Effects of Eleven Flavonoids from the Osteoprotective Fraction of *Drynaria fortunei* (Kunze) J. Sm. on Osteoblastic Proliferation Using an Osteoblast-Like Cell Line

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***Drynaria fortunei* (Kunze) J. Sm. (DFE) is one of the most frequently used traditional Chinese medicines prescribed for the treatment of osteoporosis in China. The present study was designed to investigate the osteoprotective constituents from *Drynaria fortunei*. The 60% ethanol extract of the rhizome of *D. fortunei* (DFE) was chromatographed on a D-101 macroporous resin column (ϕ25×150 cm); three fractions (DFA eluted with water, DFB eluted with 30% and 50% ethanol, and DFC eluted with 95% ethanol) were obtained and their osteoprotective activity as examined both *in vivo* and *in vitro*. DFB showed significant activity on both the proliferation of UMR106 cells and promoting bone mineral density (BMD) in ovariectomized (OVX) mice. A bioactivity-guided method led to the isolation of 11 flavonoids from this fraction (DFB) with antiosteoporotic activity, including two new compounds, kaempferol 3-*O*-β-D-glucopyranoside-7-*O*-α-L-arabinofuranoside (1) and (*R*)-5,7,3′,5′-tetrahydroxy-flavanone 7-*O*-neohesperidoside (2), along with nine known ones: three flavanones (3—5), one flavone (7), one flavonol (6), two chromones (8, 10), maltol glucoside (9), and (−)-epicatechin (11). Compounds 4—11 are reported for the first time from this genus. We investigated the proliferative effects of the 11 compounds in the UMR106 osteoblastic cell line *in vitro*. All compounds exhibited the proliferative activity in the UMR106 cells at most of the concentrations tested. Most compounds are reported for the first time from the *Drynaria* genus and this was the first study of their proliferative activity in osteoblast-like cells. The main peaks in the HPLC fingerprint of the active fraction (DFB) were also identified.**

Key words *Drynaria fortunei*; flavonoid; osteoporosis; UMR 106 cell; 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2*H*-tetrazolium); ovariectomized mice

Osteoporosis is a metabolic bone disease characterized by a reduction in bone mass and microarchitectural deterioration of bone tissue, resulting in skeletal fragility and susceptibility to fractures, especially of the wrist, spine, and hip.[1,2] The approach to the design of antiosteoporotic drugs follows the two basic processes of bone remodeling including agents that target the prevention of bone resorption (estrogen, calcitonin, bisphosphonates, raloxifene) as well as agents that stimulate bone formation (fluoride, anabolic steroids).[3—9] Postmenopausal osteoporosis induced by estrogen deficiency is the most common cause of age-related bone loss. Estrogen replacement therapy (ERT) can help relieve postmenopausal syndrome,[10,11] but has a low acceptance rate due to the possible increase in the risks of breast and endometrial cancers with long-term use.[12] In addition, the most frequently used antiosteoporotic drugs were developed in affluent countries and the costs are too high to benefit a large population in the developing or even developed countries for the prevention and treatment of osteoporosis. Thus an alternative approach for the management of osteoporosis is desirable.

Chinese herbal medicine has been widely used in clinical practice to treat bone disease for thousands of years and will undoubtedly continue to be used as a cost-effective alternative to commercial pharmaceutical products by traditional users.[13—16] *Drynaria fortunei* (Kunze) J. Sm. is a folk medicine with a long history of safe use for the treatment of bone fractures and joint diseases in China. Many studies reported on the therapeutic effects of the rhizomes of Gusuibu on osteoporosis and bone fracture,[17—20] especially of the flavonoids tested in the ovariectomized (OVX) rat model.[21,22] However, few phytochemical studies have been conducted on the active constituents leading to the osteoprotective effects of the rhizome.[23] In our bioactive screening assay, the 60% alcohol extract of *D. fortunei* was found to stimulate the proliferation of osteosarcoma UMR106 cells. Activity-guided isolation was carried out, following examination in OVX models as well as UMR106 cells, which were used to test the samples *in vivo* and *in vitro*, respectively. The present study deals with the isolation and identification of 11 compounds from the active fraction of the extract of *D. fortunei* and the effects of these compounds on the proliferation of UMR106 cells. Two were new compounds and eight were reported for the first time from the *Drynaria* genus. This was the first study of their proliferative activity in osteoblast-like cells, except for naringin.[23] The main components of the active fraction were clarified using HPLC.

Results and Discussion

Effects of 60% Ethanol Extract of DFE and Extract Fractions Chromatographed on D-101 Resin on the Proliferation of UMR106 Cells and Bone Mineral Density in Mice The UMR106 cell line is a rat osteosarcoma cell

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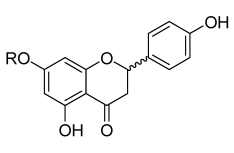
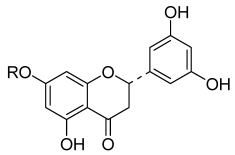
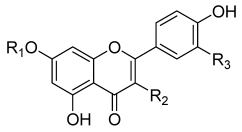


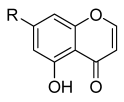
3 [(2R)-naringin]
R= neohesperidosyl
4 [(2S)-narigenin-7-*O*-*β*-D-glucoside]
R= *β*-D-glucopyranosyl



2 [(2S)-5,7,3',5'-tetrahydroxy-flavonone 7-*O*-hesperidoside]
R = neohesperidosyl
5 [(2S)-5,7,3′,5′-tetrahydroxy-flavanone 7-*O*-*β*-D-glucopyranoside]
R = *β*-D-glucopyranosyl



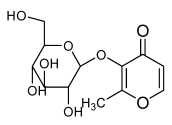
1 (kaempferol, 3-*O*-*β*-D-glucopyranoside-7-*O*-*α*-L-arabinofuranoside)
R_1=*α*-L-arabinofuranosyl,
R_2=*O*-*β*-D-glucopyranosyl,
R_3=H
6 (kaempferol, 3-*O*-*α*-L-rhamnosyl-7-*O*-*β*-D-glucoside)
R_1= *β*-*D*-glucopyranosyl,
R_2=*O*- *α*-L-rhamnopyranosyl,
R_3=H
7 (luteolin-7-*O*-*β*-D-neohesperidoside)
R_1=neohesperidosyl,
R_2=H,
R_3=OH



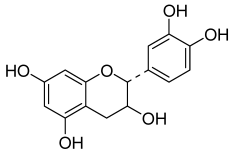
8 (5,7-dihydroxychromone-7-*O*-*β*-D-glucopyranoside)
R= *O*-*β*-D-glucopyranosyl

10 (4H-1-benzopyran-4-one,7-[[2-*O*-(6-deoxy-α-L-mannopyranosyl)- *β*-D-glucopyranosyl]oxy]-5-hydroxy)
R= *O*-neohesperidosyl

neohesperidose= *α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl



9 (maltol glucoside)



11 [(-)-epicatechin]

Chart 1. The Structures of the Isolated Compounds from *Drynaria fortunei* (Kunze) J. Sm

line with osteoblast-like phenotypic properties. Insulin-like growth factor (IGF)-I is a peptide growth factor that can stimulate bone formation and was used as a positive control in the present study. The 60% ethanol extract of *D. fortunei* (DFE) stimulated the proliferation of UMR106 cells by 11.79% ($p<0.05$, Table 1) and 12.75% ($p<0.05$, Table 1) at concentrations of 1 μg/ml and 100 ng/ml, respectively. DFE was chromatographed on a D-101 macroporous resin column and eluted with gradients of water, and 30%, 50%, and 95% alcohol to give DFA (water ethanol eluate), DFB (30% and 50% ethanol eluate), and DFC (95% ethanol eluate). Table 1 shows the effects of DFE and fractions DFA, DFB, and DFC, on the proliferation of UMR106 cells. DFB increased proliferation by 10.26% ($p<0.05$), 16.42% ($p<0.05$), and 20.73% ($p<0.01$) at concentrations of 10 μg/ml, 1 μg/ml, and 0.1 μg/ml, respectively. DFC also stimulated the proliferation of UMR106 cells by 13.8% ($p<0.05$) and 9.79% ($p<0.05$) at concentrations of 10 μg/ml and 1 μg/ml, respectively. DFA showed no effects on the proliferation of UMR106 cells. As shown in Fig. 1, DFB significantly increased both the trabecular bone mineral density (BMD) and the total BMD of the mouse femoral head.

Bioassay-Guided Separation of the Constituents of *D. fortunei* Repeated chromatography of the active fraction (DFB) on SiO_2, Sephadex LH-20, and ODS columns afforded two new flavonoids and nine known ones. Eleven flavonoids were characterized on the basis of comprehensive analyses of their mono- and bidimensional NMR (DEPT-135, ^{1}H–^{1}H COSY, HMQC, and HMBC) spectra, and the nine known compounds were subsequently identified as (2*R*)-naringin (**3**),[23] (2*S*)-narigenin-7-*O*-*β*-D-glucoside (**4**),[24] (2*S*)-5,7,3′,5′-tetrahydroxy-flavanone 7-*O*-*β*-D-glucopyranoside (**5**),[25] kaempferol 3-*O*-*α*-L-rhamnoside-7-*O*-*β*-D-glucoside (**6**),[26] luteolin-7-*O*-*β*-D-neohesperidoside (**7**),[27,28] 5,7-dihydroxychromone-7-*O*-*β*-D-glucopyranoside (**8**),[29] maltol glucoside (**9**),[30,31] 4*H*-1-benzopyran-4-one,7-[[2-*O*-(6-deoxy-*α*-L-mannopyranosyl)-*β*-D-glucopyranosyl]oxy]-5-hydroxy (**10**),[32] and (−)-epicatechin (**11**)[33] by comparison of their spectral data (UV, IR, NMR, and MS) with those reported previously. Figure 2 exhibits the HPLC fingerprint of DFB. The main components in it were compounds **1**, **2**, **3**, **6**, and **10** and they were thought to be the key principles in osteoprotective activity.

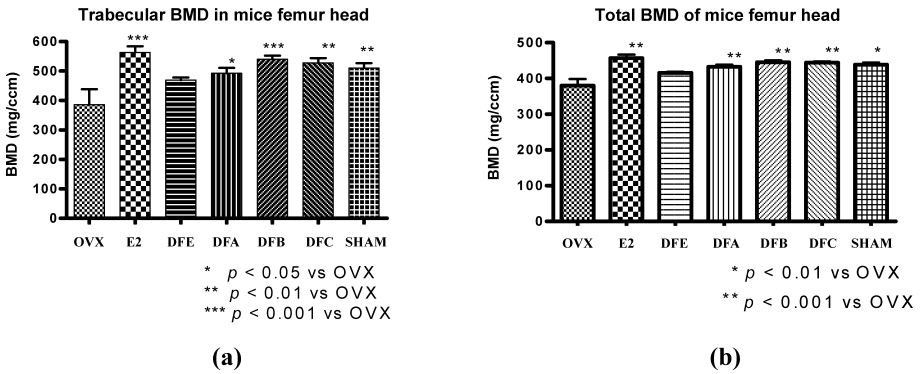
Compound **1** was isolated as pale yellow powder and shown to be a flavonoid based on TLC visualization under UV light, which was confirmed by its UV absorption maxima at 346 nm, 266 nm, and 202 nm in methanol (typical of flavonol glycosides). The IR absorption bands at 3287, 1659,

Table 1. Effects of the 60% Ethanol Extract of *Drynaria fortunei* (DFE) and the Fractions of the Extract Chromatographed with D-101 Macroporous Resin Column (DFA, DFB and DFC) on Proliferation of the UMR106 Cells

Samples	Concentration (μg/ml)	$A_{control}$ at 409 nm (mean±S.D.)	A_{sample} at 409 nm (mean±S.D.)	Proliferation ratio (%)
DFE	0.01	1.158±0.068	1.122±0.056	−3.6
	0.1	1.158±0.068	1.165±0.139	0.8
	1	1.158±0.068	1.274±0.086	11.8*
	10	1.158±0.068	1.284±0.090	12.8*
	100	1.158±0.068	1.188±0.026	3.1
DFA	0.01	0.966±0.049	0.982±0.032	−4.8
	0.1	0.966±0.049	0.949±0.042	−2.1
	1	0.966±0.049	0.985±0.049	2.0
	10	0.966±0.049	0.990±0.030	2.5
	100	0.966±0.049	0.964±0.037	−0.2
DFB	0.01	1.158±0.068	1.225±0.019	6.8
	0.1	1.158±0.068	1.259±0.030	10.3*
	1	1.158±0.068	1.320±0.033	16.4**
	10	1.158±0.068	1.362±0.035	20.7**
	100	1.158±0.068	0.129±0.018	−2.9
DFC	0.01	1.158±0.068	1.294±0.024	13.8**
	0.1	1.158±0.068	1.254±0.055	9.8*
	1	1.158±0.068	1.052±0.025	−10.7*
	10	1.158±0.068	1.053±0.030	−10.5*
	100	1.158±0.068	0.237±0.018	−93.1**
Igf-1	00.1	1.158±0.068	1.401±0.027	24.6**

$*p<0.05$ *vs.* control, $**p<0.01$ *vs.* control.

and 1589 cm^{-1} showed the presence of hydroxyl, carbonyl, and aromatic rings respectively. HR-ESI-TOF-MS showed a *pseudo* molecular ion peak at *m/z* 603.1334 $[M+Na]^+$ (Calcd 603.1326), presenting the molecular formula of $C_{26}H_{28}O_{15}$, which was also confirmed by the *quasi* molecular ion peaks (*m/z* 603 $[M+Na]^+$ and *m/z* 579 $[M-H]^-$) obtained from the positive and negative ESI ion-trap (IT)-MS experiments. In the ESI-IT-MS^n experiments, the MS^2 spectrum of the ion at *m/z* 603 $[M+Na]^+$ showed a positive fragment at *m/z* 471 $[M+Na-132]^+$ and the MS^3 spectrum of the ion at *m/z* 471 $[M+Na-132]^+$ showed a positive fragment at 309 $[(M+Na-132)-162]^+$, suggesting that there were one hexose unit and one pentose unit in **1**. The monosaccharides obtained after acid hydrolysis of **1** were derivatized into aldononitrile peracetate derivatives and analyzed with GC-MS using authentic samples as references. The absolute configurations of the sugar residues were assumed to be D-glucose and L-arabinose. The ^{1}H-NMR spectrum showed the presence of kaempferol as an agylcone, which was characterized by two doublet signals at δ_H 8.07 (H-2′, 6′) and 6.90 (H-3′, 5′) and two doublet signals at δ_H 6.76 (H-8) and 6.42 (H-6). The ^{13}C-NMR spectrum showed 26 carbon signals, corresponding to a flavanone skeleton bearing two sugar units.[34] These signals were similar to those of kaempferol, 3-*O*-β-D-glucoside, except for one sugar unit.[35] The anomeric signal of the glucose unit appeared at δ_C 100.8



(a) **(b)**

Fig. 1. Effects of the 60% Ethanol Extract (DFE) of *Drynaria fortunei* (Kunze) J. Sm. and the Fractions of the Extract Chromatographed on a D-101 Macroporous Resin Column (DFA, DFB, DFC) on OVX Mice, and Estrodiol (E_2) as a Positive Control

Values are mean±S.E.M. $*p<0.05$ in mice treated with E2, DFE, DFA, DFB, or DFC *versus* sham-operated mice. $**p<0.01$ in mice treated with E2, DFE, DFA, DFB, or DFC *versus* sham-operated mice. $***p<0.001$ in mice treated with E2, DFE, DFA, DFB, or DFC *versus* sham-operated mice. (a) Trabecular BMD in mice femoral head. (b) Total BMD of mice femoral head.

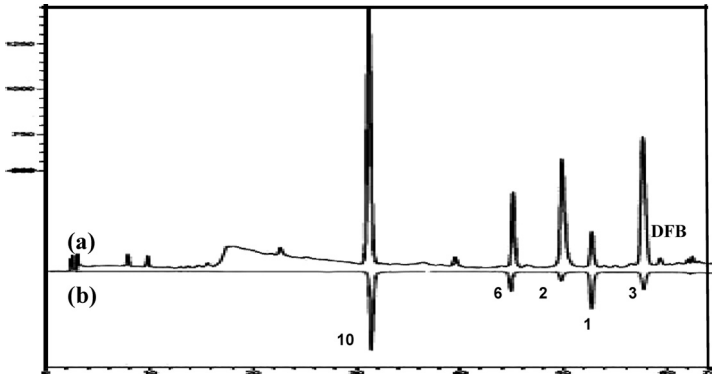


Fig. 2. HPLC Chromatogram of Antiosteoporotic Fraction (DFB) and Its Main Components

The upper line (a) is the HPLC profile of DFB, while the lower one (b) is the mixture of the five labeled compounds. HPLC system, Agilent series 1100; HPLC conditions: column, Shimadzu C_{18}; column 250×4.6 mm; mobile phase, (A) H_2O, (B) MeOH; elution program, 0—25 min, linear gradient from 10%B to 40%B in 60 min and then 100%B maintained for 10 min; flow rate, 1.00 ml/min; detection wavelength, 254 nm; injection volume: 10 μl; oven temperature, 30 °C.

and at δ_H 5.47 (1H, d, J=7.3 Hz), showing the relative configuration of β. Five additional ^{13}C-NMR signals were observed at δ 106.3, 81.9, 76.4, 85.5, and 61.0, indicating that the presence of a pentose unit which was identified as an arabinopyranosyl by analysis of the ^{1}H–^{1}H coupling constants, and these data were consistent with published data for arabinofuranosides rather than arabinopyranosides.[36,37] The HMBC correlation signals found at δ_H 5.47 (H-1″)/δ_C 133.5 (C-3) and δ_H 5.60 (H-1‴)/δ_C 162.2 (C-7) suggested that the glucopyranosyl unit was located at C-3 and that the arabinofuranosyl unit was attached to C-7. Each carbohydrate spin system was assigned by exhaustive analyses of ^{1}H–^{1}H COSY, HMQC, and HMBC experiments. On the basis of all spectroscopic data, **1** was determined to be kaempferol-3-*O*-β-D-glucopyranosyl-7-*O*-α-L-arabinofuranoside, which is a new flavonol glycoside. Table 2 gives the NMR data for **1**.

Compound **2** was obtained as a pale yellow powder. The IR absorption bands at 3425, 1647, 1585, 1519, and 1454 cm^{-1} suggested the presence of hydroxyl, carbonyl, and aromatic rings, respectively. The characteristic UV absorption bands (328, 283 nm) showed compound **2** to be a flavanone. The HR-ESI-TOF-MS showed a *pseudo* molecular ion peak at *m/z* 619.1646 $[M+Na]^+$ (Calcd 619.1639), representing the molecular formula of $C_{27}H_{32}O_{15}$, which was confirmed by the *quasi* molecular ion peaks (*m/z* 619 $[M+Na]^+$ and *m/z* 595 $[M-H]^-$) obtained from the positive and negative ESI-IT-MS experiments, respectively. In the ESI-MS^n experiments, the MS^2 spectrum of the positive *quasi* molecular ion peak *m/z* 619 $[M+Na]^+$ gave fragments at *m/z* 473 $[M-146+Na]^+$, for which the MS^3 spectrum gave fragments at *m/z* 311 $[M-146-162+Na]^+$, suggesting the attachment of two sugars, a 6-deoxyhexose and a hexose. The monosaccharides obtained after acid hydrolysis of **2** were derivatized into aldononitrile peracetate derivatives and analyzed by GC-MS using authentic samples as references. The absolute configurations of the sugar residues were assumed to be D-glucose and L-rhamnose. ^{1}H- and ^{13}C-NMR spectra (Table 3) resembled those of compound **5**, except for the additional of six signals due to a sugar moiety and also were similar to those of compound **3**, differing only in the observation of ABM-type aromatic signals instead of A_2B_2-type signals, and thus it has the same aglycone as compound **5** and the same *O*-neohesperidoside as compound **3**. The MS^2 spectrum of

Table 2. ^{1}H-NMR (400 MHz, Coupling Constant J in Parentheses), ^{13}C-NMR (100 MHz) Data in DMSO-d_6 for **1** and Data in CD_3OD for **2**

Position	**1**		**2**	
	δ_H (J Hz)	δ_C	δ_H (J Hz)	δ_C
2	—	156.7	5.23 dd (2.4, 2.0)	80.7
3	—	133.5	3.15 dd (17.2, 2.0), 2.785 dd (17.2, 2.4)	44.1
4	—	177.6		198.5
5	—	160.8		165.0
6	6.42 d (1.8)	99.3	6.15 d (2.4)	97.9
7	—	162.2		166.6
8	6.76 d (1.8)	94.5	6.17 d (2.4)	96.8
9	—	156.0		164.7
10	—	105.5		104.9
1′	—	120.7		131.5
2′	8.07 d (8.9)	130.9	6.78 br s	119.4
3′	6.90 d (8.9)	115.1		146.9
4′	—	160.2	6.91 br s	114.8
5′	6.90 d (8.9)	115.1		146.5
6′	8.07 d (8.9)	130.9	6.78 br s	116.3
	3-*O*-Glc		7-*O*-Glu-2-Rha	
1″	5.47 d (7.3)	100.8	5.09 d (7.3)	99.4
2″	3.16 m	74.2	3.62 m	79.2
3″	3.08 m	77.5	3.62 m	78.9
4″	3.09 m	69.9	3.39 m	71.2
5″	3.19 m	76.4	3.54 m	78.1
6″	3.60, 3.48, m[a)]	60.8	3.86, 3.67 m	62.3
	7-*O*-Ara		7-*O*-Glu-2-Rha	
1‴	5.60 d (1.6)	106.3	5.23 d (1.6)	102.6
2‴	4.13 m	81.9	3.93 m	72.2
3‴	3.83 m	76.4	3.58 m	72.2
4‴	3.91 m	85.5	3.38 m	73.7
5‴	3.57, 3.35 m[a)]	61.0	3.87 m	70.0
6‴	—	—	1.21 d (6.0)	18.2

a) Coupling constant not clearly defined or obscured by overlap.

Table 3. The MTS Assay of Eleven Pure Compounds Isolated from *Drynaria fortunei* on UMR106 Cells (Mean±S.D.)

Samples	Concentration (M)	$A_{control}$ (mean±S.D.)	A_{sample} (mean±S.D.)	Proliferation ratio (%)
1	10^{-8}	0.651±0.040	0.883±0.048	35.6***
	10^{-7}	0.651±0.040	0.786±0.051	20.7**
	10^{-6}	0.651±0.040	0.928±0.029	42.5***
	10^{-5}	0.651±0.040	0.788±0.771	21.0**
2	10^{-8}	0.495±0.020	0.534±0.018	7.9*
	10^{-7}	0.495±0.020	0.519±0.016	4.8
	10^{-6}	0.495±0.020	0.548±0.037	10.7*
	10^{-5}	0.495±0.020	0.550±0.007	11.1***
3	10^{-8}	0.495±0.020	0.558±0.014	12.7**
	10^{-7}	0.495±0.020	0.593±0.073	19.8*
	10^{-6}	0.495±0.020	0.584±0.020	18.0***
	10^{-5}	0.495±0.020	0.577±0.021	16.6***
4	10^{-8}	0.495±0.020	0.543±0.042	9.7*
	10^{-7}	0.495±0.020	0.538±0.025	8.7*
	10^{-6}	0.495±0.020	0.544±0.042	9.9*
	10^{-5}	0.495±0.020	0.527±0.031	6.5
5	10^{-8}	0.495±0.020	0.520±0.012	5.1*
	10^{-7}	0.495±0.020	0.501±0.034	1.2
	10^{-6}	0.495±0.020	0.538±0.034	8.7*
	10^{-5}	0.495±0.020	0.537±0.040	8.5
6	10^{-8}	0.851±0.030	0.961±0.065	12.9*
	10^{-7}	0.851±0.030	0.951±0.058	11.8*
	10^{-6}	0.851±0.030	0.908±0.055	6.7
	10^{-5}	0.851±0.030	0.889±0.078	4.5
7	10^{-8}	0.851±0.030	0.883±0.061	3.8
	10^{-7}	0.851±0.030	0.939±0.028	10.3**
	10^{-6}	0.851±0.030	0.935±0.034	9.9**
	10^{-5}	0.851±0.030	0.959±0.032	12.7**
8	10^{-8}	0.630±0.031	0.678±0.022	7.6*
	10^{-7}	0.630±0.031	0.715±0.021	13.5**
	10^{-6}	0.630±0.031	0.683±0.006	8.4*
	10^{-5}	0.630±0.031	0.691±0.010	9.7*
9	10^{-8}	0.851±0.030	0.919±0.037	8.0*
	10^{-7}	0.851±0.030	0.965±0.026	13.4***
	10^{-6}	0.851±0.030	0.946±0.051	11.2*
	10^{-5}	0.851±0.030	0.866±0.023	1.8
10	10^{-8}	0.630±0.031	0.703±0.009	11.6**
	10^{-7}	0.630±0.031	0.713±0.027	13.2**
	10^{-6}	0.630±0.031	0.688±0.033	9.2*
	10^{-5}	0.630±0.031	0.681±0.061	8.1
11	10^{-8}	0.851±0.030	0.877±0.067	3.1
	10^{-7}	0.851±0.030	0.897±0.074	5.4
	10^{-6}	0.851±0.030	0.947±0.017	11.3***
	10^{-5}	0.851±0.030	0.928±0.071	9.0*

* $p<0.05$ *vs.* control, ** $p<0.01$ *vs.* control, *** $p<0.001$ *vs.* control.

the *quasi* molecular ion peak *m/z* 595 $[M-H]^-$ gave fragment *m/z* 459 $[M-H-136]^-$, and the loss of 136 mass units is characterisitc of a flavanoid 7-*O*-hesperidose, as shown by a retro-Diels-Alder (RDA) fragmentation in the C-ring of the aglycone involving a cleavage of bonds 1 and 3 (bonds 1 and 3 refer to the O–C-2 and C-3–C-4 bonds of the C-ring).[38] This is also confirmed by the loss of 120 mass units in the MS^2 of naringin (compound **3**) in the negative mode in the same RDA fragmentation. In the B-ring, 3,5-substitution were confirmed by the ABM system protons at δ_H 6.91 (1H, br s) and 6.78 (2H, br s). The CD spectrum showed a positive Cotton effect at 328 nm and a negative one at 283 nm, consistent with the *S*-configuration at C-2.[25,39] Based on the information mentioned above, compound **2** was identified as (2*S*)-5,7,3′,5′-tetrahydroxy-flavanone 7-*O*-neohesperidoside, which is a new flavanone glycoside because of the 3′,5′-substitute mode in the B ring of the aglycone.

Proliferative Effects of Eeleven Flavonoids from Rhizomes of *D. fortunei* Table 3 shows the proliferative effects of the 11 flavonoids from the rhizomes of *D. fortunei* on UMR106 cells. Kaempferol, 3-*O*-*β*-D-glucopyranoside-7-*O*-*α*-L-arabinofuranoside (**1**), potently stimulated the proliferation of UMR106 cells by 35.9% and 42.6% at the concentrations of 10^{-8} M and 10^{-6} M, respectively, compared with control values, was the most effective compound. Comparing the four flavanoids, the compounds with two sugar moieties [(2*S*)-5,7,3′,5′-tetrahydroxy-flavonone 7-*O*-neohesperidoside (**2**) and (2*R*)-naringin (**3**)] were more effective than those with one sugar moiety [(2*S*)-narigenin-7-*O*-*β*-D-glucoside (**4**) and (2*S*)-5,7,3′,5′-tetrahydroxy-flavanone 7-*O*-*β*-D-glucopyranoside (**5**)]. The others exhibited proliferative activity in UMR106 cells to a certain degree at most concentrations.

Experimental

General The optical rotations were measured with a P-1020 digital polarimeter (Jasco, Japan). The UV and IR spectra were recorded on UV2401PC (Shimadzu, Japan) and FT/IR-8400 (Shimadzu, Japan) spectrophotometers, respectively. NMR (^{1}H-NMR: 400 MHz, ^{13}C-NMR: 100 MHz) spectra were measured on an Avance 400 Fourier transform spectrometer (Bruker). Chemical shifts are expressed in δ (ppm) and coupling constants (*J*) are reported in Hertz (Hz). The ESI-MS were obtained on a Bruker Esquire 2000 mass spectrometer. HR-ESI-MS spectra were recorded using a Micromass Q-TOF mass spectrometer. CD data were recorded on a JASCO J-715 instrument. GC-MS were obtained on a Shimadzu GC-MS-2010 gas chromatograph mass spectrometer with AOC-20i autoinjection and AOC-20s autosampling. Preparative HPLC and analytical HPLC were performed on a Shimadzu Pak, UV detector for RP-HPLC. Silica gel 60 (Qingdao Haiyang Chemical Co., Ltd., China), Sephadex LH-20 (Advanced Technology Industrial Co., Ltd.), ODS (40—75 μm, Fuji Silysia Chemical Ltd., Japan), and D-101 macroporous resin (Tianjin Chemical Co., Ltd., China) were used as column chromatography stationary phases. D-glucose, L-arabinose, and L-rhamnose were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

The UMR106 cell line was purchased from the American Type Culture Collection, no. CRL-1661. Dulbecco's modified Eagle's medium (DMEM), FBS, phenol red-free DMEM, charcoal-stripped FBS, and 0.5% trypsin-EDTA 5.3 mM (10×) were purchased from Gibco BRL. Penicillin–streptomycin–glutamine (100×) was obtained from Invitrogen Corporation. Ninety-six-well plates and plastic dishes (100×20 mm) for cell culture were obtained from Falcon (U.S.A.). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2*H*-tetrazolium) (MTS) was a product of USBTM (Austria). All compounds were dissolved in DMSO at a concentration of 10^{-2} M as stock solution, diluted with DMSO to gradients of 10^{-3} M, 10^{-4} M, 10^{-5} M and then freshly prepared at a dilution of 1 : 1000 in phenol red-free DMEM supplemented with 1% charcoal-stripped FBS.

Seventy 6-month-old female C57BL/6J mice were purchased from Guangzhou University of Traditional Chinese Medicine (Guangzhou, China). Bone mass was measured with peripheral quantitative computerized tomography (pQCT) with an XCT2000 machine (Norland Stratec Medizintechnik GmbH, Birkenfeld, Germany). Regular diet (0.6% Ca) was purchased from Harlan Teklad (Madison, WI, U.S.A.) and the formula was referenced as the normal Ca diet in a previous study.[40]

Plant Materials Rhizomes of *D. fortunei* were collected in Guizhou province, China, in July 2005 and were identified by Professor Rong-Han Zhou (Chinese Pharmaceutical University, Nanjing, Jiangsu, China). A voucher specimen is preserved in the Shenzhen Research Center of Traditional Chinese Medicines and Natural Products, Shenzhen, China.

Extraction and Isolation The rhizomes of *D. fortunei* (50 kg) were cut and extracted with 60% ethanol (400 l, reflux, 2 h, ×2) to yield a 60% ethanol extract (DFE, 3.4 kg). DFE was chromatographed on a D-101 macroporous resin column (ϕ 25×150 cm) and eluted with gradients of water and 30%, 50%, and 95% alcohol to give DFA (water ethanol eluate, 2.2 kg), DFB (30% and 50% ethanol eluate, 0.6 kg), and DFC (95% ethanol eluate, 0.072 kg). The 0.2 kg DFB fraction was subjected to silica gel column chromatography (ϕ 7.5×60 cm) with $CHCl_3$/MeOH to yield seven fractions: fr. 1 (100 : 0—95 : 5 $CHCl_3$/MeOH eluate, 3.7 g), fr. 2 (90 : 10 $CHCl_3$/MeOH eluate, 2.0 g), fr. 3 (80 : 20 $CHCl_3$/MeOH eluate, 10.3 g), fr. 4 (70 : 30 $CHCl_3$/MeOH eluate, 40.0 g), fr. 5 (70 : 30 $CHCl_3$/MeOH eluate, 72.0 g), fr. 6 (60 : 40 $CHCl_3$/MeOH, 31 g), fr. 7 (MeOH eluate, 19.6 g). Fr. 3 (10.3 g) was chromatographed on MPLC on ODS (ϕ 2×20 cm) with MeOH/H_2O (10—100%) to yield five subfractions. Subfraction 3-1 (6.3 g) was subjected to another MPLC on ODS (ϕ 2×20 cm) with MeOH/H_2O (10%) to give three subfractions: 3-1a (1.8 g), 3-1b (1.2 g), and 3-1c (2.0 g). 3-1a (1.8 g) was subjected to Sephadex LH-20 column chromatography (ϕ 2×39 cm) with MeOH/H_2O (1 : 1) to yield three subfractions, 3-1a-1 (400 mg), 3-1a-2 (1.0 g), and 3-1a-3 (200 mg), which were then subjected to preparative RP-HPLC eluted with MeOH/H_2O (ρ=0.966) to afford **9** (15 mg), **8** (60 mg), and **11** (8 mg), respectively. 3-1c (2.0 g) was subjected to Sephadex LH-20 column chromatography (ϕ 2×37 cm) with MeOH/H_2O (1 : 1) to yield five subfractions, 3-1c-1—3-1c-5. 3-1c-3 was then subjected to preparative RP-HPLC eluted with MeOH/H_2O (ρ=0.942) to afford **4** (10 mg). Fr. 4 (40.0 g) was chromatographed on MPLC on ODS (ϕ 2×20 cm) with MeOH/H_2O (10—100%) to yield five subfractions. Subfraction 4-3 (9 g) was subjected to silica gel column chromatography (ϕ 7.5×60 cm) with $CHCl_3$/MeOH (0—100%) to yield six subfractions. 4-3-5 was subjected to Sephadex LH-20 column chromatography (ϕ 3×41 cm) with MeOH/H_2O (3 : 7) to yield eight subfractions. Compound **10** (50 mg) was crystallized from the fifth subfraction. Subfraction 4-4 (2.0 g) was subjected to Sephadex LH-20 column chromatography (ϕ 3×41 cm) with MeOH/H_2O (3 : 7) to yield 13 subfractions: 4-3-1—4-3-12. Subfraction 4-3-12 (17.8 mg) was then subjected to preparative RP-HPLC eluted with MeOH/H_2O (ρ=0.946) to afford **5** (8 mg). Fr. 5 (72 g) was chromatographed on MPLC on ODS (ϕ 2×20 cm) with MeOH/H_2O (10—100%) to yield five subfractions. Subfraction 5-1 (8 g) was subjected to Sephadex LH-20 column chromatography (ϕ 3×41 cm) with $CHCl_3$/MeOH (30%) to yield nine subfractions. Subfraction 5-1-4 was then subjected to preparative RP-HPLC eluted with MeOH/H_2O (ρ=0.949) to afford **6** (100 mg), **2** (300 mg), **1** (12 mg), and **3** (100 mg) Fr. 6 (20 g) was chromatographed on MPLC on ODS (ϕ 2×20 cm) with MeOH/H_2O (10—100%) to yield five subfractions. Subfraction 6-2 (6.4 g) was then subjected to preparative RP-HPLC eluted with MeOH/H_2O (ρ=0.958) to afford four subfractions. Subfraction 6-2-3 was subjected to Sephadex LH-20 column chromatography (ϕ 1.5×41 cm) with MeOH/H_2O (70%) to yield **7** (34 mg).

Compound **1** (kaempferol 3-*O*-*β*-D-glucopyranoside-7-*O*-*α*-L-arabinofuranoide): pale yellow powder; $[\alpha]_D^{25}$ −93.9° (*c*=1.0, DMSO); UV λ_{max} [MeOH, 346 nm (log ε 3.84), 266 nm (log ε 3.87), 202 nm (log ε 4.17)]; IR (KBr) cm^{-1}: 3287, 2889, 2361, 2341, 1659, 1589, 1489, 1342, 1288, 1265, 1211, 1169, 999, and 945; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ_H and ^{13}C-NMR (DMSO-d_6, 100 MHz) δ_C given in Table 2. ESI-MS *m/z* 603 $[M+Na]^+$, 579 $[M-H]^-$; HR-ESI-TOF-MS: *m/z* 603.1334 $[M+Na]^+$ (Calcd for $C_{26}H_{28}O_{15}Na$: 603.1326).

Compound **2** [(2*S*)-5,7,3′,5′-Tetrahydroxy-flavonone 7-*O*-Neohesperidoside]: Pale yellow powder; $[\alpha]_D^{25}$ −10.8° (*c*=1.3, MeOH); UV λ_{max} [MeOH, 325 nm (log ε 3.58), 284 nm (log ε 4.35)]; IR (KBr) cm^{-1}: 3425, 1647, 1585, 1519, 1454; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ_H and ^{13}C-NMR (DMSO-d_6, 100 MHz) δ_C was given in Table 2; CD (MeOH): $[\theta]_{328\,nm}$ $+2.25\times10^4$, $[\theta]_{283\,nm}$ -5.0×10^4; ESI-MS: *m/z* 619 $[M+Na]^+$, 595 $[M-H]^-$; HR-ESI-TOF-MS: *m/z* 619.1646 $[M+Na]^+$ (Calcd for $C_{27}H_{32}O_{15}Na$: 619.1639).

Determination of the Absolute Configuration of Sugars Compounds **1** and **2** (each 2 mg) were dissolved in 1 N HCl (2 ml) and then heated to 80 °C for 2 h. The solvent was removed under N_2. After extraction with

acetic ether, the aqueous layer was concentrated to dryness using N_2. The residue was dissolved in 0.1 ml of dry pyridine and then L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 ml) was added to the solution. The reaction mixture was heated at 60 °C for 2 h. After drying with N_2, 0.1 ml of *N*-(trimethylsilyl)imidazole was added, followed by heating at 60 °C for 1 h. The product was partitioned between cyclohexane and H_2O (0.1 ml each), and the organic layer was analyzed using gas chromatography (GC).[41,42] GC-MS analysis conditions were: column DB-50, 25 mm×30 m; column oven temperature, 150 °C (keep 2 min)→300 (keep 10 min); rate, 15 °C/min; injection temperature, 250 °C; and carrier gas, He (1.0 ml/min). Peaks of the hydrolyzate of **1** were detected at 14.50 min (L-arabinose) and 16.45 min (D-glucose); and peaks of the hydrolyzate of **2** were detected at 15.28 min (L-rhamnose) and 16.45 min (D-glucose). Treated in the same way, standard D-glucose (Sigma) gave a peak at t_R (min) 16.45, L-rhamnose (Sigma) gave a peak at t_R (min) 15.28, and L-arabinose (Sigma) gave a peak at t_R (min) 14.50.

Cell Culture UMR106 cells were maintained at 37 °C in humid air containing 5% CO_2 in DMEM supplemented with 5% FBS and 100 U/ml of penicillin/100 μg/ml of streptomycin. The cells were subcultured every 4 d using 0.05% trypsin-EDTA 0.53 mM. For the experiments, phenol red-free DMEM was used to rule out the possibility of estrogen binding to the phenol red in the culture medium.

Cell Proliferation Assay The proliferative effects of the samples against the UMR106 cell line was determined in the MTS colorimetric assay. The cells were trypsinized (0.05% trypsin–EDTA 0.53 mM) and seeded into 96-well tissue culture plates at a concentration of 7.5×10^3 cells/well. The cells were allowed to attach for 48 h. The medium was removed and replaced with the experimental medium (1% CDT-FBS in phenol red-free DMEM) containing various concentrations of each sample. After 48 h of incubation, 60 μl of MTS (5 mg/ml) was added to each well. The cells were incubated at 37 °C in darkness for 1 h, and then the optical density was measured on POLAR star Galaxy at 490 nm. The negative control was 0.1% DMSO. Optical density of the samples at different concentrations without cells was also determined at 409 nm to correct the effect of the sample color. Thus, $A_{\text{sample}}=A_{\text{sample with cells}}-A_{\text{sample without cells}}$. The proliferation ratio was expressed as the percentage of the difference of the optical density at 490 nm of the cells treated with test samples minus that of control relative to that of control, *i.e.*, proliferation ratio$=(A_{\text{sample}}-A_{\text{control}})/A_{\text{control}}$.

Statistical Analysis All data are expressed as mean±S.D. of at least three independent experiments, each performed in tetraplicate. One-way ANOVA with *post-hoc* multiple comparisons was performed for statistical analysis. A p value of less than 0.05 was regarded as significant.

Animal Study Design After 1 week of acclimatization, 60 mice were ovariectomized (OVX, $n=60$) and 10 other mice were sham-operated (sham). After 10 d of recovery, OVX mice were randomly and equally divided into six groups: OVX+vehicle; OVX+17β estradiol (2 mg/kg); OVX+DF (880 mg/kg); OVX+DFA (567 mg/kg); OVX+DFB (155 mg/kg); and OVX+DFC (18 mg/kg). The oral administration to all rats continued for 6 weeks. All rats were fed the regular diet in the experimental period. After killing, femurs were collected and stored at −20 °C. When measuring BMD, femurs were scanned and the trabecular BMD and total BMD in femoral heads were measured using pQCT.[24]

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