seen from the PI results) and is also cytostatic, as evidenced by a decrease in viability in the MTS data (Figure 1). The selectivity of the most potent cytotoxic compounds (**7** and **8**) was tested using the CCD-112CoN human normal colon cell line. The results indicated that compounds **7** and **8** were highly selective to HT-29 cells (Table 4).

The cancer chemopreventive potential of certain flavanones and rotenoids has been documented widely.^{25,37–42} Accordingly, induction of the phase II enzyme, quinone reductase, was evaluated for compounds **2–11**, using the Hepa 1c1c7 murine hepatoma cell line. Compounds **2–4** and **6–11** were active with CD (the concentration required to double quinone reductase activity) values of less than 10 μM. The most potent compounds were the new flavanone, (+)-5-methoxypurpurin (**2**), and the rotenoid, tephrosin (**9**), which showed greater activity than the positive control, L-sulphoraphane (Table 5). In addition to the high quinone reductase induction activity of the new compound **2**, it also showed low toxicity with high IC₅₀ and CI values (Table 5), and it may represent a promising lead as a cancer chemopreventive agent. The rotenoids, *cis*-(6a, 12a)-hydroxyrotenone (**7**) and rotenone (**8**), showed potent quinone reductase induction activities with low CD values but high toxicities with low IC₅₀ and CI values (Table 5). No report of potential cancer chemopreventive activity has been described for *cis*-(6a, 12a)-hydroxyrotenone (**7**). The new compounds, (2*S*)-2,3-dihydrotephroglabrin (**3**), and (2*S*)-2,3-dihydrotephroapollin C (**4**), showed moderate quinone reductase induction activity with low to moderate toxicities (Table 5). A

close analogue of compound **3**, (+)-tephrorin A, isolated from *Tephrosia purpurea*, has been reported to induce quinone reductase activity.⁴⁰ The known compounds, (+)-tephropurpurin (**10**), and the semi-synthetic compound, (+)-tephrosone (**11**), isolated previously from *Tephrosia purpurea*, moderately induced quinone reductase activity, in agreement with previous reports (Table 5).^{25,40}

Compound **7** had not been tested previously in vivo, so it was evaluated in a hollow fiber assay^{43,44} using colon (HT-29), breast (MCF-7), and melanoma (MDA-MB-435) human cancer cells. The treatment of mice with intraperitoneal (ip) injections of **7** at 5, 10, 15, and 30 mg/kg were found to be lethal, whereas mice treated with doses of 0.5, 1, and 2 mg/kg presented with no noticeable toxicity to mice. A 20% relative net reduction in cell growth was observed for MDA-MB-435 melanoma cells with 2 mg/kg injection of *cis*-(6a, 12a)-hydroxyrotenone (**7**). No effect at any dose was observed in the net reduction of relative percent cell growth for HT-29 cells.

EXPERIMENTAL SECTION

General Experimental Procedures

Optical rotations were recorded on a Perkin-Elmer 343 automatic polarimeter. UV spectra were measured with a Hitachi U-2910 UV/vis spectrometer. CD spectra were measured with a JASCO J-810 spectropolarimeter. IR spectra were obtained on a Thermo Scientific Nicolet 6700 FT-IR spectrometer. NMR spectra were run at room temperature on Bruker Avance DRX-400 or 800 MHz spectrometers, and the data were processed using MestReNova 6.0 software (Mestrelab Research SL, Santiago de Compostela, Spain). Accurate mass values were performed on a Micromass Q-ToF II ESI spectrometer. Sodium iodide was used for mass calibration for a calibration range of *m/z* 100–2000. Column chromatography (CC) was carried out with silica gel (65–250 or 230–400 mesh; Sorbent Technologies, Atlanta, GA). Analytical TLC was conducted on precoated 200 μ m thickness silica gel UV₂₅₄ aluminum-backed plates (Sorbent Technologies). Waters XBridge[®] or SunFire[®] analytical (4.6 $\times$ 150 mm), semi-preparative (10 $\times$ 150 mm), and preparative (19 $\times$ 150 mm) OBD C₁₈ (5 μ m) columns were used for HPLC, as conducted on a Waters system comprised of a 600 controller, a 717 Plus autosampler, and a 2487 dual wavelength absorbance detector. Cytotoxicity assays with 697 and Raji cells were performed using the CellTiter 96 Aqueous Cell Proliferation (MTS) assay (Promega, Madison, WI). Propidium iodide (PI) (BD Biosciences, San Jose, CA) experiments were conducted on a FC500 flow cytometer.

Plant Material

The combined flowers, fruits, leaves, and twigs of *I. spicata* were collected in January 2010 at Nui Chua National Park (11°42.482 N; 109° 11.098 E.) in Southern Vietnam by two of the authors (T. N. Ninh and D. D. Soejarto), who also identified this plant. A voucher specimen (*DDS et al.* 14530) has been deposited in the John G. Searle Herbarium of the Field Museum of Natural History, Chicago, Illinois.

Extraction and Isolation

The air-dried and milled flowers, fruits, leaves, and twigs of *I. spicata* (1.2 kg) were extracted by maceration with MeOH (4 $\times$ 3 L) at room temperature overnight. After removing the solvent under reduced pressure, the combined and concentrated methanol extract (129 g) was suspended in a mixture of 90% MeOH/H₂O (1 L), then partitioned with hexane and CHCl₃, in turn, to afford hexane- (5 g) and CHCl₃ (32 g)-soluble extracts. Bioactivity-guided fractionation was performed on the cytotoxic CHCl₃ extract of the flowers, fruits, leaves, and twigs of *I. spicata* (IC₅₀ values against HT-29, 697, and Raji cells of 4.3, 2.2, and 5.0 μ g/mL, respectively), leading to the isolation of ten compounds (**1–10**).

The cytotoxic CHCl_3 -soluble extract was subjected to CC over silica gel eluted with a CH_2Cl_2 -acetone gradient to afford seven fractions (F1-F7). Fraction F2 was active against HT-29 and 697 cells with IC_{50} values of 16.4 and 6.9 $\mu\text{g/mL}$, respectively, whereas F3 was active against HT-29, Raji, and 697 cells (IC_{50} values of 1.3, 3.2, and 0.5 $\mu\text{g/mL}$, respectively). Fraction F2 (6.8 g) was purified by a silica gel CC eluted with a gradient of hexane-acetone (15:1, 8:1, 0:1), from which only one subfraction, F2.5, was active. Subfraction F2.5 (168 mg), with IC_{50} values of 15.8 and 4.4 $\mu\text{g/mL}$ against HT-29 and 697 cells, respectively, was subjected to HPLC using a preparative RP-18 column and eluted with $\text{CH}_3\text{CN-H}_2\text{O}$ (50:50) to yield compounds **1** (1.4 mg) and **7** (1.3 mg). Fraction F3 (4.7 g) was chromatographed over silica gel by elution with a CH_2Cl_2 -acetone gradient to afford three active subfractions (F3.2, F3.3, and F3.4). Subfraction F3.2 (1.2 g) exhibited IC_{50} values of 14.5, 2.8, and 1.6 $\mu\text{g/mL}$ against Raji, HT-29 and 697 cells, respectively, and was further subjected to silica gel CC using hexane-acetone (15:1, 10:1, 5:1, 0:1) solvent mixtures to obtain compound **5** (61.3 mg) in crystalline form. Subfraction F3.3 (2.2 g), with IC_{50} values of 4.4, 0.4, and 0.3 $\mu\text{g/mL}$ against Raji, 697 and HT-29 cells, respectively, was then subjected to silica gel CC, using hexane-acetone mixtures (8:1, 4:1, 0:1) for elution, to obtain three active subfractions (F3.3.6, F3.3.7, and F3.3.8). Subfraction F3.3.6 (372.9 mg) was further purified by HPLC using a preparative RP-18 column eluted with $\text{CH}_3\text{CN-H}_2\text{O}$ (40:60) to yield compound **8** (17.1 mg). Subfraction F3.3.6.2 (20.3 mg) was further purified by HPLC using a semi-preparative RP-18 column eluted with $\text{MeOH-H}_2\text{O}$ (50:50) to yield compound **3** (9.7 mg). Subfraction F3.3.7 (788 mg) was subjected to silica gel CC using a CHCl_3 - MeOH gradient, from which subfraction F3.3.7.2 (59.8 mg) was purified by HPLC using a semi-preparative RP-18 column eluted with $\text{CH}_3\text{CN-H}_2\text{O}$ (68:32), to yield compound **6** (33.0 mg). Subfraction F3.3.7.3 (298.6 mg) was purified by HPLC using a preparative RP-18 column eluted with $\text{MeOH-H}_2\text{O}$ (52:48) to yield compound **9** (68.9 mg), and a further quantity of compound **7** (105 mg). Subfraction F3.3.8 (28.3 mg) was purified by HPLC using a semi-preparative RP-18 column, eluting with $\text{CH}_3\text{CN-H}_2\text{O}$ (27:73), to yield compound **2** (1.8 mg), and additional compound **7** (1.4 mg). Subfraction F3.4 (1.3 g), with IC_{50} values of 15.2 and 4.7 $\mu\text{g/mL}$ against HT-29, and 697 cells, respectively, was subjected to silica gel CC using a CHCl_3 - MeOH gradient. Of these, subfraction F3.4.2 was purified by HPLC with a semi-preparative RP-18 column, eluting with $\text{MeOH-H}_2\text{O}$ (55:45) to afford compound **10** (2.7 mg) as yellow needles. Subfraction F3.4.2.2 (23.7 mg) was subjected to HPLC purification using a semi-preparative RP-18 column, eluting with $\text{CH}_3\text{CN-H}_2\text{O}$ (25:75), to yield compound **4** (3.1 mg).

(+)-**5-Deacetylporpurin (1)**: yellow amorphous solid; $[\alpha]_D^{20} +50.0$ (c 0.1, CHCl_3); UV (MeOH) λ_{max} (log ϵ) 216 (4.55), 240 (sh, 4.19), 274 (4.06), 310 (sh, 3.69) nm; ECD (CHCl_3) 306 (ϵ -4.14), 330 (2.82) nm; IR (film) ν_{max} 3408, 2920, 2848, 1679, 1610, 1461, 1214, 1090, 1062, 751 cm^{-1} ; ^1H NMR (800 MHz, CDCl_3) and ^{13}C NMR (200 MHz, CDCl_3) data, see Tables 1 and 2; HRESIMS m/z 375.1197 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{21}\text{H}_{20}\text{O}_5\text{Na}$, 375.1208).

(+)-**5-Methoxypurpurin (2)**: white powder; $[\alpha]_D^{20} +14.0$ (c 0.18, CHCl_3); UV (MeOH) λ_{max} (log ϵ) 240 (sh, 4.14), 282 (3.96), 320 (sh, 3.54) nm; ECD (MeOH) 240 (ϵ -3.34), 284 (ϵ -3.15), 329 (1.54) nm; IR (film) ν_{max} 3455, 2930, 2851, 1743, 1676, 1619, 1594, 1458, 1375, 1340, 1230, 1100, 1033, 960, 815, 758, 701 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3) and ^{13}C NMR (100 MHz, CDCl_3) data, see Tables 1 and 2; HRESIMS m/z 447.1439 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{24}\text{H}_{24}\text{O}_7\text{Na}$, 447.1420).

(*2S*)-**2,3-Dihydrotephroglabrin (3)**: white powder; $[\alpha]_D^{20} -24.0$ (c 0.1, CHCl_3); UV (MeOH) λ_{max} (log ϵ) 225 (sh, 4.24), 278 (3.80), 325 (sh, 3.26) nm; ECD (MeOH) 249 (ϵ -1.19), 303 (ϵ -2.21), 337 (1.72) nm; IR (film) ν_{max} 3459, 2962, 2927, 2857, 1695, 1591, 1401, 1432, 1340, 1271, 1201, 1116, 1087, 805, 764, 701 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3)

and ^{13}C NMR (100 MHz, CDCl_3) data, see Tables 1 and 2; HRESIMS m/z 365.1390 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{22}\text{H}_{20}\text{O}_5 + \text{H}$, 365.1389).

(2*S*)-2,3-dihydrotephroapollin **C** (**4**): white powder; $[\alpha]_{\text{D}}^{20}$ -40.0 (c 0.09, CHCl_3); UV (MeOH) λ_{max} (log ϵ) 225 (sh, 4.35), 283 (3.94), 325 (sh, 3.46) nm; ECD (MeOH) 263 (-9.47), 302 (-5.01), 339 (3.92) nm; IR (film) ν_{max} 3440, 2965, 2924, 2653, 2363, 1679, 1591, 1423, 1337, 1277, 1223, 1112, 1084, 799, 704 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3) and ^{13}C NMR (100 MHz, CDCl_3) data, see Tables 1 and 2; HRESIMS m/z 361.1406 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{21}\text{H}_{22}\text{O}_4\text{Na}$, 361.1416).

Semi-synthesis of (+)-Tephrosone (**11**)

Compound **5** (8.0 mg, 0.02 mmol) was dissolved in 1 mL of HPLC grade MeOH, and mixed with LiOH (27 mg, 1.13 mmol) (Aldrich, 98% purity), dissolved in 1 mL of distilled H_2O . The mixture was sealed and left stirring overnight at room temperature. The following day, the solution was partitioned between CHCl_3 and H_2O . The CHCl_3 partition was evaporated under reduced pressure to obtain compound **11** (7.9 mg), $[\alpha]_{\text{D}}^{20}$ $+73.0$ (c 0.1, CHCl_3) (lit.¹⁷ $[\alpha]_{\text{D}}^{20}$ $+157.4$ (c 1%)). The ^1H and ^{13}C NMR values of **11** were comparable to the values reported in the literature for this compound.¹⁷

Cell Culture and Cytotoxicity Assays

The cytotoxicity of the plant CHCl_3 extract, chromatographic fractions, and the purified compounds (**3–11**) was evaluated against the HT-29 human colon cancer cell line using the sulforhodamine B (SRB) assay.⁴⁵ Samples were also examined against the 697 human acute lymphoblastic leukemia and Raji human Burkitt's lymphoma cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS) assay. The 697 and Raji cell lines were cultured as previously described.⁴⁶ The 697 pre-B acute lymphoblastic leukemia cell line was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Braunschweig, Germany), and the Raji human Burkitt's lymphoma was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The density of 697 and Raji cells was adjusted to 200,000 cells/mL. Cells (95 μL /well) and test compounds (5 μL /well), after proper dilution, were placed in a 96-well microplate. Test compounds were initially dissolved in 100% DMSO to a final stock solution of 2 mM. Serial dilutions were made from the stock solution using 10% DMSO as solvent. For the CHCl_3 extract and chromatographic fractions, four five-fold dilutions were performed to obtain the final concentrations ($\mu\text{g/mL}$): 10, 2, 0.4, 0.08, 0. For the pure compounds, eight three-fold dilutions were prepared to obtain the following final concentrations (μM): 10, 3.33, 1.11, 0.37, 0.123, 0.041, 0.014, 0.005, and 0. Vincristine sulfate at 25 nM, purchased from Sigma-Aldrich (St. Louis, MO, USA), was used as the positive control. The final DMSO concentration for all test samples was 0.5%. Samples, in quadruplicate, were incubated for 48 and 72 h in a humidified 5% CO_2 atmosphere at 37°C , and analysis proceeded as previously described.⁴⁶

Compounds **7** and **8** were also evaluated against the CCD-112CoN human normal colon cell line using the SRB assay, as previously described.

MTS Assay Conducted Simultaneously with a Propidium Iodide (PI) Assay

A MTS assay carried out simultaneously with a PI assay was performed with tephrosin (**9**) using 697 cells and the results read at 48 and 72 h. Cell density was adjusted to 200,000 cells/mL. Serial dilutions of the tephrosin (**9**) stock solution (2 mM) was performed to obtain the following final test concentrations (μM): 10, 1.11, 0.123, 0.014, 0. Sample dilutions were initially made in 10% DMSO, and the final DMSO concentration in all test samples was 0.5%. Vincristine sulfate at 25 and 5 nM was used as a positive control. The same dilutions

(test samples and vincristine) were used for both the MTS and PI testing. The MTS experiment was performed as previously described.⁴⁶ For the PI experiment, cells (95 μ L/well), test compounds (5 μ L/well), and vincristine (25 and 5 nM), were placed in eight wells (one column) of a 96-well microplate, and incubated for 48 and 72 h. After the incubation period, each test sample was mixed by re-suspension and transferred to flow tubes, then centrifuged at 1200 rpm for 6 min. Supernatant was decanted and the pellet re-suspended in 200 μ L of the PI/PBS solution, consisting of 50 μ L of PI and 2.0 mL of PBS. For the untreated/unstained control, 200 μ L of PBS were added to the cells. An untreated/stained sample was used as a negative control. Test samples were incubated for 15 min in the dark at room temperature, and immediately read by flow cytometry.

Quinone Reductase Induction Assay

The quinone reductase induction activities of compounds **2–11** were evaluated using the Hepa 1c1c7 murine hepatoma cell line, according to a standard procedure.^{48,49} L-Sulforaphane was used as the positive control.

In Vivo Hollow Fiber Assay

The potential in vivo antineoplastic activity of *cis*-(6a, 12a)-hydroxyrotenone (**7**) was evaluated using human colon cancer (HT-29), breast cancer (MCF-7), and melanoma (MDA-MB-435) cells grown in hollow fibers (HT-29: 1×10^6 ; MCF-7: 5×10^6 and MDA-MB-435: 2.5×10^6 per mL), and implanted intraperitoneally (ip) and subcutaneously (sc) in immunodeficient female NCr *nu/nu* mice, according to an established procedure.^{43,44} Compound **7** was dissolved in 30% cyclodextrin (CDT, Inc., Alachua, FL), and administered through ip injections to mice at 5, 10, 15, and 30 mg/kg, once daily. The compound was lethal at the two highest doses after one injection and at the two lower doses after two injections. Bortezomib, dissolved in 30% cyclodextrin and used as the positive control, was administered through ip injections to mice at 1 mg/kg, once daily.

The in vivo hollow fiber assay was repeated using lower doses of **7** and with hollow fibers containing HT-29 (1×10^6 per mL) and MDA-MB-435 (2.5×10^6 per mL), that were implanted ip and sc in immunodeficient female NCr *nu/nu* mice.^{43,44} *cis*-(6a, 12a)-hydroxyrotenone (**7**) was administered to mice through once daily ip injections at 0.5, 1, and 2 mg/kg. A once daily ip injection of paclitaxel at 5 mg/kg dose, dissolved in 10% (1:1 EtOH and Tween 20) and 90% water, was used as a positive control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. MTS assay conducted simultaneously with a PI Assay to assess cell death caused by tephrosin (**9**) in 697 cells. Error bars represent the standard deviation of three independent experiments.

- 1 $R_1 = \text{OH}$, $R_2 = \text{H}$
2 $R_1 = \text{OCOCH}_3$, $R_2 = \text{OCH}_3$
5 $R_1 = \text{OCOCH}_3$, $R_2 = \text{H}$

- 7 $R = \text{OH}$
8 $R = \text{H}$

9

- 10 $R_1 = \text{OCOCH}_3$, $R_2 = \text{OCH}_3$
11 $R_1 = \text{OH}$, $R_2 = \text{H}$

Table 1¹H NMR Chemical Shifts (δ) of Compounds 1–4

position	1 ^{a,b}	2 ^{b,c}	3 ^{b,c}	4 ^{b,c}
2	5.56, dd (12.8, 3.0)	5.53 ^d	5.48, d (13.0)	5.49, dd (12.7, 3.0)
3a	2.91, dd (16.9, 3.1)	2.87, dd (16.5, 3.5)	2.88, d (17.6)	2.91, m
3b	3.05, dd (16.9, 12.9)	2.97, dd (16.4, 12.5)	3.01, m	3.05, dt (24.8, 12.4)
5	7.89, d (8.5)	-	7.95, d (8.7)	7.92, d (8.8)
6	6.58, d (8.5)	6.12, s	6.70, d (8.5)	6.68, m
2'-6'	7.43, m	7.41, m	7.39, m	7.44, m
2	6.51, d (6.4)	6.50, d (6.3)	-	-
5	4.25, s	5.50 ^d	8.19, s	-
6	3.95, m	3.96, d (6.6)	-	-
7	-	-	-	6.00, d (12.5)
8	-	-	-	5.88, d (12.5)
Me ₂	1.06, s 1.37, s	1.12, s 1.26, s	1.41, s 1.42, s	1.21, s 1.22, s
OMe	-	3.90, s	3.87, s	3.92, s
OAc	-	2.09, s	-	-

^a Measured at 800 MHz, obtained in CDCl₃ with TMS as internal standard.^b *J* values (Hz) are given in parentheses. Assignments are based on ¹H-¹H COSY, HSQC, HMBC, and NOESY spectroscopic data.^c Measured at 400 MHz, obtained in CDCl₃ with TMS as internal standard.^d Overlapping signals.

Table 2¹³C NMR Chemical Shifts (δ) of Compounds 1–4

position	1 ^{a,b}	2 ^{b,c}	3 ^{b,c}	4 ^{b,c}
2	80.2	79.5	78.4	80.2
3	44.6	46.6	43.2	44.5
4	190.0	189.0	190.1	191.4
4a	115.9	106.6	114.5	115.9
5	130.3	164.6	127.7	128.2
6	105.3	88.7	104.1	105.5
7	165.7	165.7	162.3	162.4
8	113.8	105.2	104.8	115.8
8a	158.3	159.3	159.5	159.3
1'	138.9	139.2	137.9	138.9
2', 6'	126.0	126.0	124.8	126.6
3', 5'	129.1	129.1	127.5	129.2
4'	128.9	128.8	127.3	126.2
2	112.6	113.1	86.8	-
3	-	-	203.2	-
4	88.0	88.3	108.9	-
5	80.7	80.8	173.7	-
6	55.3	52.8	-	-
7	-	-	-	117.2
8	-	-	-	142.2
9	-	-	-	71.9
Me ₂	23.0, 27.4	23.5 27.9	21.8 21.8	29.9 30.3
OMe	-	56.9	55.2	56.5
OAc	-	170.0 21.2	-	-

^a Measured at 200 MHz obtained in CDCl₃ with TMS as internal standard.^b Assignments are based on HSQC, and HMBC NMR spectra. Multiplicity obtained from the DEPT spectrum.^c Measured at 100 MHz obtained in CDCl₃ with TMS as internal standard.

Table 3
Cytotoxicity of Compounds Isolated from *I. spicata* Against Three Cancer Cell Lines ^a

compound	IC ₅₀ ^b (μM)				
	HT-29 ^c		697 ^d		Raji ^d
	72 h	48 h	72 h	48 h	72 h
7	0.1 ± 0.02	0.7 ± 0.14	0.5 ± 0.05	5.6 ± 2.02	2.4 ± 2.62
8	0.3 ± 0.04	0.3 ± 0.09	0.3 ± 0.10	4.2 ± 0.78	1.1 ± 0.87
9	> 10	5.4 ± 0.73	9.0 ± 3.62	> 10	> 10
paclitaxel ^e	0.0006 ± 0.001	-	-	-	-

^aCompounds **1–6**, **10** and **11** were not cytotoxic against the cell lines (IC₅₀ > 10 μM).

^bIC₅₀ is the concentration that inhibits 50% cell growth.

^cThe values represent the average ± standard deviation (SD) from a triplicate.

^dThe values represent the average ± SD from three independent experiments.

^eUsed as a positive control for HT-29 cytotoxicity testing.

Table 4

Cytotoxicity of Compounds 7 and 8 Against the HT-29 and CCD-112CoN Cell Lines

compound	IC ₅₀ ^a (μM)		selectivity ^b
	HT-29	CCD-112CoN	
7	0.1 ± 0.02	102 ± 7.07	1020
8	0.3 ± 0.04	> 127	> 423
paclitaxel ^c	0.0006 ± 0.001	23.0 ± 2.62	38333

^aIC₅₀ is the concentration that inhibits 50% cell growth. The values represent the average ± SD from a triplicate.

^bThe fold selectivity was calculated by dividing the IC₅₀ value of the normal human colon cell line (CCD-112CoN) by the IC₅₀ value of the human colon cancer cell line (HT-29).

^cPositive control.

Table 5Quinone Reductase Induction Activities of Compounds Isolated from *I. spicata*^{a-c}

compound	CD ^d (μM)	IC ₅₀ ^e (μM)	CI ^f
2	0.2 ± 0.02	63.9 ± 8.24	376.7
3	6.4 ± 0.99	23.1 ± 4.39	3.6
4	4.2 ± 0.40	47.4 ± 3.62	11.3
6	8.3 ± 1.12	49.0 ± 7.41	5.9
7	0.4 ± 0.10	0.2 ± 0.05	0.6
8	0.8 ± 0.11	0.7 ± 0.08	0.9
9	0.4 ± 0.07	13.9 ± 1.58	35.3
10	0.8 ± 0.09	18.1 ± 2.21	23.9
11	3.8 ± 0.70	37.6 ± 5.97	9.9
L-sulforaphane ^g	0.5 ± 0.07	12.1 ± 1.73	23.9

^aCompound **1** was not tested due to limited amount isolated.^bCompound **5** did not induce quinone reductase activity against Hepa 1c1c7 cells (CD values > 20 μM)^cThe values represent the average ± SD from three independent experiments.^dCD is the concentration required to double quinone reductase activity.^eIC₅₀ is the concentration that inhibits 50% cell growth.^fCI is the chemopreventive index (= IC₅₀/CD).^gL-Sulforaphane was used as the positive control.