Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 68 (2007) 1448–1458
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Taxonomic, genetic, chemical and estrogenic characteristics of *Epimedium* species

P. Shen [a], B.L. Guo [b], Y. Gong [a], Deborah Y.Q. Hong [c], Y. Hong [c], E.L. Yong [a,*]

[a] *Department of Obstetrics and Gynecology, National University Hospital, Yong Loo Lin School of Medicine, Lower Kent Ridge Road, Singapore 119074, Republic of Singapore*
[b] *Institute for Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing, PR China*
[c] *Temasek Life Sciences Laboratory, National University of Singapore, Singapore 119074, Republic of Singapore*

Received 24 June 2006; received in revised form 16 February 2007
Available online 16 April 2007

Abstract

To understand the factors contributing to estrogenic properties of extracts from the genus *Epimedium* L. (Berberidaceae), we performed taxonomic, genetic and chemical characterization on 37 specimens from 18 species and related these to estrogen receptor (ERα and ERβ) bioactivity, as measured by reporter genes in stable human cells. Boot strap values derived from amplified fragment length polymorphisms indicated that specimens of *E. koreanum*, *E. brevicornum*, *E. myrianthum*, *E. leishanense*, and *E. membranaceum* were genetically distinct and this was supported by their very similar ERα activities. In contrast, specimens from *E. pubescens* and *E. sagittatum* were diverse both genetically, chemically and in terms of ERα and ERβ bioactivities. Strikingly, a genetic cluster comprising six rare *Epimedium* species exhibited strongest ERα and ERβ activity, and this bioactivity was positively correlated with content of trace flavonoid aglycones (kaempferol, apigenin, quercetin, luteolin and breviflavone B). In contrast, there was no association between estrogenic activity and the major flavonol glycoside constituents (icariin and epimedin A–C). Although they exhibited equally strong ERα and ERβ activity, *E. koreanum* can be clearly differentiated from *E. pubescens* and *E. brevicornum* by genetic distance and its significantly lower content of epimedin C. Our morphologic, genetic, chemical and bioactivity profiling provide the basis for the production of extracts with reproducible estrogenic properties. Such reproducibility will be critical for the standardization of *Epimedium*-based products.

Keywords: *Epimedium* species; Berberidaceae; Taxonomy; Genetic; Chemical; Estrogenic activity

1. Introduction

The traditional Chinese medicinal herb, *Epimedium* L. (Berberidaceae), is a popular botanical supplement used to improve menopausal symptoms and bone health, amongst other indications. Major *Epimedium* species used for medicinal purposes are *E. koreanum* Nakai, *E. pubescens* Maxim., *E. brevicornum* Maxim, *E. sagittatum* (Sieb. Et Zucc) Maxim, and *E. wushanense* T.S. Ying (The State Pharmacopoeia Commission of PR China, 2000). *Epimedium* species have a particularly high content of the prenylated flavonol glycosides (Liang et al., 1997), the most prominent of which is icariin (**1**), a flavone with two glycoside moieties (Akai, 1935). Icariin and related flavonoids (Fig. 1) were reported to enhance the osteogenic differentiation of rat primary bone marrow stromal cells (Chen et al., 2005), increase osteoblastic proliferation (Meng et al., 2005), reduced osteoclastic bone resorption (Yu et al., 1999) and to increase mineral content, and prevent osteoporosis in ovariectomized rats (Zhang et al., 2006). Because of these properties, icariin and epimedin A (**2**), B

Abbreviations: AFLP, amplified fragment length polymorphisms; ER, estrogen receptor; *E*, *Epimedium*; PCR, polymerase chain reaction; RE, relative efficacy – the ratio between maximum activity of extract and the maximum activity of estradiol; RP, relative potency – ratio between EC_{50} of extract and estradiol.

* Corresponding author. Tel.: +65 67724261; fax: +65 67794753.
E-mail address: obgyel@nus.edu.sg (E.L. Yong).

0031-9422/$ - see front matter
doi:10.1016/j.phytochem.2007.03.001

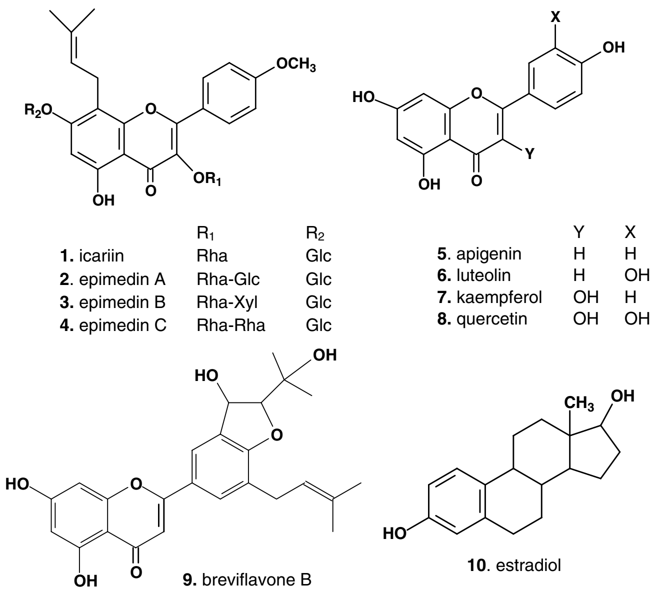

Fig. 1. Structures of major flavonoid glycosides (**1**–**4**), bioactive aglycones (**5**–**9**) from *Epimedium*, compared to the physiological estrogen, estradiol (**10**). Rha, rhamnose; Glc, glucose; Xyl, xylose.

(**3**), and C (**4**), (all glycosides of anhydroicaritin) are frequently used as marker compounds for the quality control of *Epimedium* and its medicinal extracts (Liu et al., 2006).

Flavonoids can activate the estrogen receptors (ER), part of a 48-member family of transcription factors, which control sexual differentiation and regulate skeletal health in women. The two estrogen receptors, ERα and ERβ, are similar in their DNA-binding domains but have significant differences in their C-terminal ligand binding domains, sharing only 56% homology (Koehler et al., 2005). These structural differences cause ligands to bind with differing affinity to ERα and ERβ resulting in different conformations of the activated receptor and differential recruitment of coregulators and selective estrogen modulator activity (McDonnell, 2003). The predominant receptor in the uterus is ERα, while ERβ is expressed at high levels in the ovary, vascular endothelium, smooth muscle and the central nervous system. When the receptors are expressed in the same tissue, such as the breast, they may cause different actions. Thus ERα stimulates, whereas ERβ inhibits, cells obtained form lactating mammary glands. (Koehler et al., 2005). Ligands which are selective for either ERα or ERβ is a subject of intense pharmaceutical research.

Recently extracts of *Epimedium* were found to be potent and specific estrogenic activity (Yap et al., 2005; De Naeyer et al., 2005). Estrogenic activity was not caused by flavonoid glycosides, but was due to the presence of flavonoid aglycones (Yap et al., 2005). These aglycones belong to a recently described class of prenyl flavonoids with potent estrogenic properties (Milligan et al., 2000). Intriguingly prenyl flavones exhibit dose-dependent anti-estrogenic properties in human breast cancer cells (Yap et al., 2005; Pedro et al., 2005) and may be specific inhibitors of the breast cancer resistant protein ABCG2 (Ahmed-Belkacem et al., 2005). Estrogenic activity in *Epimedium* extracts may also be contributed by known flavonoids such as apigenin (**5**), luteolin (**6**), kaempferol (**7**), and quercetin (**8**) (Wu et al., 2003). The estrogenic properties of *Epimedium* coupled with these anti-proliferative effects on breast cancer cells suggest its possible utility for menopausal estrogen replacement therapy but without adverse effects on breast health associated with current estrogen–progesterone formulations (Rossouw et al., 2002). Clinical studies are required to establish the role, if any, of *Epimedium* extracts for estrogen-deficient conditions.

However the prerequisite for a scientific study is the procurement of high quality, standardized drugs with reproducible pharmacological properties. In common with many traditional medicinal plants, a major problem with *Epimedium* is the absence of a rigorous method to authenticate species. Chinese taxonomists and geneticists have variably reported numbers ranging from 20 to 50 species (Sun et al., 2005). Traditional herbalists do not differentiate among *Epimedium* species, but rather use a mixture of species together as *Herba Epimedii* (Yang, 1985). These species differ significantly in concentrations of major and minor constituents (Wu et al., 2003). Another concern is that *Epimedium*, like the majority of traditional Chinese herbs, is not cultivated but is collected from the wild, which increases the dangers of wrong species identification, genetic diversity, and possible differences in levels of bioactive compounds due to soil and climate (Guo and Xiao, 2003; Chen et al., 1996; Mizuno et al., 1989). Other factors that may cause variations include differences in processing, packaging and storage of raw materials. These multitudes of unknowns render scientific evaluation of *Epimedium* extracts for menopausal and bone health problematic.

The aims of this study are to define the taxonomic, genetic and chemical characteristics of species from *Epimedium*, and determine their relative contributions to estrogenic properties of resultant extracts. Specimens of 18 *Epimedium* species, including the five major medicinal species, were taxonomically identified and subjected to amplified fragment length polymorphism genetic analysis. The content of major flavonol glycosides (**1**–**4**) and trace estrogenic aglycones (**5**–**9**) (Fig. 1) in various samples of the same species were determined, and compared to ERα and ERβ activity as measured with reporter genes in stable cell lines. The utility of using such a combined taxonomic, genetic, chemical and bioresponse profiling to obtain reproducible extracts from complex medicinal plants, like *Epimedium* is discussed.

2. Results

Leaves from 18 *Epimedium* species, collected from Central and Northern Mainland China, were obtained from the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing (Table 1). These were supplemented with *Epimedium* specimens purchased

Table 1
Estrogenic effects of bioactive flavonoid aglycones (**5**–**8**), and *Epimedium* specimens used in this study

Code	Ligand		ERα		ERβ	
			RE[a]	RP[b]	RE[a]	RP[b]
E2	Estradiol		1.00	1.00	1.00	1.00
Bioactive flavonoid aglycones						
A	Apigenin		2.37	4.95E−4	3.33	1.74E−3
K	Kaempferol		1.21	2.36E−4	1.07	1.43E−3
L	Luteolin		1.05	6.83E−4	0.76	1.71E−3
Q	Quercetin		0.15	9.28E−5	0.26	3.87E−4
Epimedium extract[c]						
Code	Botanical name	Location/source				
A1	*E. acuminatum*	Anlong, Guizhou province	1	5.1E−5	0.04	0.00043
A2	*E. acuminatum*	Ziyun, Guizhou province	0.96	–	0.34	–
SL	*E. stelluatum*	Shiyan, Hubei province	2.21	2.4E−6	0.42	1.7E−6
FG	*E. fargesii*	Wuxi, Chongqing	1.49	5.3E−6	1.29	2.8E−6
FH	*E. franchetii*	Shennongjia, Hubei province	2.3	1.8E−6	0.9	1.1E−6
ZH	*E. zhushanense*	Zhushan, Hubei province	1.76	3.3E−6	0.81	2.1E−6
LI	*E. leishanense*	Leishan, Guizhou province	1.22	–	0.47	–
L2	*E. leishanense*	Leishan, Guizhou province	1.7	1.3E−6	0.2	5.5E−7
DO	*E. dolichostemen*	Lichuan, Hubei province	0.64	6.3E−6	0.71	2.4E−6
S2	*E. sagittatum*	Xinning, Hunan province	0.41	2.5E−6	0.21	8.2E−7
M1	*E. myrianthum*	Duyun,Guizhou province	0.61	–	0.23	–
M2	*E. myrianthum*	Huitong,Hunan province	0.99	8.7E−6	0.26	2.2E−6
S3	*E. sagittatum*	NK, Schwabe[2]	1.18	1.2E−5	0.18	–
LT	*E. leptorrhizum*	Lichuan, Hubei province	0.77	3.3E−5	0.07	1.1E−6
P1	*E. pubescens*	Nazhen, Shanxi province	0.72	4.7E−5	0.47	2.2E−6
ZB	*E. zhenbaense*	Zhenba, Shanxi province	0.54	8.8E−6	0.37	1.1E−6
WU	*E. wushanense*	Badong, Chongqing	0.33	2.7E−6	0.12	7.4E−7
B1	*E. brevicornum*	NK, Schwabe[2]	0.65	1.4E−6	0.01	6.6E−7
B3	*E. brevicornum*	Henan province, STPT[3]	0.7	1.4E−5	0.17	2.5E−6
B2	*E. brevicornum*	Zhejiang province, STPT[3]	0.52	2.7E−6	0.08	1.6E−6
B4	*E. brevicornum*	NK, Schwabe[2]	0.73	1.6E−5	0.15	–
P8	*E. pubescens*	NK, Schwabe[2]	1.1	5.7E−5	0.55	–
P3	*E. pubescens*	NK, Schwabe[2]	1.37	4.7E−5	1.35	3.8E−6
P2	*E. pubescens*	Sichuan province, STPT[3]	1.28	1.2E−5	1.1	2.1E−6
P4	*E. pubescens*	Bazhong, Chongqing	0.87	5E−5	0.38	–
P5	*E. pubescens*	Qingchuan, Sichuan province	0.7	9.9E−6	0.06	–
P6	*E. pubescens*	Dujiangyan, Sichuan province	0.71	4.8E−5	0.25	–
P7	*E. pubescens*	Ya An, Sichuan province	0.66	1.7E−5	0.09	–
DA	*E. davidii*	Baoxing, Sichuan province	0.3	3.3E−6	0.19	1.1E−6
PA	*E. pauciflorum*	Maoxian, Sichuan province	0.27	2E−6	0.28	7.5E−7
ME1	*E. membranaceum*	Maoxian, Sichuan province	0.22	2.3E−6	0.06	1E−6
ME2	*E. membranaceum*	Shifang, Sichuan province	0.29	3.5E−6	0.19	1.3E−6
S1	*E. sagittatum*	NK, Schwabe[3]	0.26	–	0.08	–
K4	*E. koreanum*	NK, Schwabe[3]	0.67	9E−6	0.55	–
K2	*E. koreanum*	Liaoning-Shenyang[4]	0.7	7.5E−6	0.26	1.6E−6
K3	*E. koreanum*	NK, Schwabe[2]	0.67	6.9E−6	0.11	1.2E−6
K1	*E. koreanum*	Jilin-Yan Bian[5]	0.67	6.9E−6	0.4	1.9E−6

[a] Relative efficacy (RE): ratio between maximum activity of extract and maximum activity of estradiol.
[b] Relative potency (RP): ratio between EC_{50} of extract and EC_{50} of estradiol.
[c] Specimens are arranged in according to genetic relatedness as depicted in Fig. 2. All specimens from herbarium of Institute of Medicinal Plant Development, unless otherwise indicated. Other sources: Schwabe, GmbH, Germany; STPT, China; Shenyang Company, China; Yan Bian Company, China; NK, place of collection not known.

from commercial sources in Singapore and PR China (Table 1). A total of 37 *Epimedium* specimens, including eight species with multiple specimens, were taxonomically identified by one of the authors (B.L.G.). All specimens were extracted with 100% ethanol at 37 °C for 7 days, resulting in an average yield of 1.29 ± 0.07 g/100 g (dried wt/wt). All botanical specimens and extracts were subjected to genetic, chemical, and estrogenic bioresponse profiling.

2.1. Genetic characterization of Epimedium species

To genetically identify *Epimedium* species, fluorescent AFLP analysis was used to examine differences across the entire plant genome. DNA was extracted from leaves, digested with *Eco*RI and *Mse*I, ligated to the adaptor, amplified with polymerase chain reaction (PCR) primers, one of which is end-labeled with FAM fluorescent dye

Table 2
Similarity for AFLP patterns

	A	B	D0	DA	FG	FH	S	K	L	LT	M	ME	P	PA	SL	WU	ZB	ZH
A(2), *E. acuminatum*	**0.537**																	
B(4), *E. brevicornum*	0.432	**0.569**																
D0, *E. dolichostemen*	0.406	0.383	**1.000**															
DA, *E. davidii*	0.358	0.382	0.338	**1.000**														
FG, *E. fargesii*	0.461	0.413	0.426	0.347	**1.000**													
FH, *E. franchetii*	0.450	0.418	0.448	0.372	0.471	**1.000**												
S(3), *E. sagittatum*	0.365	0.358	0.357	0.326	0.373	0.374	**0.295**											
K(4), *E. koreanum*	0.255	0.268	0.261	0.227	0.277	0.280	0.272	**0.500**										
L(2), *E. leishanense*	0.439	0.443	0.420	0.358	0.463	0.412	0.369	0.249	**0.603**									
LT, *E. leptorrhizum*	0.414	0.432	0.378	0.332	0.451	0.452	0.354	0.239	0.434	**1.000**								
M(2), *E. myrianthum*	0.427	0.393	0.423	0.332	0.425	0.405	0.348	0.246	0.423	0.377	**0.485**							
ME(2), *E. membranaceum*	0.435	0.431	0.379	0.397	0.444	0.474	0.365	0.247	0.420	0.417	0.407	**0.536**						
P(8), *E. pubescens*	0.420	0.410	0.370	0.368	0.429	0.424	0.349	0.257	0.398	0.411	0.380	0.420	**0.458**					
PA, *E. pauciflorum*	0.430	0.432	0.371	0.431	0.413	0.436	0.372	0.242	0.393	0.406	0.381	0.472	0.428	**1.000**				
SL, *E. stelluatum*	0.446	0.425	0.412	0.346	0.417	0.461	0.366	0.229	0.409	0.420	0.419	0.404	0.395	0.388	**1.000**			
WU, *E. wushanense*	0.432	0.415	0.422	0.368	0.437	0.411	0.361	0.257	0.452	0.399	0.405	0.419	0.388	0.387	0.417	**1.000**		
ZB, *E. zhenbaense*	0.418	0.436	0.390	0.415	0.390	0.437	0.361	0.239	0.420	0.373	0.405	0.410	0.415	0.444	0.444	0.462	**1.000**	
ZH, *E. zhushanense*	0.475	0.434	0.456	0.359	0.508	0.515	0.379	0.251	0.482	0.451	0.411	0.472	0.419	0.450	0.465	0.459	0.423	**1.000**

Jaccard values of band sharing were averaged over pairwise comparisons. Figures in parentheses indicate the number of individuals analyzed for that species. Bold indicate intraspecific comparisons.

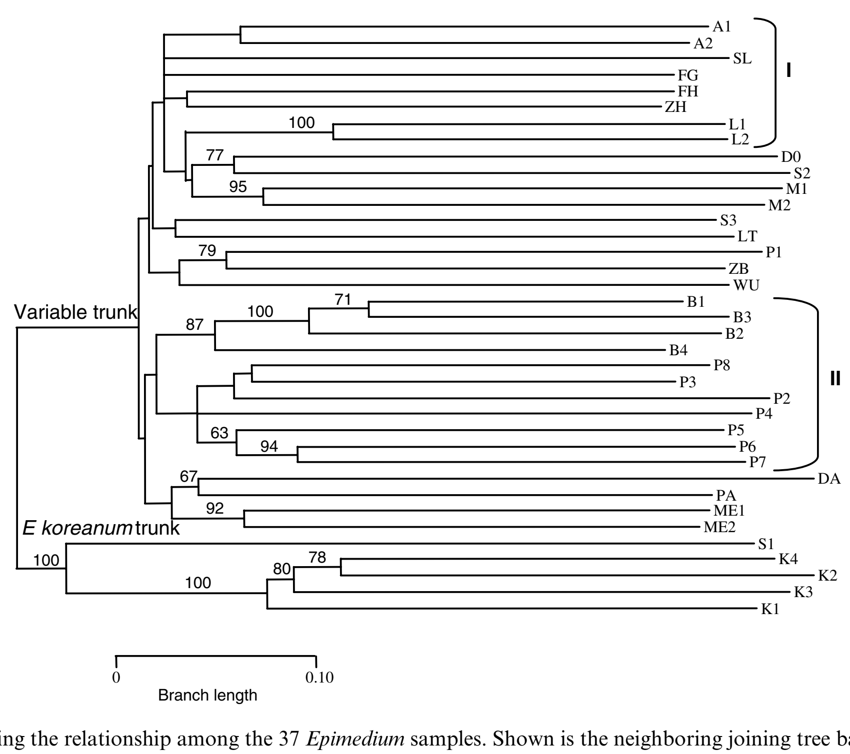

Fig. 2. Dendrogram describing the relationship among the 37 *Epimedium* samples. Shown is the neighboring joining tree based on the similarity of AFLP banding profiles. Numbers indicate the proportion of 1000 bootstrap samples in which a particular node was found. Specimens were collected from locations all over China as listed in Table 1. Scale bar indicates 10% branch length.

(blue). PCR fragments were resolved in an Applied Biosystems 3730xl DNA analyzer. Fragment sizes were calculated by mixing samples with DNA size markers in the range of 75–500 bp end labeled with Rox (red). The four primer combinations yielded a total number of 840 polymorphic markers among the samples analyzed. Table 2 shows pairwise Jaccard genetic similarity indices which were used to construct a neighbor joining phylogenetic tree (Fig. 2). Bootstrap values >50 were labeled along the nodes that they support. Genus *Epimedium* was divided clearly into two main genetic trunks; a well defined trunk consisting of mainly one *Epimedium* species, *E. koreanum*; and a more variable trunk comprising all the other species (Fig. 2). The eight *Epimedium* species with multiple samples were subjected to bootstrap analysis to evaluate intraspecies diversity (Efron et al., 1996). Jaccade values of samples from *E. myrianthum*, *E. leishanense*, *E. brevicornum*, *E. membranaceum* and *E. koreanum* strongly supported their grouping into the same species. For these species, average value of intraspecies similarity was higher than any of interspecies similarity, suggesting higher level of variation between species (Table 2). Clear identification of herbal materials for *E. myrianthum*, *E. leishanense*, *E. brevicornum*, *E. membranaceum* and *E. koreanum* through comparison of AFLP patterns was therefore more straightforward. However samples for *E. acuminatum*, *E. sagittatum* and *E. pubescens*, could not form a group per species, suggesting the possibility that the majority of genetic variation resides within species. For these species, intraspecies similarity is close or even lower than interspecies similarity and hence AFLP pattern may not be suitable for species identification. For the species with only one sample analyzed, no strong conclusion can be reached despite generally low level of interspecies similarity.

2.2. Chemical profiling of Epimedium

Major compounds used for standardization of *Epimedium* products and extracts are the flavonol glycosides, icariin, epimedin A, B and C (Fig. 1) (Yap and Yong, 2004). The relative abundance of these flavonol glycosides in all 37 plant extracts were determined by liquid chromatography tandem mass spectrometry. The major flavonoid glycosides in order of abundance were epimedin C (7.41 ± 0.91 g/100 g), icariin (3.04 ± 0.30 g/100 g), epimedin B (1.91 ± 1.0 g/100 g), and epimedin A (1.88 ± 0.33 g/100 g) (Table 3). These four flavonoid glycosides constitute an average of 10.4 ± 1.0 g/100 g of *Epimedium* ethanol extracts. The average sum of major constituents in *E. koreanum* was significantly lower than *E. brevicornum* and *E. pubescence* (4.66 ± 0.55 versus 9.09 ± 1.21 and 13.30 ± 1.05 g/100 g respectively, $P < 0.01$), due mainly to lower levels of the most abundant glycoside, epimedin C (1.31 ± 0.28 versus 5.17 ± 0.81 and 9.35 ± 0.63 g/100 g respectively, $P < 0.01$). In contrast, concentrations of the second most abundant glycoside, icariin, were similar in *E. koreanum*, *E. brevicornum* and *E. pubescens* (3.34 ± 0.28, 3.92 ± 0.41, and 3.95 ± 0.81 g/100 g respectively).

Table 3
Major flavonoid glycosides (**1**–**4**) in *Epimedium* specimens[a]

Code	Epimedin A[b]	Epimedin B[b]	Epimedin C[b]	Icariin[b]
A1	1.2758	1.4027	6.8413	3.1154
A2	0.8671	1.0324	6.3863	2.6273
SL	0.0520	0.1960	0.2071	0.1137
FG	2.2304	2.1474	10.5556	3.9104
FH	0.0474	0.5178	0.1440	0.0932
ZH	0.0439	0.0401	0.1770	0.0376
LI	1.4047	1.4504	16.6478	1.9580
L2	1.1182	1.4022	17.9569	1.3670
DO	0.7129	0.8472	5.7090	2.2029
S2	0.4397	0.5374	15.7854	6.2445
M1	6.1859	3.9292	11.5630	2.2282
M2	2.5819	1.1806	22.1945	0.8395
S3	0.2902	0.4303	16.6333	0.8557
LT	0.0633	0.1458	0.4512	0.2300
P1	2.3832	2.8676	6.3187	4.0355
ZB	0.9069	1.1287	7.5597	3.0058
WU	0.5382	0.6731	6.4458	0.7365
B1	1.5024	5.6980	3.2119	2.7782
B3	1.3286	5.0952	5.3778	3.9265
B2	1.5556	4.5492	4.9236	4.2382
B4	1.4876	2.0251	7.1848	4.7455
P8	2.0552	3.3589	9.6943	2.6149
P3	1.4620	2.1029	8.2134	2.3273
P2	1.4318	2.0737	9.9739	3.6700
P4	1.6242	2.0561	9.2314	2.9885
P5	3.1375	2.8485	12.0893	3.4422
P6	4.0989	4.0738	9.6384	8.6503
P7	2.6874	3.0065	6.6212	3.9868
DA	9.4539	1.1183	4.4325	3.9508
PA	7.6014	2.5513	11.4250	5.0796
ME1	1.0279	1.2288	7.5661	6.5034
ME2	1.3437	1.4148	7.1720	3.7776
S1	0.4728	0.3446	0.6598	3.0959
K4	1.8507	2.0609	1.5569	3.4961
K2	1.9802	2.2844	1.8966	4.0939
K3	1.6269	1.8296	1.2204	2.9763
K1	0.9336	1.1829	0.5719	2.8297

[a] Specimens were arranged in order of genetics relatedness as depicted in Fig. 2.
[b] Concentrations expressed as g/100 g of dried ethanol extracts.

Concentrations of bioactive minor compounds, the flavonoid aglycones apigenin, kaempferol, luteolin, quercetin and breviflavone B (**9**) (Fig. 1) were also determined (Table 4). These five flavonoid aglycones form an average of 0.038 ± 0.0061 g/100 g of the ethanol extract. The average concentrations of flavonoids in *Epimedium* species were in the order quercetin > luteolin > breviflavone B > apigenin > kaempferol with concentrations of 0.013 ± 0.003, 0.0080 ± 0.002, 0.0075 ± 0.0010, 0.0062 ± 0.0019, and 0.0035 ± 0.0009 g/100 g, respectively.

2.3. Estrogenic activity of Epimedium extracts and correlations with genetic clusters

In view of *Epimedium* being advertized as a drug for male impotence, we proceeded to examine the effects of

Table 4
Minor bioactive flavonoid aglycones (**5**–**9**) in *Epimedium* specimens[a]

Code	Apigenin[b]	Kaempferol[b]	Luteolin[b]	Quercetin[b]	Breviflavone B[b]
A1	0.0043	0.0000	0.0000	0.0020	0.0105
A2	0.0103	0.0002	0.0002	0.0036	0.0030
SL	0.0655	0.0270	0.0338	0.0385	0.0084
FG	0.0129	0.0021	0.0026	0.0075	0.0000
FH	0.0124	0.0114	0.0114	0.0888	0.0106
ZH	0.0096	0.0092	0.0011	0.0114	0.0102
LI	0.0102	0.0056	0.0190	0.0055	0.0107
L2	0.0014	0.0000	0.0031	0.0018	0.0137
DO	0.0120	0.0034	0.0226	0.0033	0.0216
S2	0.0081	0.0010	0.0036	0.0026	0.0074
M1	0.0044	0.0025	0.0026	0.0015	0.0011
M2	0.0049	0.0062	0.0015	0.0013	0.0064
S3	0.0057	0.0001	0.0026	0.0025	0.0055
LT	0.0012	0.0014	0.0094	0.0195	0.0000
P1	0.0225	0.0063	0.0020	0.0060	0.0034
ZB	0.0242	0.0011	0.0019	0.0025	0.0000
WU	0.0045	0.0007	0.0000	0.0033	0.0042
B1	0.0017	0.0001	0.0131	0.0051	0.0086
B3	0.0011	0.0000	0.0060	0.0062	0.0249
B2	0.0010	0.0000	0.0013	0.0424	0.0252
B4	0.0016	0.0002	0.0066	0.0031	0.0096
P8	0.0014	0.0023	0.0326	0.0030	0.0091
P3	0.0000	0.0000	0.0690	0.0566	0.0042
P2	0.0014	0.0061	0.0177	0.0352	0.0091
P4	0.0000	0.0000	0.0004	0.0038	0.0000
P5	0.0008	0.0027	0.0004	0.0039	0.0111
P6	0.0000	0.0020	0.0000	0.0014	0.0000
P7	0.0000	0.0168	0.0010	0.0038	0.0118
DA	0.0013	0.0000	0.0000	0.0045	0.0095
PA	0.0022	0.0132	0.0000	0.0223	0.0111
ME1	0.0000	0.0000	0.0109	0.0175	0.0000
ME2	0.0010	0.0011	0.0013	0.0103	0.0047
S1	0.0000	0.0000	0.0000	0.0165	0.0000
K4	0.0000	0.0000	0.0000	0.0020	0.0030
K2	0.0000	0.0003	0.0015	0.0175	0.0108
K3	0.0009	0.0002	0.0015	0.0022	0.0026
K1	0.0008	0.0046	0.0137	0.0183	0.0060

[a] Specimens were arranged in order of genetic relatedness as depicted in Fig. 2.
[b] Concentrations expressed as g/100 g of dried ethanol extracts.

the extract on the androgen receptor and related members of the steroid receptor family. Despite its colloquial name of "horny goat weed", extracts of *Epimedium* did not increase androgen, glucocorticoid or progesterone receptor activities; but instead dose-dependently increased ERα activity in transient transfections (Fig. 3). To increase the accuracy and reproducibility of bioassays, we constructed permanently transfected cells incorporating either ERα or ERβ, and an estrogen responsive reporter gene in its chromosomes (pERE4-Luc$_{hygro}$). Dose–response studies were performed for the *Epimedium* extracts and estradiol (**10**) (Fig. 4). For easy comparability, estrogenic activity of each extract was expressed as relative efficacy (RE), the ratio between maximum activity of extract and the maximum activity of estradiol; and relative potency (RP), the ratio between EC_{50} of extract and estradiol. Generally *Epimedium* species were ERα-selective in terms of potency (Table 1). Thus whereas the phytoestrogens apigenin, kaempferol, luteolin and quercetin had RP which were higher for ERβ compared to ERα, the reverse was generally observed for *Epimedium* extracts. Extracts also displayed higher maximal activity (higher RE) for ERα compared to ERβ.

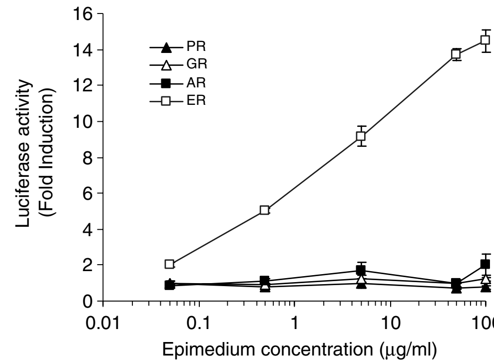

Fig. 3. Effect of *Epimedium* extracts on estrogen, androgen, progesterone and glucocorticoid receptor activities. HeLa cells, transiently transfected with plasmids encoding for ERα, androgen (AR), progesterone (PR) and glucocorticoid (GR) receptors and their cognate response elements driving luciferase-reporter genes, were exposed to an *E. pubescence* extract at the indicated doses. Data (mean ± s.e. of at least three replicates) represent increase in luciferase activity over replicates exposed to vehicle only.

The four samples from the most genetically distinct group, *E. koreanum*, exhibited remarkably similar ERα activity (mean RE of 0.67 ± 0.007, and mean RP of 7.5 ± 0.4) (Table 1). In contrast their ERβ activities were relatively weaker with mean RE of 0.33 ± 0.094. In the same way, the four samples from the closely linked *E. Brevicornum* specimens exhibited similar and strong ERα (mean RE of 0.65 ± 0.046) and relatively weaker ERβ (mean RE of 0.10 ± 0.036) activities. Although having only two specimens each, ERα activities of *E. leishanense*, *E. myrianthum*, and *E. membranaceum* specimens were very similar suggesting that estrogenic activity may have utility to differentiate these *Epimedium* species.

In contrast, the three specimens from the genetically diverse species, *E. sagittatum*, exhibited correspondingly divergent estrogenic activities with RE for ERα ranging from 1.18 to 0.26 (mean: 0.61 ± 0.28) (Table 1). Similarly the eight specimens form the genetically divergent *E. pubescens* species displayed ERα maximum activities ranging from RE of 1.37 to 0.66. In contrast the three *E. pubescens* specimens that were genetically linked (P5, P6, P7) exhibited similar RE ranging from 0.66 to 0.71. This was remarkable as they were collected from different locations at different times.

Intriguingly *E. acuminatum*, *E. stelluatum*, *E. fargesii*, *E. franchetii*, *E. zhushanense* and *E. leishanense* clustered at one end (genetic cluster I) of the phylogenetic tree (Fig. 2) exhibited highest ERα (mean RE: 1.58 ± 0.25) and ERβ (mean RE: 0.55 ± 0.20) activity. These species exhibited maximum ERα activities that was higher than that of estradiol itself, suggesting the presence of compounds which may augment estrogenic activity.

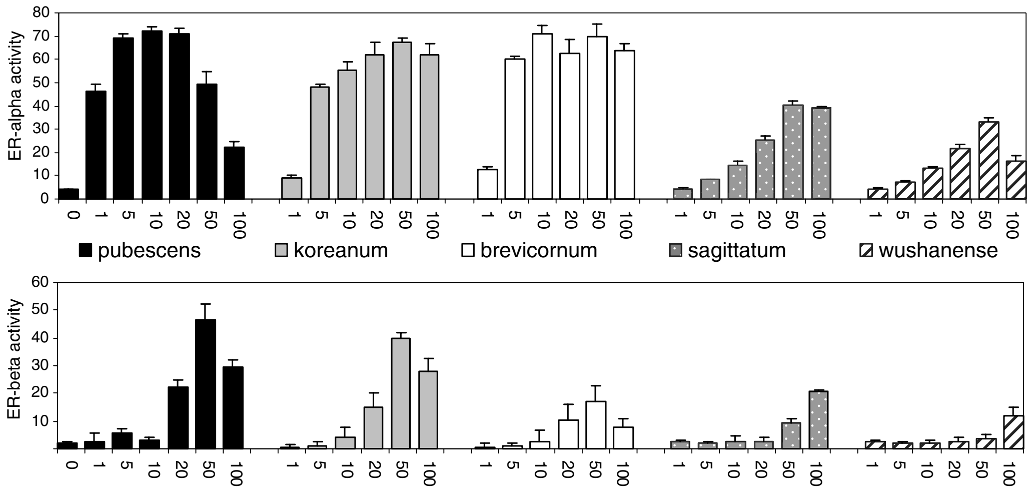

Fig. 4. Estrogenic activity of major *Epimedium* species. HeLa cells stably transfected with ERα (upper panel) or ERβ (lower panel) and an estrogen-responsive reporter gene were exposed to increasing doses (1–100 μg/ml) of ethanol extracts of indicated *Epimedium* species. Relative estrogenic activity (mean ± s.e.) was expressed as percentages of maximal estradiol activity.

2.4. Relationship between chemical constituents, genetic clusters and bioactivity

2.4.1. Major constituents

There were no simple correlations between the abundance of major flavonoid glycosides and estrogenic activity (Tables 1 and 3). This was not unexpected as the major glycosides (icariin, epimedin A, B, C) were inactive in our estrogenic assays (data not shown).

2.4.2. Minor bioactive flavonoid aglycones

In contrast, dose–response studies indicated that minor flavonoid aglycones such as apigenin, kaempferol, luteolin and quercetin exerted significant estrogenic effects (Fig. 5). As expected, phytoestrogens were several fold more ERβ selective in terms of relative potency (Table 1). However *Epimedium* species appear ERα selective, suggesting that combined activity of extracts was different from that expected from their flavonoid constituents. However within species, some correlations between ERα activity and sum of the concentrations of bioactive flavonoids (apigenin, kaempferol, luteolin, quercetin, and breviflavone B) (Fig. 1) were observed (Table 4). The eight specimens from genetic cluster I with highest ERα activity had the highest concentration of bioactive flavonoids (0.059 ± 0.02%). In this cluster, ERα activity correlated positively ($R_2 = 0.78$) with the sum of minor bioactive flavonoids (Fig. 6A). The *E. pubescens* group had the next higher concentration of bioactive flavonoids (0.044 ± 0.016%) and reflecting their greater genetic diversity had a lesser degree of correlation ($R_2 = 0.55$) between minor components and ERα activity (Fig. 6B). No definite correlations were observed between ERβ activity and flavonoid content.

3. Discussion

This systematic study of 37 taxonomically-identified specimens revealed differences in the genetic, chemical, and ERα/ERβ activity profiles that may be used to stan-

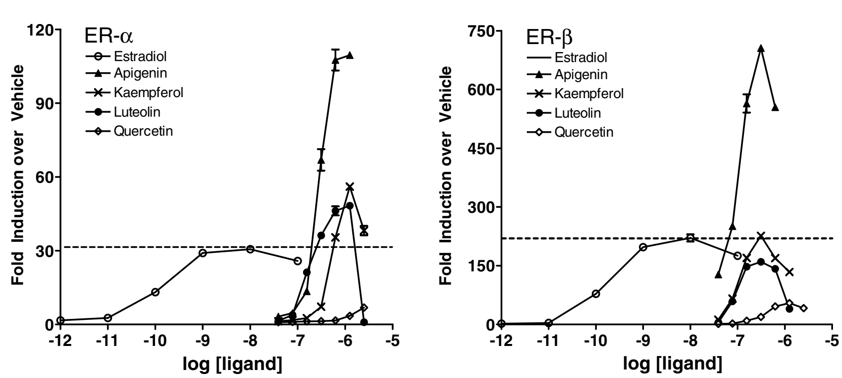

Fig. 5. Estrogenic effects of estradiol and bioactive flavonoid aglycones present in *Epimedium* extracts. Stable cells, permanently transfected with plasmids encoding ERα and ERβ and the pERE$_4$-Luc$_{hygro}$ estrogen-responsive reporter gene, were exposed to indicated doses of estradiol, apigenin, kaempferol, luteolin and quercetin. Dotted line indicates maximum activity of estradiol. Data (mean ± s.e. of at least three replicates) represent increase in luciferase activity over replicates exposed to vehicle only.

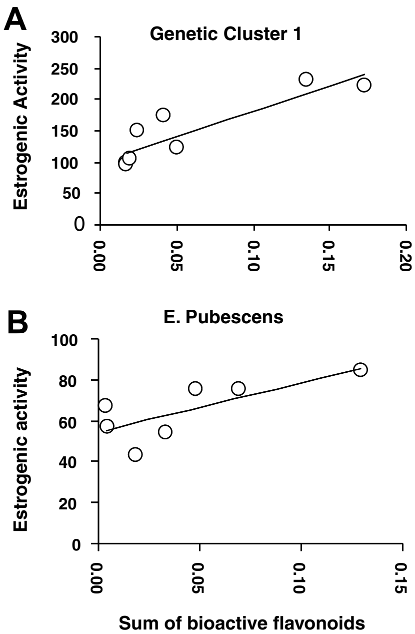

Fig. 6. Relationship between minor bioactive flavonoid aglycones and estrogenic activity. Scatter plot relating bioactivity (ERα activity, Table 1) of ethanol extracts of specimens from, (A) genetic cluster I and, (B) *E. pubescens* to the sum of the concentrations of bioactive flavonoids (**5**–**9**). Relative estrogenic activity (mean ± s.e.) was expressed as percentages of maximal estradiol activity.

dardize *Epimedium* species, and medicines derived from their extracts.

For DNA analysis, the use of 840 AFLP bands covering the entire plant genome provides rich genetic data adding to previous phylogenetic analyses based on limited specimens (Nakai et al., 1996) or less informative ribosomal DNA sites (Rossouw et al., 2002). Dendrogram analyses confirm the uniqueness of *E. koreanum*, a plant with an exclusive geographical footprint in the colder regions of Northern China and Korea (Guo and Xiao, 2003). In addition, *E. myrianthum*, *E. leishanense*, *E. brevicornum*, and *E. membranaceum* had samples of the same species consistently grouped together. Their average value of intraspecies similarity is higher than interspecies similarity. For these five species, AFLP can be reliably used for identification with Jaccard genetic similarity >50% and >90 bootstrap value. On the other hand, specimens of *E. acuminatum*, *E. sagittatum* and *E. pubescens* demonstrated greater intra-genetic variability, suggesting that conclusive identification of this species, and medicines derived from it, may be problematic. Differences in genomic DNA are regarded as definitive means of botanical identification compared to morphology. Genetic profiles like that generated by AFLP analyses may help in the difficult task of standardization and quality assurance for wildcrafted medicinal plants (like *Epimedium*) with many species, a situation that is not uncommon for many herbs in the Chinese herbal pharmacopoeia.

To examine whether marker compounds may aid standardization of extracts, we measured the concentrations of four major flavonoid glycosides and five minor bioactive flavonoid aglycones (Fig. 1) using chromatographic tandem mass spectrometry. The content of the major glycoside epimedin C was significantly lower in *E. koreanum* compared to *E. brevicornum* and *E. pubescens*. Thus *E. koreanum* can be differentiated from other major *Epimedium* species by its AFLP profiles and its content of Epimedin C. In contrast no differences were observed in the content of icariin, the next most abundant glycoside.

To explore mechanism(s) underlying relationships between genetic, chemical profiles and estrogenic activity, we devised cellular tools to capture summated estrogenic effects of plant extracts. In our bioassay, coding sequences for ERα or ERβ and a reporter gene, containing a promoter with estrogen-response elements driving a luciferase gene, were stably incorporated into the chromosomes of ER-negative HeLa lines. Such permanent cell lines provide accurate, sensitive, and reproducible biomarkers for the action of estrogens. They have near-linear responses over several orders of magnitude (Wang et al., 2005) and have been shown to correlate with estrogen-driven outcome parameters such as increases in thickness of uterine lining in rats (Sonneveld et al., 2006). Generally *Epimedium* species exhibited high ERα activity with maximal activity that was about 70% of that observed with estradiol. The estrogenicity of *Epimedium* extracts were consistent with findings from rat studies where *Epimedium* flavonoids enhances the osteogenic differentiation of rat primary bone marrow stromal cells (Chen et al., 2005) and can prevent OVX-induced osteoporosis independent of its enhancement of intestinal calcium absorption (Zhang et al., 2006).

Unlike common phytoestrogens, *Epimedium* extracts were ERα-selective, probably reflecting their content of prenyl-flavonoids (Wu et al., 2003; Yap and Yong, 2004), a new class of phytoestrogens with potent ERα activity isolated from hop (*Humulus lupulus* L.) extracts. Furthermore, the ERα activity of genetically related *Epimedium* specimens was consistent, more so than was observed with ERβ. Specimens that were genetically distinct such as *E. koreanum* and *E. brevicornum* induced remarkably similar ERα activities. In contrast divergent species such as *E. sagittatum* induced correspondingly diverse estrogenic activities. The remarkable correlation between bioassay and genetic classification suggest that their combined use may result in greater accuracy for identification of specimens. The validity of this conclusion has to be tested in a larger study.

Interestingly a cluster comprising six relatively rare *Epimedium* species (*E. acuminatum*, *E. stelluatum*, *E. fargesii*, *E. franchetii*, *E. zhushanense* and *E. leishanense*), exhibited ERα activity that was up to twofold higher than that observed with saturating doses of the physiological estrogen, estradiol. Further studies are needed to investigate whether such ''super-agonist'' activity may be due to the presence of unknown compounds that activate pathways that synergize steroid receptor signaling, such as the mitogen-activated protein kinase pathway (Jansen et al., 2004).

Although ERβ generally correlated with ERα activity, some striking differences were also observed. The two specimens of *E. acuminatum* showed high ERα activity, but almost negligible ERβ activity. In addition to its strong ERα activity (RE: 1.49), *E. fargesii* displayed exceptionally strong ERβ activity (RE: 1.29), suggesting the possibility that ERβ-specific compounds may be isolated from this *Epimedium* species. Whether these differences between ERα and ERβ activities can aid species identification of *E. acuminatum* and *E. fargesii* needs to be determined with larger numbers of specimens. Among major *Epimedium* species, specimens of *E. koreanum*, *E. pubescens* and *E. brevicornum* exhibited high ERα and ERβ activities. However *E. koreanum* was the most distinct genetically, chemically and in their ERα and ERβ activities, suggesting that this well-defined species may have value as the source of estrogenic medicines with consistent activity.

4. Concluding remarks

Legend has it that *Epimedium* received its colloquial name, "horny goat weed", when goats grazing on the herb were observed to have excessive copulating behaviors. In contrast to reports that *Epimedium* extracts may elicit penile erections in rats (Chen and Chiu, 2006), we found extracts of *Epimedium* to be strong activators of ERα and ERβ but not the androgen receptor. Like other dietary supplements, *Epimedium* is currently sold to consumers in dozens of different preparations. In many preparations, the source materials, chemical contents and bioactivities are not known. We have examined the estrogenicity of ethanol extracts of taxonomically identified *Epimedium* species prepared under standardized conditions, established a reproducible hierarchy of ERα and ERβ bioactivities, and defined their relationships to phylogenetic origins and chemical content. Although more specimens from each of the *Epimedium* species would be required to completely validate our conclusions, nevertheless our approach, combining taxonomic, genetic chemical and bioresponse profiling, may be generally relevant for the standardization of botanical extracts affecting processes mediated by steroid/nuclear receptors. This integrated approach to the standardization and authentication of herbal raw materials, and products derived from them, may lead to botanical medicines of pharmaceutical quality.

5. Experimental

5.1. General experimental procedures

Genomic DNA was isolated using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Pre-amplification and amplification steps were performed according to instructions of AFLP Plant Mapping Kit (Applied Biosystems, Foster City, USA). Capillary electrophoresis was performed with an ABI PRISM 3730xl and size of DNA fragments determined with GeneMapper 3.7 software (Applied Biosystems, Singapore). Chromatographic separation was performed on a Cadenza CD-C18 column (150 × 2 mm, Imatakt, Japan). Concentrations of major constituents and minor bioactive components were quantified using an Agilent 1100 LC/MSD system (Ion-trap, Agilent, Germany). Major flavonol glycosides icariin, epimedin A–C were purchased from Chromadex, USA. Bioactive flavonoids, luteolin, apigenin, kaempferol were purchased from Fluka Chemie AG, Germany; and quercetin from Sigma, Switzerland. Isolation and characterization of breviflavone B have been described (Yap et al., 2005).

5.2. Epimedium raw materials

Specimens of *Epimedium* were collected under the supervision of one of the coauthors (B.L.G.). Reference specimens were archived at Institute for Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing (Guo and Xiao, 2003). Sample collection was done at spring time, when the corolla characteristics of flower petals can be used to aid identification. Some samples were obtained from commercial sources and subsequently taxonomically identified (B.L.G.). Dried *Epimedium* leaves were grounded into powder and soaked in 100% ethanol (1:10, wt/vol) at 37 °C for 7 days, filtered and the supernatant dried *in vacuo*. The dried ethanol residue were weighed and re-dissolved in DMSO to give various concentrations for chemical and bioactivity profiling.

5.3. Genomic DNA amplification and fluorescent AFLP analyses

Genomic DNA was isolated from 2 to 5 g of dried leaves and DNA quantity and quality verified using 1% agarose gel electrophoresis. Plant DNA (∼0.5 μg) was digested with *Eco*RI and *Mse*I and ligated to adaptor. For each sample, four specific PCR amplifications were performed with *Eco*RI-ACA/*Mse*I-CACC (B2 + C), *Eco*RI-AGG/*Mse*I-CACC (B7 + C), *Eco*RI-ACT/*Mse*I-CAC (B1) and *Eco*RI-ACT/*Mse*I-CTG (G1). The *Eco*RI primers were labeled with the fluorescent dye FAM (blue) or HEX (green) at the 5′ end. After mixing with DNA size standards (50–500 bp) labeled with ROX (red), amplified fragments were resolved in an Applied Biosystems 3730xl DNA analyser. Fragment data were collected by using GENEMAPPER 3.7 software.

5.4. Chemical profiling

The concentrations of major constituents and minor bioactive components were quantified with liquid chromatography tandem mass spectrometry. Following chromatographic separation, concentrations of major flavonoid glycosides (icariin, epimedin A, B, C) were determined under full scan mode using 4-hydroxylbenzophenone as internal standard (RSD < 5%). Minor bioactive flavonoid aglycones were determined by multiple-reaction monitoring (m/z 269 → 225 for apigenin, m/z 285 → 185, 239 for kaempferol, m/z 285 → 175 for luteolin, m/z 301 → 151, 179 for quercetin, m/z 437 → 351 for breviflavone B) (RSD < 8%). The MSD settings for the maximum signal of the selected ion pairs for the each of the standard compounds were optimized by flow injection analysis. Concentrations for each bioactive compound were quantified using a six-point calibration curve of peak area ratio for compound to internal standard against the concentration. The concentrations of each compound were determined on three separate occasions. Results are the mean of these determinations and concentrations expressed as g per 100 g of original dried ethanol extract.

5.5. Measurement of estrogenic activity using stably transfected ERα and ERβ-responsive cell lines

5.5.1. Plasmids

pEGFP-ERα_{neo} and pEGFP-ERβ_{neo} were constructed by excising the full length hERα or hERβ, and inserting into the eukaryotic pEGFP-N2 expression plasmid (Clontech, Palo Alto, CA) with neomycin resistance gene. The pERE$_4$-Luc$_{hygro}$ reporter gene consists of four tandem copies of consensus vitellogenin ERE cDNA (5′-AGGTCA-CAGTGACCT-3′) cloned into a pGL3-basic plasmid (Promega, Madison, WI), upstream of the luciferase reporter gene. The hygromycin resistance gene was inserted into the vector as selection marker. All plasmids were sequenced to check fidelity of construction. Transient transfections for ERα, ERβ, androgen, progesterone and glucocorticoid receptors were preformed as described previously (Lim et al., 2006).

5.5.2. Determination of LD_{90} for hygromycin and neomycin

HeLa (ATCC) cells plated in 96-well plates with a cell density of 10,000 cells/well were exposed to increasing concentrations of hygromycin B (Stratagene, CA) or neomycin (G418 sulfate, Stratagene). HeLa cells exposed to vehicle alone served as controls. After 10 days of incubation in antibiotics containing media, the number of viable cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction assay to draw the kill curves and determine the LD_{90} for hygromycin B and neomycin.

5.5.3. Stable transfection and clone selection

HeLa cells plated in 24-well plates were sequentially transfected with pERE$_4$-Luc$_{hygro}$ and pEGFP-ERα_{neo}. Selection of stably transfected clones was done with LD_{90} concentration of hygromycin B and neomycin respectively. Hygromycin/neomycin-resistant foci were identified, subcultured and screened for estradiol-induced luciferase activity. A highly inducible clone αC3, stably expressing ERα, and βC3, stably expressing ERβ were obtained and used for subsequent experiments.

5.5.4. ERα and ERβ-responsive bioassays

ERα and ERβ stable cells were plated in 24-well plates at an optimized density of 4×10^4 cells in RMPI medium supplemented with 10% dextran-coated charcoal-stripped fetal bovine serum for 12 h. After 30 h of incubation with test samples, luciferase activity of lysates was measured with luminometer. Each data point was mean ± s.e. of at least triplicate samples. Dose–response curves were performed for each extract to determine maximal and EC_{50} values.

5.6. Data and statistical analyses

For AFLP data analysis, electrophoretic patterns were converted into binary matrixes (1 for presence, 0 for absence of a band). Jaccard similarity matrix (number of shared bands/total number of bands) was calculated for each pair-wise comparison with the software package NTSYSpc 2.1 (Exeter software, NY). The same software was used to generate a neighbor joining phylogenetic tree. Phylogenetic consistency was evaluated by bootstrapping analysis of 1000 replicates with neighbor-joining search by using PAUP* 4.0b10 (Sinauer, MA). Comparisons of differences in chemical contents between genetic groups were performed with Student's t-test after ANOVA. $P < 0.05$ was regarded as significant. Regression analysis was performed using SPSS (SPSS Inc., Chicago, IL). Maximum activity (C_{max}) and the dose that elicited half maximum activity (EC_{50}) were calculated from dose–response curves using Graphpad prism V4. In those instances where there was insufficient extract for a full dose response curve, maximum activity was measured at a dose of 50 μg /ml.

Acknowledgements

Study supported by National University of Singapore Academic Research Fund, and US National Institutes of Health and National Center for Complimentary and Alternative Medicines (Planning Grant: 1 R21 AT001945-01). We thank Dr. Li Jun and Dr. Yap Sook Peng for data and technical help.

References

Ahmed-Belkacem, A., Pozza, A., Munoz-Martinez, F., et al., 2005. Flavonoid structure–activity studies identify 6-prenylchrysin and tectochrysin as potent and specific inhibitors of breast cancer resistance protein ABCG2. Cancer Res. 65, 4852–4860.

Akai, S., 1935. Constituents of *Epimedium macranthum* Morr and Decne I. Chemical constitution of a new glucoside of *Epimedium macranthum* Morr and Decne I. J. Pharm. Soc. Jpn. 55, 537–599.

Chen, K.K., Chiu, J.H., 2006. Effect of *Epimedium brevicornum* Maxim extract on elicitation of penile erection in the rat. Urology 67, 631–635.

Chen, C., Sha, M., Yang, S., 1996. Quantitative changes of flavonoids in *Epimedium koreanum* Nakai in different collecting periods. Zhongguo Zhong Yao Za Zhi 21, 86–88.

Chen, K.M., Ge, B.F., Ma, H.P., Liu, X.Y., Bai, M.H., Wang, Y., 2005. Icariin, a flavonoid from the herb *Epimedium* enhances the osteogenic differentiation of rat primary bone marrow stromal cells. Pharmazie 60, 939–942.

De Naeyer, A., Pocock, V., Milligan, S., De Keukeleire, D., 2005. Estrogenic activity of a polyphenolic extract of the leaves of *Epimedium brevicornum*. Fitoterapia 76, 35–40.

Efron, B., Halloran, E., Holmes, S., 1996. Bootstrap confidence levels for phylogenetic trees. Proc. Natl. Acad. Sci. USA 93, 13429–13434.

Guo, B.L., Xiao, P.G., 2003. Comment on main species of herba epimedii. Zhongguo Zhong Yao Za Zhi 28, 303–307.

Jansen, M.S., Nagel, S.C., Miranda, P.J., Lobenhofer, E.K., Afshari, C.A., McDonnell, D.P., 2004. Short-chain fatty acids enhance nuclear receptor activity through mitogen-activated protein kinase activation and histone deacetylase inhibition. Proc. Natl. Acad. Sci. USA 101, 7199–7204.

Koehler, K.F., Helguero, L.A., Haldosen, L.A., Warner, M., Gustafsson, J.A., 2005. Reflections on the discovery and significance of estrogen receptor beta. Endocr. Rev. 26, 465–478.

Liang, H.R., Siren, H., Jyske, P., Reikkola, M.L., 1997. Characterization of flavonoids in extracts from four species of *Epimedium* by micellar electrokinetic capillary chromatography with diode-array detection. J. Chromatogr. Sci. 35, 117–125.

Lim, L.S., Shen, P., Gong, Y.H., Yong, E.L., 2006. Dimeric progestins from rhizomes of *Ligusticum chuanxiong*. Phytochemistry 67, 728–734.

Liu, J.J., Li, S.P., Wang, Y.T., 2006. Optimization for quantitative determination of four flavonoids in *Epimedium* by capillary zone electrophoresis coupled with diode array detection using central composite design. J. Chromatogr. A 1103, 344–349.

McDonnell, D.P., 2003. Mining the complexities of the estrogen signaling pathways for novel therapeutics. Endocrinology 144, 4237–4340.

Meng, F.H., Li, Y.B., Xiong, Z.L., Jiang, Z.M., Li, F.M., 2005. Osteoblastic proliferative activity of *Epimedium brevicornum* Maxim. Phytomedicine 12, 189–193.

Milligan, S.R., Kalita, J.C., Pocock, V., Van De Kauter, V., Stevens, J.F., Deinzer, M.L., Rong, H., De Keukeleire, D., 2000. The endocrine activities of 8-prenylnaringenin and related hop (*Humulus lupulus* L.) flavonoids. J. Clin. Endocrinol. Metab. 85, 4912–4915.

Mizuno, M., Iinuma, M., Tanaka, T., Iwashima, S., Sakakibara, N., 1989. Seasonal fluctuation of flavonol glycosides in *Epimedium* species. Yakugaku Zasshi 109, 271–273.

Nakai, R., Shoyama, Y., Shiraishi, S., 1996. Genetic characterization of *Epimedium* species using random amplified polymorphic DNA (RAPD) and PCR-restriction fragment length polymorphism (RFLP) diagnosis. Biol. Pharm. Bull. 19, 67–70.

Pedro, M., Lourenco, C.F., Cidade, H., Kijjoa, A., Pinto, M., Nascimento, M.S., 2005. Effects of natural prenylated flavones in the phenotypical ER (+) MCF-7 and ER (−) MDA-MB-231 human breast cancer cells. Toxicol. Lett. (Dec 17) [Epub ahead of print] PMID: 16371244.

Rossouw, J.E., Anderson, G.L., Prentice, R.L., et al., 2002. Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. JAMA 288, 321–333.

Sonneveld, E., Riteco, J.A., Jansen, H.J., et al., 2006. Comparison of in vitro and in vivo screening models for androgenic and estrogenic activities. Toxicol. Sci. 89, 173–187.

Sun, Y., Fung, K.P., Leung, P.C., Shaw, P.C., 2005. A phylogenetic analysis of *Epimedium* (Berberidaceae) based on nuclear ribosomal DNA sequences. Mol. Phylogenet. Evol. 35, 287–291.

The State Pharmacopoeia Commission of PR China. 2000. In: Pharmacopoeia of The People's Republic of China English Edition, vol.1. Chemical Industry Press, Beijing, China, p. 107.

Wang, S., Paris, F., Sultan, C.S., et al., 2005. Recombinant cell ultrasensitive bioassay for measurement of estrogens in postmenopausal women. J. Clin. Endocrinol. Metab. 90, 1407–1413.

Wu, H., Lien, E.J., Lien, L.L., 2003. Chemical and pharmacological investigations of *Epimedium* species: a survey. Prog. Drug Res. 60, 1–57.

Yang, Z.Z., 1985. Clinical applications of Yinyanghuo. Zhejiang J. Trad. Chin. Med. 20, 478–480.

Yap, S.P., Yong, E.L., 2004. *Epimedium* species. In: Packer, L., Ong, C.L., Halliwell, B. (Eds.), Herbal and Traditional Medicine: Molecular Aspects of Health. Marcel Dekker, New York, pp. 229–246.

Yap, S.P., Shen, P., Butler, M.S., Gong, Y., Loy, C.J., Yong, E.L., 2005. New estrogenic prenylflavone from *Epimedium brevicornum* inhibits the growth of breast cancer cells. Planta Med. 71, 114–119.

Yu, S., Chen, K., Li, S., Zhang, K., 1999. In vitro and in vivo studies of the effect of a Chinese herb medicine on osteoclastic bone resorption. Chin. J. Dent. Res. 2, 7–11.

Zhang, G., Qin, L., Hung, W.Y., 2006. Flavonoids derived from herbal *Epimedium brevicornum* Maxim prevent OVX-induced osteoporosis in rats independent of its enhancement in intestinal calcium absorption. Bone (January 12) [Epub] PMID: 16413840.