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Bioactive Compounds from the Fern *Lepisorus contortus*

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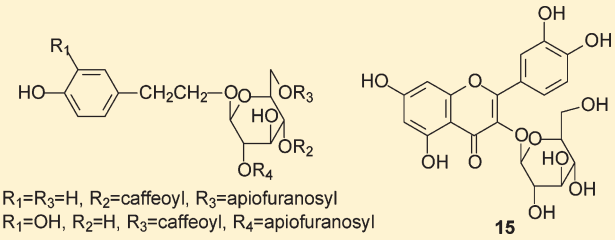
S *Supporting Information*

ABSTRACT: Phytochemical investigation of the whole plant of *Lepisorus contortus* (Christ) Ching led to the isolation of five new phenylethanoid glycosides (**1**−**5**), each containing a caffeoyl group, a new flavonoid glycoside (**10**), and 14 known compounds (**6**−**9** and **11**−**15**, syringic acid, vanillic acid, phloretic acid, diplopterol, and β-sitosterol). This is the first report of phenylethanoid glycosides from the family Polypodiaceae. Compounds **1**−**15** were evaluated for their cancer chemopreventive potential based on their ability to inhibit tumor necrosis factor alpha (TNF-α)-induced NF-κB activity, nitric oxide (NO) production, and aromatase, quinone reductase 2 (QR-2), and COX-1/-2 activities. Quercetin-3-*O*-β-D-glucoside (**15**) demonstrated inhibition against QR2 with an IC_{50} value of 3.84 μM, which confirmed kaempferol/quercetin glycosides as the active compounds to inhibit QR2. The compound also demonstrated NF-κB activity with an IC_{50} value of 33.6 μM. In addition, compounds **1**, **2**, **4**, and **6** showed aromatase activity with IC_{50} values of 30.7, 32.3, 26.8, and 35.3 μM, respectively.



Lepisorus contortus, belonging to the family Polypodiaceae, is a fern that is widely distributed in China and India. It grows on rocks and tree trunks under moist conditions at 1450−2600 m above sea level and has been used in folk medicine for trauma, burns, and scald injuries.[1,2] Although the genus *Lepisorus* contains approximately 70 species, only two have been phytochemically investigated previously, which led to the identification of five flavonoids and three steroids from *L. ussuriensis*[3,4] and two phenylpropanoids and one flavonoid from *L. thunbergianus*.[5] In our search for new cancer chemopreventive agents from medicinal plants of Yunnan, China, we isolated 20 natural compounds including five new phenylethanoid glycosides (**1**−**5**) and a new flavone glycoside (**10**) from the fern *L. contortus* (Chart 1). This is also the first report of phenylethanoid glycosides from the family Polypodiaceae. Compounds **1**−**15** have been evaluated for their cancer chemopreventive potential in the assays for TNF-α-induced NF-κB, NO production, aromatase, QR-2, and COX-1/-2 activities. Some of these compounds (**1**, **2**, **4**, and **6**) showed inhibitory activities against TNF-α-induced NF-κB and aromatase. Our study further revealed that the kaempferol/quercetin glycosides contained in this plant are QR-2 inhibitors. The current paper reports the isolation and structure identification of these compounds as well as the evaluation of their cancer chemopreventive potentials, based on their ability to inhibit TNF-α-induced NF-κB activity, nitric oxide (NO) production, and aromatase, quinone reductase 2 (QR-2), and COX-1/-2 activities.

RESULTS AND DISCUSSION

The 95% EtOH extract of *L. contortus* was sequentially partitioned with petroleum ether and EtOAc to yield two extracts, which were combined and subjected to a series of column chromatographic separations including silica gel and Sephadex LH-20 gel permeation chromatography to afford 20 compounds (**1**−**15**, syringic acid, vanillic acid, phloretic acid, diplopterol, and β-sitosterol).

Compound **1**, a brown syrup, was determined to have a molecular formula of $C_{28}H_{34}O_{14}$ by a negative FABMS deprotonated molecule at m/z 593 $[M - H]^-$ and a negative HRESIMS deprotonated molecule at m/z 593.1864 ($[M - H]^-$, calcd

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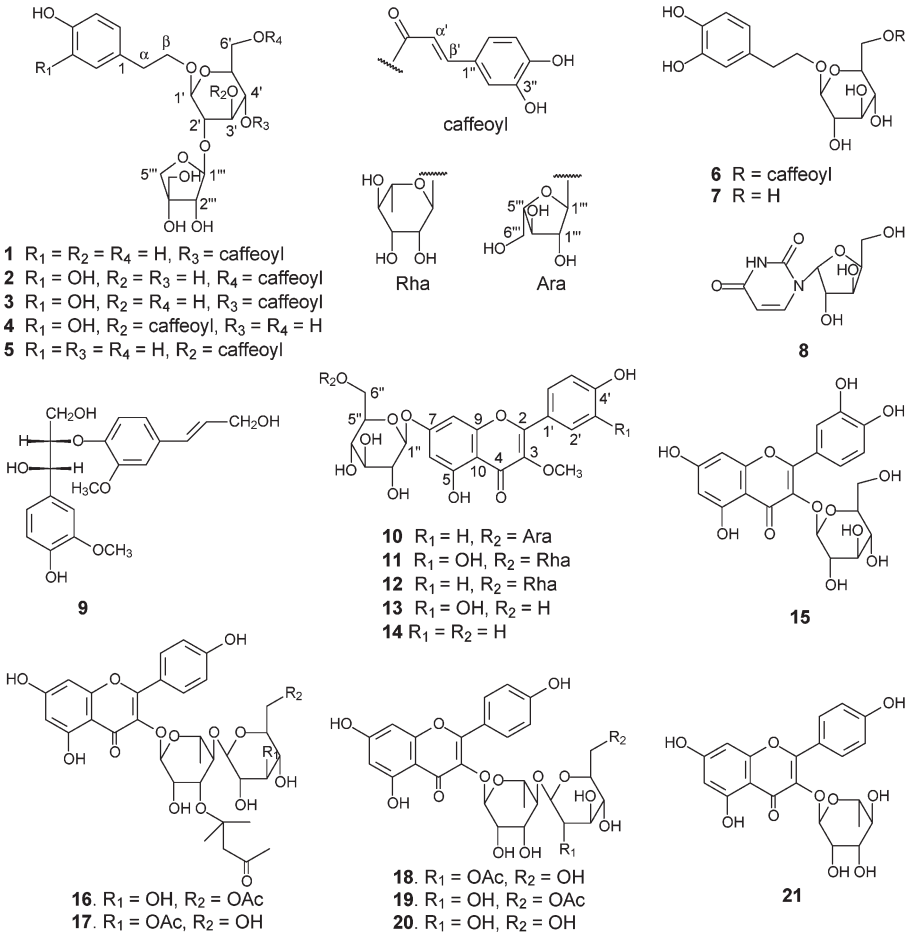
Chart 1



for 593.1870), which was supported by ^{13}C NMR and DEPT data. The ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) revealed the presence of a *para*-substituted benzene ring due to the proton and carbon signals at δ_H 7.10 (2H, d, J = 8.6 Hz, H-2 and H-6) and 6.73 (2H, d, J = 8.6 Hz, H-3 and H-5) and δ_C 131.7 (s, C-1), 131.4 (d, C-2 and C-6), 116.8 (d, C-3 and C-5), and 157.2 (s, C-4) and a caffeoyl moiety due to the aromatic and olefinic proton and carbon signals at δ_H 7.63 (1H, d, J = 16.0 Hz, H-β'), 6.32 (1H, d, J = 16.0 Hz, H-α'), 6.82 (1H, d, J = 8.1 Hz, H-5″), 6.96 (1H, dd, J = 8.1, 1.3 Hz, H-6″), and 7.08 (1H, d, J = 1.3 Hz, H-2″) and δ_C 168.6 (s, ester carbonyl carbon), 115.2 (d, C-α'), 148.1 (d, C-β'), 128.2 (s, C-1″), 115.7 (d, C-2″), 147.3 (s, C-3″), 150.2 (s, C-4″), 117.0 (d, C-5″), and 123.5 (d, C-6″).[6,7] The ^{1}H NMR, ^{13}C NMR, and DEPT spectra also showed signals of an oxygenated methylene group at δ_H 3.70 and 4.10 (each 1H, m, H_2-β) and δ_C at 72.3 (t, C-β), a methylene group at δ_H 2.86 (2H, t, J = 7.5 Hz, H_2-α) and δ_C 36.9 (t, C-α), a glucopyranosyl at δ_H 4.81 (1H, d, J = 8.5 Hz, H-1′) and δ_C 100.9 (d, C-1′), and an apiofuranosyl moiety at δ_H 5.15 (1H, d, J = 1.2 Hz, H-1‴) and δ_C 107.3 (d, C-1‴), indicating **1** to be a diglycoside. The presence of correlations between the two methylenes in the ^{1}H−^{1}H COSY and HMBC spectra indicated that the two groups were linked together as an ethylene group, which was further determined to be connected to the *para*-substituted benzene moiety to form a 4-hydroxyphenylethoxy group, evidenced by the presence of the cross-peaks between the signals of the methylenes and the 4-hydroxybenzene ring in the HMBC spectrum (Figure 1).

The glucose moiety was determined to have a β-configuration at C-1 due to a large coupling constant for the anomeric proton of the sugar unit at δ_H 4.81 (1H, d, J = 8.5 Hz), and the apiose unit was also determined to have a β-configuration at C-1 due to the chemical shift of its anomeric carbon signal in the ^{13}C NMR at δ_C 107.3.[8,9] The presence of a three-bond correlation between the glucose C-2′ signal at δ_C 75.3 and the apiose anomeric proton signal at δ_H 5.15 in the HMBC spectrum suggested that the interglycosyl linkage is apiosyl-(1→2)-glucose. The location of the caffeoyloxy group in **1** was fixed at C-4′ of the glucose residue by the presence of the HMBC correlation from the acyl carbonyl carbon signal at δ_C 168.6 to the glucosyl C-4′ proton signal at δ_H 4.82. The glycosidation position was unambiguously determined by the presence of a three-bond correlation between the glucosyl anomeric proton H-1′ at δ_H 4.81 and the oxygenated methylene group at δ_C 72.3 (C-β) in the HMBC spectrum. Thus, compound **1** was identified as β-(4-hydroxyphenyl)ethyl-4-*O*-*E*-caffeoyl-*O*-[β-D-apiofuranosyl-(1→2)]-β-D-glucopyranoside.

Compound **2** was also obtained as a brown syrup. The molecular formula was determined as $C_{28}H_{34}O_{15}$ from a negative FABMS ion at m/z 609 $[M - H]^-$ and a negative HRESIMS ion at m/z 609.1804 ($[M - H]^-$, calcd for 609.1819), suggesting the presence of an additional hydroxy group in comparison with **1**.

Table 1. ^{1}H NMR Data (500 MHz, methanol-d_4) [δ_H (J in Hz)] for 1−5

position	1	2	3	4	5
2	7.10, d (8.6)	6.70, d (1.9)	6.70, d (1.5)	6.69, d (1.5)	7.09, d (8.6)
3	6.73, d (8.6)				6.72, d (8.6)
5	6.73, d (8.6)	6.66, d (8.1)	6.73, d (8.0)	6.71, d (8.0)	6.72, d (8.6)
6	7.10, d (8.6)	6.56, dd (8.1, 1.9)	6.58, dd (8.0, 1.5)	6.58, dd (8.0, 1.5)	7.09, d (8.6)
α	2.86 t (7.5)	2.78 t (7.2)	2.79 t (7.5)	2.79 t (7.5)	2.79 t (7.4)
βa	4.10, m	3.99, m	4.09, m	4.10, m	4.09, m
βb	3.70, m	3.68, m	3.71, m	3.70, m	3.70, m
Glc-1′	4.81, d (8.5)	4.82, d (8.0)	4.82, d (8.2)	4.74, d (8.0)	4.75, d (8.0)
2′	3.59, m	3.32, m	3.60, m	3.82, m	3.82, m
3′	4.50, m	4.28, m	4.52, m	5.75, m	5.75, m
4′	4.82, m	3.57, m	4.83, m	3.76, m	3.76, m
5′	4.05, m	4.00, m	4.07, m	3.67, m	3.67, m
6′	3.76, m	4.50, m	3.75, m	3.90, m	3.89, m
	3.63, m	4.33, m	3.64, m	3.72, m	3.72, m
2″	7.08, d (1.3)	7.06, d (1.9)	7.08, d (1.3)	7.09, d (1.3)	7.08, d (1.2)
5″	6.82, d (8.1)	6.79, d (8.1)	6.82, d (8.1)	6.87, d (8.1)	6.83, d (8.0)
6″	6.96, dd (8.1, 1.3)	6.92, dd (8.1, 1.9)	6.96, dd (8.1, 1.3)	7.00, dd (8.1, 1.3)	7.00, dd (8.0, 1.2)
α′	6.32, d (16.0)	6.31, d (16.0)	6.32, d (16.0)	6.37, d (16.0)	6.38, d (16.0)
β′	7.63, d (16.0)	7.58, d (16.0)	7.63, d (16.0)	7.62, d (16.0)	7.62, d (16.0)
Api-1‴	5.15, d (1.2)	5.17, d (1.0)	5.19, d (1.5)	5.18, d (1.2)	5.22, d (1.2)
2‴	3.97, d (1.2)	4.00, d (1.0)	3.98, d (1.5)	3.78, d (1.2)	3.78, d (1.2)
4‴a	3.96, d (9.9)	4.00, d (9.8)	3.96, d (9.8)	3.89, d (9.8)	3.89, d (9.8)
4‴b	3.79, d (9.9)	3.77, d (9.8)	3.79, d (9.8)	3.74, d (9.8)	3.74, d (9.8)
5‴	3.65, s	3.63, s	3.63, s	3.58, s	3.58, s

Its ^{1}H NMR, ^{13}C NMR, and DEPT spectra (Tables 1 and 2) were similar to those of **1**, indicating that **2** was also a phenylethanoid diglycoside with a caffeoyl group. Analysis of the ^{1}H NMR, ^{13}C NMR, and DEPT data showed that the aglycone of **2** was a 3,4-dihydroxyphenylethanol group due to the signals at δ_H 6.70 (1H, d, J = 1.9 Hz, H-2), 6.66 (1H, d, J = 8.1 Hz, H-5), and 6.56 (1H, dd, J = 8.1, 1.9 Hz, H-6) and δ_C 132.1 (s, C-1), 117.6 (d, C-2), 146.5 (s, C-3), 145.0 (s, C-4), 117.0 (d, C-5), and 121.8 (d, C-6). The presence of the correlation between an acyl carbonyl carbon signal at δ_C 169.7 and the glucosyl C-6′ proton signals at δ_H 4.50 and 4.33 (each 1H, m, H_2-6′) in the HMBC spectrum (Figure 1) assigned the caffeoyloxy group at C-6′ of the glucose residue. Thus, compound **2** was identified as β-(3,4-dihydroxyphenyl)ethyl-6-*O*-*E*-caffeoyl-*O*-[β-D-apiofuranosyl-(1→2)]-β-D-glucopyranoside.

Compound **3** was obtained as a brown syrup. It was determined to have the same molecular formula ($C_{28}H_{34}O_{15}$) as that of **2** by a negative FABMS ion at m/z 609 [M − H]$^-$ and a negative HRESIMS ion at m/z 609.1807 ([M − H]$^-$, calcd for 609.1819). It showed similar ^{1}H and ^{13}C NMR data to those of **1** and **2** (Tables 1 and 2). Compound **3** differs from **1** by having a 3,4-dihydroxyphenylethanol group instead of a 4-hydroxyphenylethanol in **1**, and from **2** only by the location of the caffeoyl group. The caffeoyloxy group in **3** was determined to be located at C-4′ of the glucose residue by the presence of the HMBC correlation (Figure 1) from the acyl carbonyl resonance at δ_C 168.6 to the glucosyl C-4′ proton signal at δ_H 4.83. Thus, compound **3** was identified as β-(3,4-dihydroxyphenyl)ethyl-4-*O*-*E*-caffeoyl-*O*-[β-D-apiofuranosyl-(1→2)]-β-D-glucopyranoside.

Compound **4** was obtained as a brown syrup. The molecular formula was determined to be the same as that of **3** ($C_{28}H_{34}O_{15}$) from a negative FABMS ion at m/z 609 [M − H]$^-$ and a negative HRESIMS ion at m/z 609.1809 ([M − H]$^-$, calcd for 609.1819). The ^{1}H NMR, ^{13}C NMR, and DEPT spectra (Tables 1 and 2) of **4** were similar to those of **3**, differing only by the location of the caffeoyl group. The caffeoyloxy group in **3** was determined at C-3′ of the glucose residue by the presence of the HMBC correlation (Figure 1) from the acyl carbonyl resonance at δ_C 169.4 to the glucosyl C-3′ proton signal at δ_H 5.75. Accordingly, compound **4** was identified as β-(3,4-dihydroxyphenyl)ethyl-3-*O*-*E*-caffeoyl-*O*-[β-D-apiofuranosyl-(1→2)]-β-D-glucopyranoside.

Compound **5**, a brown syrup, was deduced to have the same molecular formula as that of **1** ($C_{28}H_{34}O_{14}$) by the negative FABMS (m/z 593 [M − H]$^-$) and the negative HRESIMS ([M − H]$^-$ m/z 593.1864, calcd for 593.1870). The ^{1}H NMR, ^{13}C NMR, and DEPT data (Tables 1 and 2) were similar to those of **1**. The difference between **5** and **1** is similar to that between **4** and **3** in that the caffeoyl group is at a different location. Analysis of the HMBC data (Figure 1) determined the caffeoyloxy group in **5** at C-3′. Thus, compound **5** was identified as β-(4-hydroxyphenyl)ethyl-3-*O*-*E*-caffeoyl-*O*-[β-D-apiofuranosyl-(1→2)]-β-D-glucopyranoside.

Compound **10** was obtained as a yellow, amorphous powder, and its molecular formula was deduced as $C_{27}H_{30}O_{15}$ by the negative HRESIMS (found 593.1493, calcd for 593.1506). The ^{1}H and ^{13}C NMR spectra (Table 3) showed typical signals of a flavonoid [δ_H 8.05 (2H, d, J = 8.5 Hz, H-2′ and H-6′), 6.96 (2H, d, J = 8.5 Hz, H-3′ and H-5′), 6.53 (1H, brs, H-6), 6.78 (1H, brs, H-8) and 15 sp^2 hybrid carbon signals including a conjugated carbonyl group at δ_C 180.6 (C-4, s)].[10,11] The presence of the two doublets corresponding to the AA′BB′ spin system at δ_H 8.05 and 6.96 suggested a *p*-substituted ring B. The proton signals of the two broad singlets at 6.53 and 6.78 were assigned, respectively, to *meta*-coupled H-6 and H-8 of ring A in **10**, which

Table 2. ^{13}C NMR Data (125 MHz, methanol-d_4) (δ_C, mult.) for **1**−**5**

position	1	2	3	4	5
1	131.7, C	132.1, C	132.2, C	132.1, C	132.2, C
2	131.4, CH	117.6, CH	117.7, CH	117.6, CH	131.4, CH
3	116.8, CH	146.5, C	146.1, C	146.5, C	116.9, CH
4	157.2, C	145.0, C	145.0, C	145.0, C	157.1, C
5	116.8, CH	117.0, CH	117.1, CH	117.0, CH	116.9, CH
6	131.4, CH	121.8, CH	121.7, CH	121.8, CH	131.4, CH
α	36.9, CH_2	37.2, CH_2	37.1, CH_2	37.2, CH_2	37.2, CH_2
β	72.3, CH_2	72.4, CH_2	72.3, CH_2	72.2, CH_2	72.2, CH_2
Glc-1′	100.9, CH	101.0, CH	100.9, CH	101.3, CH	101.3, CH
2′	75.3, CH	75.6, CH	75.2, CH	76.5, CH	76.5, CH
3′	67.4, CH	69.4, CH	67.4, CH	71.8, CH	71.9, CH
4′	71.0, CH	69.7, CH	71.0, CH	67.8, CH	67.8, CH
5′	73.7, CH	73.4, CH	73.7, CH	73.7, CH	73.8, CH
6′	62.9, CH_2	65.6, CH_2	62.9, CH_2	62.2, CH_2	63.2, CH_2
1″	128.2, C	128.2, C	128.2, C	128.3, C	128.3, C
2″	115.7, CH	115.6, CH	115.8, CH	115.7, CH	116.5, CH
3″	147.3, C	147.2, C	146.5, C	146.5, C	147.2, C
4″	150.2, C	150.0, C	150.1, C	150.1, C	150.0, C
5″	117.0, CH	116.9, CH	116.9, CH	116.8, CH	117.0, CH
6″	123.5, CH	123.6, CH	123.7, CH	123.5, CH	123.5, CH
α′	115.2, CH	115.4, CH	115.2, CH	115.7, CH	115.8, CH
β′	148.1, CH	147.6, CH	148.2, CH	147.6, CH	147.7, CH
CO	168.6, C	169.7, C	168.6, C	169.4, C	168.4, C
Api-1‴	107.3, CH	107.2, CH	107.3, CH	107.4, CH	107.4, CH
2‴	78.5, CH	78.5, CH	78.5, CH	78.2, CH	78.2, CH
3‴	81.2, C	81.2, C	81.3, C	81.2, C	81.3, C
4‴	75.9, CH_2	75.9, CH_2	75.9, CH_2	76.1, CH_2	76.1, CH_2
5‴	66.6, CH_2	66.5, CH_2	66.6, CH_2	66.9, CH_2	66.9, CH_2

was confirmed by the presence of the ^{1}H−^{1}H COSY correlation between the two protons and the presence of the HMBC correlations from H-6 to C-8 (δ_C 96.4, d) and from H-8 to C-6 (δ_C 101.3, d). Additionally, the ^{1}H NMR spectrum showed the resonance of a methoxy singlet at δ_H 3.82 (3H, s), which was assigned at C-3 (δ_C 140.2, s) due to the presence of the correlation between the methoxy protons and C-3 in the HMBC experiment (Figure 1).

The ^{1}H and ^{13}C NMR spectra also showed two anomeric protons at δ_H 5.08 (1H, d, J = 7.2 Hz, H-1″) and 4.95 (1H, d, J = 2.0 Hz, H-1‴) and 11 oxygenated carbons, suggesting **10** to be a flavonol diglycoside possessing a pentosyl and a hexosyl moiety. The anomeric configurations of the glucose and the arabinose units were determined as β and α, respectively, on the basis of the coupling constants of 7.2 Hz for H-1″ and 2.0 Hz for H-1‴.[12,13] The glucose C-6″ signal appeared at δ_C 68.5, suggesting that the interglycosyl linkage is arabinosyl-(1→6)-glucose, which was confirmed by the presence of the HMBC correlation between H-1‴ and C-6″. The glycosidation position was further determined by the presence of the three-bond HMBC correlation between the glucosyl anomeric proton H-1″ and C-7 of ring A. The structure of **10** was identified as 4′,5,7-trihydroxy-3-methoxyflavone-7-*O*-α-L-arabinofuranosyl (1→6)-β-D-glucopyranoside.

Acid hydrolysis of **1**−**5** with 5% H_2SO_4 in EtOH yielded respectively two sugars, which were separated by column chromatography. The two sugars were identified as glucose and apiose, respectively, by comparison of their ^{1}H NMR and optical rotation data with literature reports and authentic samples. Acid hydrolysis of **10** with 5% H_2SO_4 in EtOH afforded D-glucose and L-arabinose, which were separated by column chromatography and identified by comparison of their ^{1}H NMR and optical rotation data with literature reports and authentic samples. The total assignment of the ^{1}H and ^{13}C NMR spectroscopic data of compound **1**−**5** and **10** was carried out by a combination of ^{1}H−^{1}H COSY, HSQC, HMBC, and ROESY experiments.

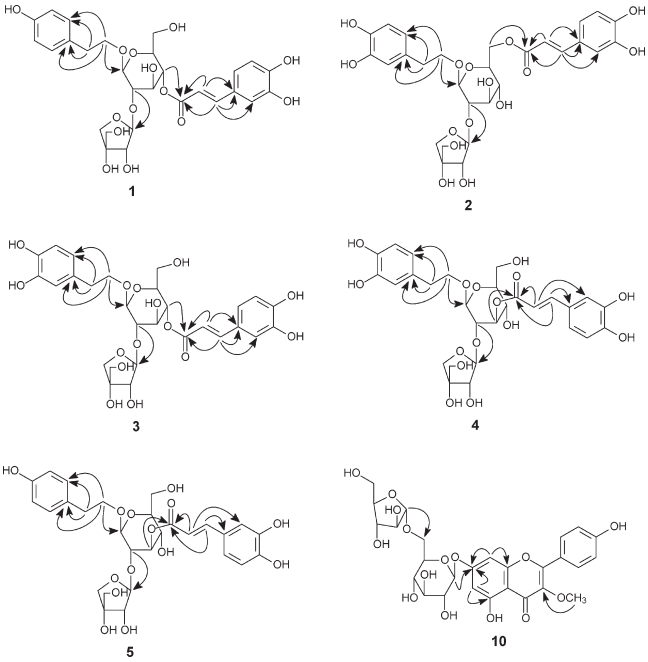


Figure 1. Key HMBC correlations for **1**−**5** and **10**.

The 14 known isolates were identified as calceolarioside B (**6**),[14] β-(3, 4-dihydroxylphenyl) β-D-glucopyranoside (**7**),[15] arauridine (**8**),[16,17] threo-1-(4-hydroxy-3-methoxyphenyl)-2-{4-[-(*E*)-3-hydroxy-1-propenyl]-2-methoxyphenoxy}-1,3-propanediol (**9**),[18] 3′,4′,5,7-tetrahydroxy-3-methoxyflavone-7-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**11**),[19] 4′,5,7-trihydroxy-3-methoxyflavone-7-*O*-rutinoside (**12**), transilin (**13**), 4′,5,7-trihydroxy-3-methoxyflavone-7-*O*-β-D-glucopyranoside (**14**),[20] quercetin-3-*O*-β-D-glucoside (**15**),[21] syringic acid,[22] vanillic acid,[23] phloretic acid,[24] diplopterol,[25,26] and β-sitosterol by comparison of their physical data with reported data.

Although flavonoids are found abundantly in ferns, phenylethanoids are rare. This is the first report of phenylethanoid glycosides from the family Polypodiaceae. Compounds **1**−**15** were evaluated for their cancer chemopreventive potential based on their ability to inhibit TNF-α-induced NF-κB activity, nitric oxide (NO) production, and aromatase, quinone reductase 2 (QR-2), and COX-1/-2 activities.

The nuclear transcription factor (NF-κB), the signaling molecule (NO), and the enzymes (aromatase, QR-2, and COX-1/-2) all play different roles in cells, respectively.[27,28] NF-κB is a ubiquitous transcription factor associated with cell apoptosis, differentiation, and migration and may promote cell proliferation and prevent cell death through antiapoptotic factors upon being activated.[29] Aromatase catalyzes the conversion of androgen to estrogen, the female sex hormone associated with proliferation of

Table 3. ^{1}H (500 MHz) [δ_{H}, (J, Hz)] and ^{13}C NMR (125 MHz) (δ_{C}) Data of 10 in Methanol-d_4

position	^{1}H	^{13}C
2		159.2, C
3		140.2, C
4		180.6, C
5		163.2, C
6	6.53, brs	101.3, CH
7		165.1, C
8	6.78, brs	96.4, CH
9		158.4, C
10		108.2, C
1′		122.9, C
2′	8.05, d (8.5)	132.1, CH
3′	6.96, d (8.5)	117.1, CH
4′		162.3, C
5′	6.96, d (8.5)	117.1, CH
6′	8.05, d (8.5)	132.1, CH
Glc-1″	5.08, d (7.2)	102.0, CH
2″	3.52, m	75.2, CH
3″	3.53, m	78.2, CH
4″	3.40, m	72.1, CH
5″	3.65, m	77.5, CH
6″a	4.11, m	68.5, CH_2
6″b	3.73, m	
Ara-1‴	4.95, d (2.0)	110.5, CH
2‴	4.08, m	83.5, CH
3‴	3.85, m	79.3, CH
4‴	4.01, m	86.5, CH
5‴a	3.73, m	63.5, CH_2
5‴b	3.63, m	
MeO-3	3.82, s	61.0 CH_3

breast cancer cells.[30,31] Although NO production has a beneficial role for protection of an organ such as the liver from ischemic damage, excessive and sustained levels of NO expression may damage tissues, which may result in vascular collapse, inflammation, or even carcinomas. Studies have shown that NO impacts many physiological and pathological processes associated with the development of cancers in the early stages.[32] QR2, which may transform certain quinone substrates into more highly reactive species, is abundantly expressed in some cancer cells,[33−39] and is also associated with various neurological disorders including Parkinson's disease[40,41] and schizophrenia.[42] Although the precise function of QR2 remains to be determined, some chemopreventive agents such as resveratrol and melatonin were found to have potent binding activities with QR2,[37,38] suggesting QR2 as a potential new target for the development of chemopreventive agents. COX-1/-2 are associated with neoplastic transformation.[43] Thus, inhibition against each of NF-κB signaling, NO production, and the enzyme activities of aromatase, QR2, and COX-1/-2 may have beneficial effects for the treatment or prevention of cancer.

Among the evaluated isolates, only quercetin-3-O-β-D-glucoside (**15**) demonstrated inhibition of TNF-α-induced NF-κB activity, with an IC_{50} value of 33.6 μM, and compounds **1**, **2**, **4**, and **6** showed aromatase activity, with IC_{50} values of 30.7, 32.3, 26.8, and 35.3 μM, respectively. Compounds **1**−**15** were also evaluated for their inhibition against NO production and COX-1/-2 enzymes, but none of them showed significant NO activity at a concentration of 20 μg/mL (in a range of 27 to 82 μM, depending on compounds), and none of them showed COX activity at a concentration of 10 μg/mL (in a range of 13 to 41 μM, depending on compounds).

Compounds **1**−**15** were further evaluated for their ability to interact with QR2 using an LC-MS ultrafiltration binding assay. Compounds **10** and **14** were shown to mediate a positive response. Since neither of the two compounds showed more than 50% inhibition against QR2, it may be suggested they interact with a binding site of the enzyme rather than the catalytic site. A similar phenomenon was also observed in our previous study for compounds **16**−**21** (Chart 1).[28] Our studies showed that only the kaempferols/quercetin with a glycoside unit at C-3 (**15**, **17**, **19**, and **21**) were able to inhibit QR2. Further, the kaempferols/quercetin with a single sugar unit at C-3 (**15** and **21**) showed stronger binding activity than other kaempferol/quercetin derivatives. To confirm the findings, we further evaluated QR2 inhibition activity for the commercially purchased compound **21** at different concentrations (Figure 2). The compound was found to have strong inhibition activity against QR2 with an IC_{50} value of 3.84 μM.

To determine cytotoxicity, compounds **1**−**15** were evaluated against Hepa1c1c7 and MCF7 cells. None of them showed any growth inhibitory effects against the two cell lines at a concentration of 20 μg/mL (in a range of 27 to 82 μM, depending on compounds). The absence of general toxicity is considered beneficial in view of cancer chemoprevention since a high therapeutic index is required for disease prevention. Further studies to elucidate the modulation of unique chemopreventive targets of these compounds, especially the kaempferol/quercetin glycosides, may provide a rationale for modification of the structures to produce more potent chemopreventive molecules.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. MS were determined on a Finnigan MAT 90 instrument and a VG Auto Spec-3000 spectrometer. NMR spectra were measured on a Bruker DRX-500 spectrometer. Silica gel (200−300 mesh, Qingdao Marine Chemical Co., China), MCI gel (Mitsubishi Chemical Corp.), and Sephadex LH-20 (25−100 μm, Pharmacia Fine Chemical Co. Ltd.) were used for column chromatography (CC), and silica gel GF_{254} was used for TLC (Qingdao Marine Chemical Co., China). Solvents were of industrial purity and distilled prior to use.

Plant Material. The whole plant of *L. contortus* was collected from Gongshan County, Yunnan, China, in October 2005 and identified by Prof. Shugang Lu, School of Life Science, Yunnan University, where a voucher specimen (No. 0510017) is deposited.

Extraction and Isolation. Air-dried powder of the whole plant (1.1 kg) was extracted with 95% EtOH (×5) at room temperature. The EtOH extract (108 g) was partitioned sequentially with petroleum ether and EtOAc to yield petroleum ether and EtOAc extracts, respectively. The two extracts were combined (17.5 g) and fractionated by CC over silica gel with a gradient from petroleum ether to EtOAc to give six fractions, fractions I−VI. Fraction II (0.1 g) was separated by silica gel CC eluted with petroleum ether−EtOAc (15:1) to afford diplopterol (21 mg) and β-sitosterol (110 mg). Fraction IV (2.5 g) was separated using a MCI gel ion exchange column eluted with a gradient of 50−90% MeOH in H_2O to afford four subfractions (IV-1−IV-4). Fractions IV-1 (50 mg), IV-2 (1.2 g), IV-3 (163 mg), and IV-4 (163 mg) were further

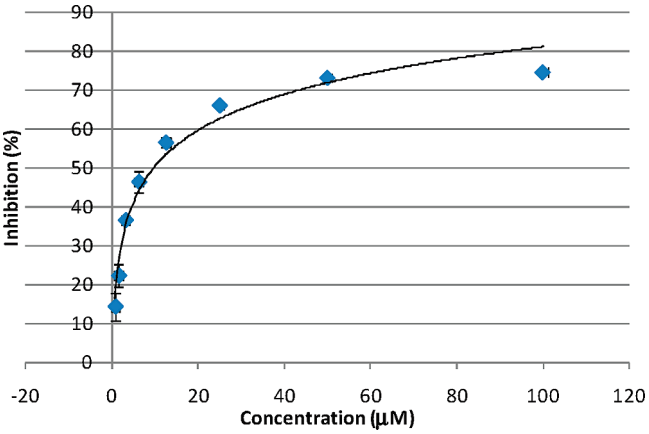


Figure 2. Titration of **21**: the compound was shown to inhibit QR2 with an IC_{50} value of 3.84 μM.

subjected to a Sephadex LH-20 column, respectively, eluting with MeOH to yield **9** (4 mg), syringic acid (5 mg), vanillic acid (8 mg), phloretic acid (6 mg), **7** (240 mg), **12** (163 mg), and **15** (13 mg). Fraction V (3.1 g) was subjected to CC separation over MCI gel ion exchange eluted with 50−90% MeOH in H_2O to yield fractions V-1−V-5. Fractions V-1 (800 mg), V-2 (600 mg), V-3 (300 mg), and V-4 (56 mg) were further chromatographed on a Sephadex LH-20 column, respectively, eluting with MeOH to afford **13** (250 mg), **14** (25 mg), **6** (4 mg), **8** (24 mg), **2** (14.5 mg), **3** (52 mg), **4** (10.5 mg), **1** (7 mg), and **5** (20 mg). Fraction VI (2.7 g) was chromatographed on a silica gel RP-18 column, eluting with MeOH−H_2O (6:4 → 1:0, v/v) in a stepwise system, to get five subfractions (VI-1−VI-5). Fractions VI-2 (20 mg) and VI-3 (590 mg) were purified by a Sephadex LH-20 column, respectively, eluting with MeOH to yield compounds **10** (12 mg) and **11** (210 mg).

*β-(4-Hydroxyphenyl)ethyl-4-O-E-caffeoyl-O-[β-D-apiofuranosyl-(1→2)]-β-D-glucopyranoside (**1**)*:. brown syrup; $[\alpha]^{25}_{D}$ −73.2 (*c* 1.5, MeOH); UV (MeOH) λ_{max} (abs.) 324 (0.30), 203.5 (0.58) nm; IR (KBr) ν_{max} 3425, 2927, 1692, 1607, 1516, 1164 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Tables 1 and 2; negative FABMS (glycerol matrix) *m/z* 593 $[M - H]^-$; negative HRESIMS *m/z* 593.1864 $[M - H]^-$ (calcd for $C_{28}H_{33}O_{14}$, 593.1870).

*β-(3,4-Dihydroxyphenyl)ethyl-6-O-E-caffeoyl-O-[β-D-apiofuranosyl-(1→2)]-β-D-glucopyranoside (**2**)*:. brown syrup; $[\alpha]^{25}_{D}$ −63.4 (*c* 5.4, MeOH); UV (MeOH) λ_{max} (abs.) 330 (0.20), 290 (0.18), 204 (0.57) nm; IR (KBr) ν_{max} 3430, 2927, 1694, 1607, 1519, 1447, 1283, 1092 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Tables 1 and 2; negative FABMS (glycerol matrix) *m/z* 609 $[M - H]^-$; negative HRESIMS *m/z* 609.1804 $[M - H]^-$ (calcd for $C_{28}H_{33}O_{15}$, 609.1819).

*β-(3,4-Dihydroxyphenyl)ethyl-4-O-E-caffeoyl-O-[β-D-apiofuranosyl-(1→2)]-β-D-glucopyranoside (**3**)*:. brown syrup; $[\alpha]^{25}_{D}$ −71.0 (*c* 6.4, MeOH); UV (MeOH) λ_{max} (abs.) 331 (0.26), 291.0 (0.21), 203.5 (0.60) nm; IR (KBr) ν_{max} 3423, 2934, 1691, 1605, 1520, 1446, 1360, 1283,1162 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Tables 1 and 2; negative FABMS (glycerol matrix) *m/z* 609 $[M - H]^-$; negative HRESIMS *m/z* 609.1807 $[M - H]^-$ (calcd for $C_{28}H_{33}O_{15}$, 609.1819).

*β-(3,4-Dihydroxyphenyl)ethyl-3-O-E-caffeoyl-O-[β-D-apiofuranosyl-(1→2)]-β-D-glucopyranoside (**4**)*:. brown syrup; $[\alpha]^{25}_{D}$ −86.4 (*c* 2.9, MeOH); UV (MeOH) λ_{max} (abs.) 328.5 (0.20), 290.0 (0.18), 203.5 (0.55) nm; IR (KBr) ν_{max} 3439, 2928, 1694, 1607, 1519, 1447, 1281, 1159 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Tables 1 and 2; negative FABMS (glycerol matrix) *m/z* 609 $[M - H]^-$; negative HRESIMS *m/z* 609.1809 $[M - H]^-$ (calcd for $C_{28}H_{33}O_{15}$, 609.1819).

*β-(4-Hydroxyphenyl)ethyl-3-O-E-caffeoyl-O-[β-D-apiofuranosyl-(1→2)]-β-D-glucopyranoside (**5**)*:. brown syrup; $[\alpha]^{25}_{D}$ −65.1 (*c* 4.6, MeOH); UV (MeOH) λ_{max} (abs.) 318.0 (0.22), 291.5 (0.21), 203.5 (0.52) nm; IR (KBr) ν_{max} 3424, 2927, 1694, 1606, 1516, 1446, 1268, 1159 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Tables 1 and 2; negative FABMS (glycerol matrix) *m/z* 593 $[M - H]^-$; negative HRESIMS *m/z* 593.1864 $[M - H]^-$ (calcd for $C_{28}H_{33}O_{14}$, 593.1870).

*4′,5,7-Trihydroxy-3-methoxyflavone-7-O-α-L-arabinofuranosyl (1→6)-β-D-glucopyranoside (**10**)*:. yellow, amorphous powder; $[\alpha]^{25}_{D}$ −41.6 (*c* 1.6, MeOH); UV (MeOH) λ_{max} (abs.) 332.5 (0.22), 268.5 (0.25), 205 (0.45) nm; IR (KBr) ν_{max} 3415, 2921, 1652, 1609, 1341, 1180 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 3; negative HRESIMS *m/z* 593.1493 $[M - H]^-$ (calcd for $C_{27}H_{29}O_{15}$, 593.1506).

Acid Hydrolysis of Compounds 1−5 and 10. Each compound (5 mg) was individually refluxed in 5% sulfuric acid in EtOH (5.0 mL) on a water bath for 4 h. After cooling, the reaction mixture was neutralized with 8% NaOH and concentrated under reduced pressure. The residue was separated on a silica gel column, eluting with MeCN−H_2O (8:1), to yield D-glucose (0.7 mg), $[\alpha]^{25}_{D}$ +43.3 (*c* 0.7, H_2O), and D-apiose (0.5 mg), $[\alpha]^{25}_{D}$ +5.6 (*c* 0.5, H_2O), for **1**, D-glucose (0.6 mg), $[\alpha]^{25}_{D}$ +40.6 (*c* 0.6, H_2O), and D-apiose (0.4 mg), $[\alpha]^{25}_{D}$ +3.4 (*c* 0.5, H_2O), for **2**, D-glucose (0.5 mg), $[\alpha]^{25}_{D}$ +41.6 (*c* 0.5, H_2O), and D-apiose (0.5 mg), $[\alpha]^{25}_{D}$ +4.4 (*c* 0.5, H_2O), for **3**, D-glucose (0.4 mg), $[\alpha]^{25}_{D}$ +40.2 (*c* 0.4, H_2O), and D-apiose (0.5 mg), $[\alpha]^{25}_{D}$ +3.8 (*c* 0.5, H_2O), for **4**, D-glucose (0.5 mg), $[\alpha]^{25}_{D}$ +41.3 (*c* 0.5, H_2O), and D-apiose (0.5 mg), $[\alpha]^{25}_{D}$ +3.6 (*c* 0.5, H_2O), for **5**, and D-glucose (0.5 mg), $[\alpha]^{25}_{D}$ +37.5 (*c* 0.5, H_2O), and L-arabinose (0.4 mg), $[\alpha]^{25}_{D}$ +93.5 (*c* 0.4, H_2O), for **10**, respectively.

Evaluation of Biological Activity. NF-κB luciferase, aromatase, quinone reductase 2 (QR2), cyclooxygenase-1 (COX-1), and cyclooxygenase-2 (COX-2), ultrafiltration LC-MS for QR2, and cytotoxicity assays were conducted as previously described. Below is a brief description of each assay.

NF-κB Luciferase Assay. Panomic (Fremont, CA) has established a number of stably transfected NF-κB reporter cell lines. We have employed human embryonic kidney cells 293 for monitoring any changes occurring along the NF-κB pathway. Cells were seeded into sterile 96-well plates at a density of 20×10^3 cells per well. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Co., Carlsbad, CA), supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. After a 48 h incubation, medium was replaced and various concentrations of test compounds were added (dissolved in PBS). Human recombinant TNF-α (2 ng/mL; 0.14 nM; Calbiochem, Gibbstown, NJ) was used as activator. The plate was incubated for 6 h. Spent media were discarded, and the cells were washed once with PBS. Then, the cells were lysed by adding 50 μL/well of reporter lysis buffer (diluted 5-fold with water) (Promega, Madison, WI) and incubating for 5 min on a shaker. At this point, plates can be stored at −80 °C for subsequent analysis. The luciferase assay was performed using the Luc assay system from Promega. The gene product, luciferase enzyme, reacts with luciferase substrate, emitting light, which was detected using a luminometer (LUMIstar Galaxy BMG). Dose−response curves were constructed, and data were expressed as IC_{50} values (i.e., concentration of tested sample required to inhibit TNF-α-induced NF-κB activity by 50%). Na-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (IC_{50} = 3.76 μM) was used as a positive control.[44]

Aromatase Assay. Test compounds (3.5 μL) were preincubated with 30 μL of a NADPH-regenerating system (2.6 mM $NADP^+$, 7.6 mM glucose 6-phosphate, 0.8 U/mL glucose 6-phosphate dehydrogenase, 13.9 mM $MgCl_2$, and 1 mg/mL albumin in 50 mM potassium phosphate buffer, pH 7.4) for 10 min at 37 °C. The enzyme and substrate mixture [33 μL of 1 μM CYP19 enzyme (BD Biosciences), 0.4 μM dibenzylfluorescein, 4 mg/mL albumin, in 50 mM potassium phosphate, pH 7.4] were added, and the plate was incubated for 30 min at 37 °C before quenching with 25 μL of 2 N NaOH. After termination of the reaction

and shaking for 5 min, the plate was further incubated for 2 h at 37 °C. This enhances the ratio of signal to background. Fluorescence was measured at 485 nm (excitation) and 530 nm (emission). IC_{50} values and dose−response curves were based on three independent experiments performed in duplicate using five concentrations of test substance. Naringenin (IC_{50} = 0.23 μM) was used as a positive control.[45]

Nitric Oxide (NO) Assay. The blocked production of NO is a potential mechanism for chemoprevention. RAW 264.7 cells were incubated in a 96-well culture plate for 24 h. The cells were treated with various concentrations of compounds dissolved in phenol red-free DMEM for 30 min, followed by 1 μg/mL of LPS treatment for 24 h. NO was oxidized to the stable end product, nitrite, by the addition of Griess reagent [1:1 mixture (v/v) of 1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine in 2.5% H_3PO_4], and absorbance was measured at 540 nm. A standard curve was created by using known concentrations of $NaNO_2$. Na-L monomethyl arginine (L-NMMA) (IC_{50} = 19.7 μM) was used as a positive control.[46]

QR2 Assay. The activity of QR2 under steady-state conditions was evaluated on a Molecular Devices SpectraMax Plus 384 UV−visible spectrophotometer by monitoring the decrease in absorbance of the enzyme co-substrate NMeH (*N*-methyldihydronicotinamide) at 360 nm at 25 °C. Reactions were carried out in a 96-well plate and were initiated by the addition of QR2 to the assay buffer (50 mM Tris/HCl, pH 8.0, 100 mM NaCl, and 0.1% Triton X-100) containing various concentrations of menadione (5−75 μM) and various concentrations of NMeH (10−140 μM). Stock QR2 enzyme concentrations were determined using the Bio-Rad protein assay. The final enzyme concentration was 5 nM in a reaction volume of 200 μL. The plate was shaken vigorously for 5 s to mix reagents, and the loss in absorbance upon oxidation of NMeH was monitored until the reaction reached completion. Reaction rates were converted into specific activity using ϵ_{360} = 7060 $M^{-1}\cdot cm^{-1}$ for NMeH, with a well path length of 0.445 cm. The specific activity of QR2 is expressed in μM of NMeH oxidized per mL per min per mg of QR2 added (units/mg). One unit of activity is defined as 1 μL of NMeH oxidized per min. Data were expressed as percentage of inhibition or IC_{50} values (concentration required to inhibit QR2 activity by 50%). Resveratrol was used as a positive control, which showed 50% inhibition against QR2 at a concentration of 0.96 μM.[38]

COX-1 and COX-2 Assays. COX-2 (0.2 μg) or COX-1 (0.2 μg) was activated by adding 146 μL of Tris-HCl buffer (pH 8.0), 2 μL of hematin (1 μM final), and 10 μL of L-epinephrine (2 mM final) at room temperature for 2 min on ice. Then, 2 μL of each test solution was added and preincubated for 10 min in a water bath at 37 °C. Negative control incubations were identical except that 2 μL of Tris-HCl buffer was added instead of the test solution. Celecoxib and indomethacin were used as positive controls in the COX-2 and COX-1 inhibition assays, respectively. The reactions were initiated by adding 20 μL of arachidonic acid (5 μM, final concentration) and terminated after 2 min by adding 10 μL of 2.0 M HCl. A 20 μL amount of d_4-[PGE_2] at 50 ng/mL was then added as internal standard. Both PGE_2 and [d_4]-PGE_2 were extracted from incubates using 800 μL of H_2O saturated with EtOAc. The EtOAc phase was then collected, evaporated to dryness, and reconstituted in 100 μL of MeOH−H_2O (50:50, v/v). The formation of the COX product prostaglandin E_2 was measured using the LC-MS-MS method as described previously (Cao et al., 2008). An Applied Biosystems (Foster City, CA) API 4000 triple quadrupole mass spectrometer with negative ion electrospray and a collision energy of 22 eV equipped with a Shimadzu (Columbia, MD) Prominence UFLC system with a Waters (Milford, MA) XTerra MS C18 (2.1 × 50 mm, 3.5 μm) analytical column was used for PGE_2 measurement. Indomethacin was used as a positive control for the COX-1 inhibition assay. At 10 μM, indomethacin produced 85% inhibition, and 125 nM produced 65% inhibition of ovine COX-1. For assays of COX-2 inhibition, celecoxib was used as a positive control at two different concentrations. At 33 μM, celecoxib inhibited human COX-2 93%, and at 46 nM (approximately the IC_{50} value), celecoxib produced 49% inhibition.[47]

Ultrafiltration LC-MS Binding Assay for QR2. Test compounds were incubated with ovine QR2 for 1 h at 37 °C. The mixture was then filtered through a 30 000 Da molecular weight cutoff ultrafiltration membrane. After washing each sample three times with buffer, the ligands were dissociated from QR2 using methanol. The ligand ultrafiltrates were dried under nitrogen and reconstituted in 50% aqueous methanol prior to LC-MS analysis.[48]

Cytotoxicity Assay. Hepa1c1c7 cells were maintained in MEM-α (minimum essential medium alpha) medium. MCF-7 cells were maintained in MEME (Eagle's minimum essential medium) containing 10 mg/L of insulin. In each case, PSF (100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, 250 ng/mL amphotericin B) was added. All media were supplemented with 10% heat-inactivated FBS. The 190 μL cell suspension (3×10^4 cells in 1 mL of media) was incubated with 10 μL sample solutions, in triplicate, in 96-well tissue culture plate at 37 °C in a humidified atmosphere of 5% CO_2 in air for 72 h. Aqueous (10%) DMSO (10 μL) was used as a control group. Then the cells were fixed to a plastic substratum by the addition of 100 μL of cold 20% aqueous trichloroacetic acid and washing with H_2O after incubation at 4 °C for 30 min. After staining cells with 100 μL of 0.4% sulforhodamine B in 1% aqueous HOAc for 30 min, unbound dye was removed by rinsing with 1% aqueous HOAc. The bound dye was solubilized with 200 μL of 10 mM unbuffered Tris base, pH 10, and the optical density was measured at 515 nm using an ELISA plate reader. The average data were expressed as a percentage, relative to the negative control. Paclitaxel and vinblastine were used as positive controls. At 23.4 μM, paclitaxel produced 64% and 57% inhibition of MCF-7 and Hepa1c1c7, respectively. At 49.4 μM, vinblastine produced 74% and 90% inhibition of MCF-7 and Hepa1c1c7, respectively.[49]

■ ASSOCIATED CONTENT

Supporting Information. Spectroscopic data (1D and 2D NMR spectra) of compounds **1**−**5** and **10** are available free of charge via the Internet at http://pubs.acs.org.

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