



ORIGINAL ARTICLE

Comparative study of two types of herbal capsules with different *Epimedium* species for the prevention of ovariectomised-induced osteoporosis in rats



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Received 4 March 2015; received in revised form 24 June 2015; accepted 7 July 2015
Available online 29 August 2015

KEYWORDS

Epimedium;
osteoporosis;
ovariectomised rats;
treatment efficacy

Summary *Background/Objective:* *Epimedium Folium* is the most important osteogenic herb formulated for the traditional Chinese Medicine Xian Ling Gu Bao (XLGB) capsule. The present study compared XLGB capsules containing two different *Epimedium* species, i.e., either *Epimedium pubescens* (XEP) or *Epimedium koreanum* (XEK), with the focus being on the chemical constituents and antiosteoporotic efficacy.

Methods: Ultra performance liquid chromatography was used to demonstrate the different chemical constituents. Biomechanical tests, histological, and cytological evaluation were performed to characterise and compare the bone mineral density, bone strength, microstructure

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of bone tissue, and biological activity between XEP and XEK using an established ovariectomised (OVX) rat model.

Results: Six flavonoids with different contents between XEK and XEP were identified. As compared with the OVX group, significantly higher bone mineral density, elastic-modulus, and compressive strength were found in both the XEK group and XEP group ($p < 0.05$ for all, $n = 8$). Histomorphometric data presented significantly higher osteoblast surface ratio and osteoid area accompanied by significantly lower values of erosion surface and adipocytes area in two treatment groups ($p < 0.05$, $n = 6$). XLGB Fufang with either XEK or XEP all showed significant preventive effects in OVX-induced osteoporosis and deterioration of bone mechanical properties.

Conclusion: The significance of the current preclinical experimental study was that these two *Epimedium* species used for formulating XLGB capsules were equally effective for the prevention of oestrogen-depletion induced osteoporosis.

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Introduction

Osteoporosis and osteoporotic fractures occur at metabolically more active and faster deteriorating trabecular regions such as at the spine and hip [1,2]. Prevention and treatment of osteoporosis is essential in avoiding osteoporotic fractures, especially in postmenopausal women. Hormone (oestrogen) replacement therapy is one of the first-line effective countermeasures for relieving menopausal symptoms and for the prevention and treatment of postmenopausal bone loss [3]. However, the Women's Health Initiative trial data caused concerns being raised both in postmenopausal women and their physicians due to the increased risk of breast cancer and cardiovascular events [4].

Herbal Fufang or formula is a popular alternative in traditional Chinese Medicine (TCM) developed for the prevention and treatment of osteoporosis and related bone metabolic diseases [5,6]. Xian Ling Gu Bao Fufang (XLGB) was developed based on the modification of the empirical "Miao minority" medicine, which was traditionally used to tone the "kidney system" and nourish bones in oriental herbal medicine [7]. XLGB capsule was officially approved by the Chinese State Food and Drug Administration and was served as an over-the-counter drug for the treatment of osteoporosis, osteoarthritis, aseptic osteonecrosis, and fractures [5]. As a phytoestrogen-rich TCM [8], preclinical studies showed that XLGB improved bone mineral density (BMD) and mechanical properties in ovariectomised (OVX) rat models [9]. Clinical data demonstrated its positive effects in promoting fracture healing [10] and in the treatment of osteoporosis [10]. Recently, a multicentre, double blind, placebo-controlled, and dose-effect clinical trial further confirmed the safety and efficacy of XLGB in postmenopausal women with osteoporosis [5].

As a herbal Fufang, XLGB consists of six herbs with different percentages in weight as follows: *Herba Epimedii folium* (70%), *Dipsaci Radix* (10%), *Salvia Miltiorrhizae Radix et Rhizoma* (5%), *Anemarrhenae Rhizoma* (5%), *Psoraleae Fructus* (5%), and *Rehmannia Radix* (5%) [5]. Undoubtedly, as the "monarch" herb in XLGB Fufang based on TCM theory, the source of *Herba Epimedii* is of great importance for its long-

term sustainable development. In Chinese Pharmacopoeia before 2005, there were five species of *Herba Epimedii*: *Epimedium brevicornum* Maxim., *Epimedium sagittatum* (Sieb. et Zucc.) Maxim., *Epimedium pubescens* Maxim., *Epimedium wushanense* T.S. Ying, and *Epimedium koreanum* Nakai [11]. Icariin was the index component for the quality control of the antiosteoporotic herbal Fufang [11]. Since the 2010 Chinese Pharmacopoeia, *E. wushanense* T.S. Ying has been separated as a new TCM and epimedin-C has been selected as the index component for its quality control [12]. Different species of *Herba Epimedii* have varying flavonoids with different concentrations. Even the same species may have different compositions if they are from different locations, e.g., *E. sagittatum* in the Guizhou province contains more icariin and epimedin-C than that in Henan or Hubei province [13]. It is mandatory to specify the species and locations of herbs for making herbal Fufang in TCM. XLGB contains *E. pubescens* from the Sichuan province in its formulation, where its index component icariin has at least 1.4% of the total flavonoids for quality control [13]. With the increasing demand for XLGB as preventive herbal Fufang against osteoporosis in an aging society in China, it is indispensable to seek other species of *Herba Epimedii* for formulating XLGB herbal medicine for clinical applications. *E. koreanum* in the Chinese Pharmacopoeia became a potential candidate with following three distinguished advantages: (1) large resources available in Liaoning province [12]; (2) almost all of the flavonoids are found in *E. pubescens* although the content of each flavonoid may vary [13]; and (3) the content of icariin, the index component in XLGB for its quality control, in *E. koreanum* is found more than that in *E. pubescens* from Sichuan used for formulating commercial XLGB [13].

In order to evaluate the potential of *E. koreanum* from Liaoning for potential replacement of *E. pubescens* from Sichuan used for formulating XLGB Fufang, we prepared XLGB particles containing *E. koreanum* (XEK) using the same manufacturing process as the commercial ones made of *E. pubescens* (XEP). Ultra performance liquid chromatography (UPLC) was used to demonstrate the different chemical constituents in XEK and XEP; and then those compounds with different contents were tested for their bioactivity *in vitro*.

Lastly, an *in vivo* experimental efficacy study was designed based on a null-hypothesis, i.e., to prove or disprove the similarity in the treatment efficacy of XEP and XEK. State-of-the-art imaging techniques, biomechanical tests, histological, and cytological evaluation were adopted to characterise and compare the BMD, bone strength, and microstructure of bone tissue between the two treatment groups using an established OVX rat model [9,14].

Materials and methods

Materials

Two kinds of XLGB particles formulated with either of the two species of *Epimedium* were provided by Guizhou Tongjitang Pharmaceutical Co., Ltd. (Lot no. of XEP: 110618). Chromatography grade acetonitrile and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Acetic acid was obtained from Sigma-Aldrich (St. Louis, USA). All reference standards were isolated from commercial available XLGB extracts by various columns chromatography and were unambiguously identified by hydrogen-1, carbon-13 nuclear magnetic resonance technique. Cell culture reagents were obtained from Gibco (USA). Cell Counting Kit-8 (CCK-8) was purchased from DOJINDO Lab (Tokyo, Japan). Alkaline phosphatase kit (COD 11592) was obtained from Biosystems (Spain).

UPLC chromatographic conditions for XLGB

XLGB with a weight of 5 g was extracted with 70% methanol with 50 mL [13]. Firstly, ultrasound was performed twice that lasted for 30 minutes each time when 70% methanol was supplemented to the original volume. Thus, the final concentration was 100 mg/mL for both XEP and XEK.

UPLC analyses were performed using a Thermo UPLC Ultimate 3000 system (Thermo Fisher Scientific, USA) equipped with a binary solvent system, an automatic sample manager and photodiode array detector [15]. The chromatographic separation was carried out on an Acquity UPLC BEH C18 column (2.1 mm × 5 mm, 1.7 μm) (Waters, USA) at a temperature of 30°C. The mobile phase consisted of eluent A (0.1% acetic acid in water, v/v) and eluent B (0.1% acetic acid in acetonitrile, v/v) was delivered at a flow rate of 0.4 mL/min by using a liner gradient program as follow: 2–30% B from 0 minutes to 20 minutes, 30–48% B from 20 minutes to 30 minutes, 48–80% B from 30 minutes to 32.5 minutes, 80–100% B from 32.5 minutes to 35 minutes. After holding 100% B for the following 5 minutes, the column was returned to its starting condition. Detection wavelength was at 270 nm in the ultra-violet detector. The chromatographic peaks were identified by comparing their retention time with that of each reference compound, which was eluted in parallel with a series of mobile phases [15].

In vitro bioactivity assay of six compounds

The six compounds were identified and named by C1: hexandraside F, C2: epimedin A, C3: epimedin B, C4: epimedin C, C5: icariin, and C6: 2''-O-rhamnosylcariside II. Rat

osteogenic UMR 106 cells were used for proliferation, alkaline phosphatase (ALP) activity, and receptor activator of nuclear factor-kappaB ligand/osteoprotegerin (RANKL/OPG) assay.

UMR 106 osteoblastic cells were purchased from American Type Culture Collection, Manassas, VA, USA. They were maintained in Dulbecco's modified Eagle's medium with 10% foetal bovine serum in an incubator with 5% CO₂ at 37°C. MTT, ALP activity, and real-time polymerase chain reaction were tested according to our previously published protocol [16]. Quantitative real-time polymerase chain reaction was performed using the following primer sets: RANKL-R-T forward, 5'-CATCGGGTCCCATAAAGTC-3'; RANKL-R-T reverse, 5'-CTGAAGCAAATGTTGGCGTA-3'; OPG-R-T forward, 5'-CACCATGTACCGATTGTATC-3'; OPG-R-T reverse, 5'-AGCCCAGTGACCATTCTAA-3'; GAPDH-R-T forward, 5'-CAAGTTCACGGCACAGTCA-3'; GAPDH-R-T reverse, 5'-CCATTGATGTTAGCGGGAT-3'.

Experimental animals and study design

The Animal Experimental Ethics Committee of the principal investigator's institution approved the study protocol (Ref. no: 11/065/MIS-5). Thirty-two adult female Sprague-Dawley rats (12-week old with body weight of 220 ± 20 g) were obtained from the Laboratory Animal Services Centre of the Chinese University of Hong Kong. All animals were allowed to acclimate in a temperature-controlled room (25 ± 2°C) under a 12-hour reversed light-dark schedule. The rats were used either as sham-operated (sham, *n* = 8) or ovariectomised groups (OVX, *n* = 24). Sham-operated rats were only subjected to laparotomy under general anaesthesia by intraperitoneal injection (0.2 mL/100 g body weight) of ketamine combined with xylazine, while other rats were anaesthetised for performing OVX. Upon recovery from operation, animals were body-weight-matched and randomly assigned into the following four experimental groups: Sham group; OVX group; OVX + XEK (XEK group); and OVX + XEP (XEP group). According to the clinically effective dosage of XLGB, the converted and equivalent effective dosage from human to rats with XLGB powder was 225 mg/kg/day [17]. Herbal Fufang particles dissolved in the distilled water were all administered orally through a custom-made stomach tube, which started on Day 4 after OVX for 12 weeks. Sham and OVX groups were given normal saline throughout the experimental period.

After treatment, rats were given subcutaneous injection of calcein green (5 mg/kg) and xylenol orange (90 mg/kg) in a time sequence of 10 days and 3 days before euthanasia for studying bone mineral apposition [14]. Before euthanasia, blood was collected via cardiac puncture for serum isolation that were then stored at -80°C before the biomarkers assay. The left tibiae and the fourth lumbar vertebrae were dissected for measurement of trabecular microarchitecture followed by three-point bending test for the tibia and compressive strength test for the vertebra.

Micro-computerised tomography

The fourth lumbar (L4) vertebra and the proximal tibia were scanned by micro-CT system (μCT-40, Scanco Medical, Brüttisellen, Switzerland) using our established protocol

[4,18]. The entire scan length was set for the whole fourth lumbar vertebra or for 5 mm from top of tibia to distal direction of tibia in a spatial resolution of 17 μm per voxel with a 1024 \times 1024 image matrix. For separating the signals of the mineralised tissue from the background signal, background noise was removed using a low-pass Gaussian filter (Sigma = 1.2, Support = 2) with mineralised tissue being defined at a threshold of 220. Three-dimensional structures of trabecular bone were then reconstructed. BMD, bone tissue volume fraction (BV/TV), connective density, trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) were measured separately using built-in software [14,19].

Mechanical testing

A material test machine (H25KS; Hounsfield Test Equipment Ltd. UK) with a 250 N load cell was used for the biomechanical tests. An axial compression test was performed to analyse the mechanical strength of the lumbar vertebra (L4) [20]. For the compression test, planoparallel surfaces were obtained by removing the cranial and caudal ends of the vertebral specimen. From the vertebral body, a central cylinder with planoparallel ends and a height of approximately 5 mm was obtained. A craniocaudal compression force was applied to the specimen using a steel disk at a deformation rate of 2.5 mm/min. Compressive strength (Mpa), E-modulus (Mpa), and energy at yield (J) parameters were recorded for statistical analysis.

Three-point bending test was performed to analyse the mechanical strength of tibia midshaft [21]. The left tibia was positioned horizontally with the anterior surface upwards on a special holding device with supports located with 12 mm apart. A displacement rate of 5 mm/min was selected for applying the loading vertically to mid-diaphysis on the anterior surface upward until a fracture occurred using our established protocol [22]. Failure force (N) and stiffness (N/mm) was recorded for statistical comparison.

Serum PINP, CTX, and E₂ measurement

Bone formation marker, including amino-terminal propeptide of type I collagen (PINP) and bone resorption marker, carboxy-terminal telopeptide (CTX), as well as estradiol (E₂), were assayed using enzyme-linked immunosorbent assay (ELISA) kits (IDS Ltd., Boldon, UK) [23]. Six calibrators at different PINP, CTX, or E₂ concentrations supplied by the company were measured for the standard curve.

Bone histomorphometric analysis

Sequential fluorescence labelling was used to study bone dynamic bone remodelling within the trabecular bone of proximal tibiae using our established protocol [23,24]. Samples were fixed in 10% neutral buffered formaldehyde for 3 days. The fixed samples were then dehydrated, cleared and embedded in methyl methacrylate (MMA, Meccck-Schuchardt, Germany). After hardening, midcoronal sections for trabecular bone were prepared for proximal tibiae at a thickness of 5 μm (LEICA SM2500E, Leica Instruments, Nussloch, Germany). The trabecular sections

with Goldner's Trichrome staining were used for histological and cytological evaluation, and the fluorescence images were taken and evaluated as well using professional imaging and bone histomorphometry software (Osteometrics, Atlanta, GA, USA) under fluorescence microscope (Leica image analysis system, DM5500, Germany) [23,25]. The region of 1.67 mm \times 1.10 mm in trabecular bone below growth plate was defined for quantification. Bone histomorphometric parameters were calculated and expressed by the ratio of osteoblasts surface and bone surface (Ob.S/BS), the ratio of erosion surface and bone surface (ES/BS), the ratio of osteoid surface and bone surface (Os.S/BS), fat cells area (Fc.Ar), osteoid area (Os.Ar), mineralizing surface (MS), mineral apposition rate (MAR), bone formation rate per unit of bone surface (BFR/BS), and bone formation rate per unit of bone volume (BFR/BV) according to the published guideline for bone histomorphometry [26].

Statistical analysis

All quantitative data were presented as mean \pm standard deviation. Differences in bone density and micro-architecture, bone mechanical properties, bone turnover biochemical markers, histomorphometry, as well as the *in vitro* evaluations were compared using one-way analysis of variance with Bonferroni *post hoc* test to determine group differences using SPSS statistical software (SPSS version 17.0, Chicago, IL, USA). Statistical significance was set at * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

Results

Similarity and differences of chemical compounds in XEK and XEP

Figure 1A showed the UPLC profiles of XEK and XEP. Figure 1B, C, and D presented the enlargement of the profile (Figure 1A) at 0–16 minutes, 16–31 minutes, and 31–37 minutes, respectively. The different chemicals between XEK and XEP were mainly found on the peaks at 16–31 minutes (Figure 1C). By comparing the retention time with the reference standard isolated from XEP, six compounds (peaks were indicated with the red 1–6 numbers in Figure 1E) were identified as C1: hexandraside F, C2: epimedin A, C3: epimedin B, C4: epimedin C, C5: icariin and C6: 2''-O-rhamnosylcariside II. There were higher content of C1, C2, C3, and C5 in XEK and higher content of C4 and C6 in XEP. The peak at 19.18 minutes was speculated to be icaritin-3-O- α -L-rhamnoside, which was identified using high performance liquid chromatography-mass spectrometry [27]. The retention time, peak area, and the related peak area were listed in Table 1, and the total relative peak areas of these seven compounds are 22.57 and 22.19 in XEK and XEP, respectively.

In vitro bioassay of six identified compounds

Cell proliferation assay

For test cells proliferation, four concentrations of all six compounds were used for assay, including 0.01 μM , 0.1 μM , 1 μM , and 10 μM . No significant difference was found

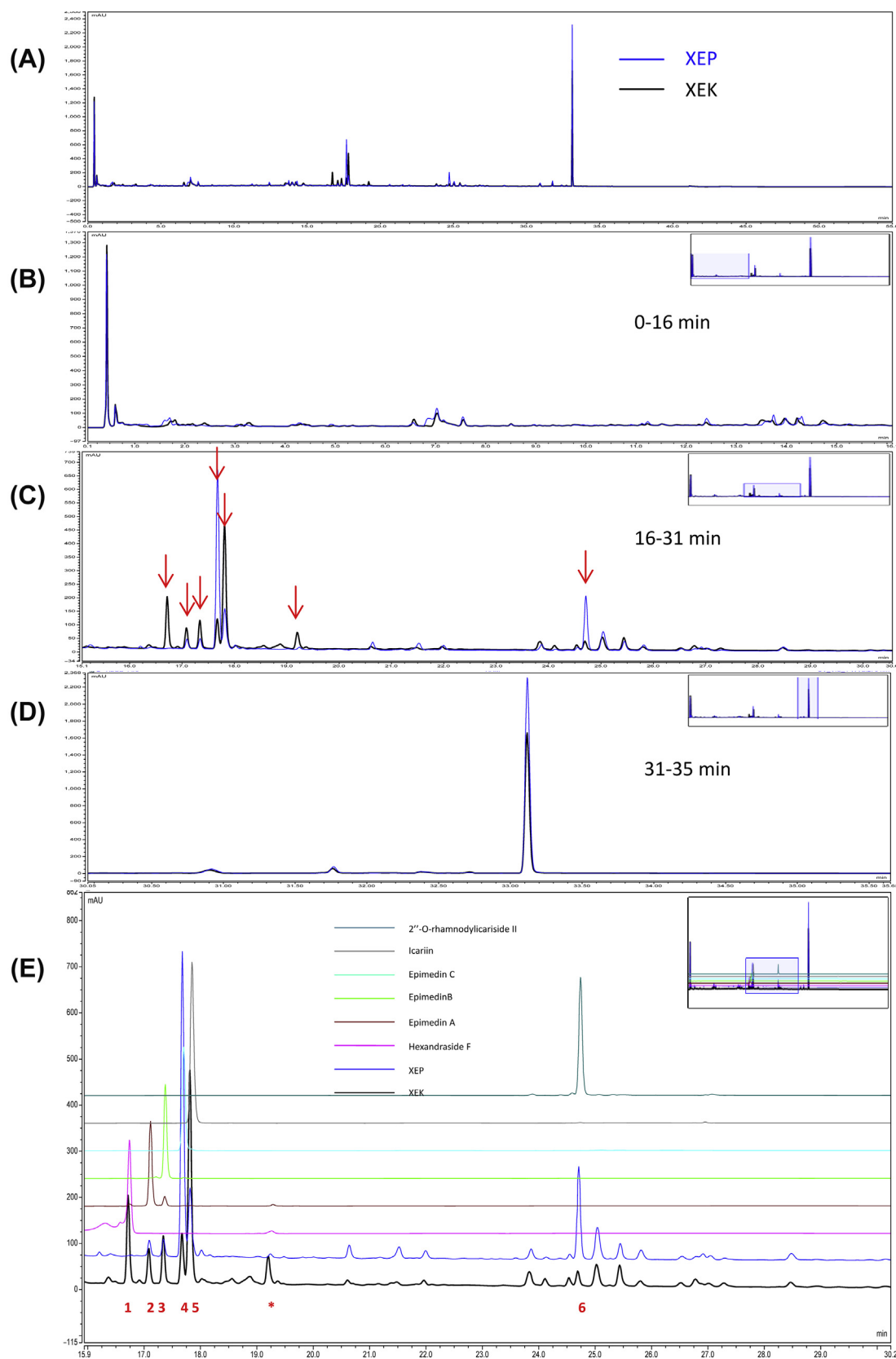


Figure 1 Ultra performance liquid chromatography profiles of different chemical constituents in *Epimedium koreanum* (XEK) and *Epimedium pubescens* (XEP). (A) Ultra performance liquid chromatography profile of XEK and XEP; (B,C,D) enlargement of the profile at 0–16 minutes, 16–31 minutes, and 31–37 minutes, respectively. The differences between XEK and XEP mainly found on the peaks (indicated with the red arrows) at 16–31 minutes; and (E) by comparing the retention time with the reference standard

Table 1 Retention time, area, and relative area of the identified peaks in *Epimedium pubescens* and *Epimedium koreanum*.

Peak no.	Peak name	XEK			XEP		
		Ret. time (min)	Area (mAU*min)	Rel. area (%)	Ret. time (min)	Area (mAU*min)	Rel. area (%)
1	Hexandraside F	16.677	11.1703	3.74	/	/	/
2	Epimedin A	17.033	4.5455	1.52	17.093	1.8292	0.56
3	Epimedin B	17.31	6.0568	2.03	17.353	2.1253	0.65
4	Epimedin C	17.64	7.1863	2.4	17.673	43.1011	13.24
5	Icariin	17.777	31.6835	10.6	17.817	10.5053	3.23
*	Icariitin-3-O- α -L-rhamnoside	19.177	4.2537	1.42	19.243	0.5576	0.17
6	2''-O-rhamnosylcariside II	24.7	2.5743	0.86	24.71	14.1174	4.34
Total rel. area		N.a.		22.57	N.a.		22.19

Rel. area = relative area; Ret. time = retention time; N.a. = Not applicable; XEK = *Epimedium koreanum*; XEP = *Epimedium pubescens*.

between each treatment group and control group ($p > 0.05$). (Figure 2A).

ALP activity assay

The results showed the increased ALP activity in C4 (160.6%), C5 (141.5%), and C6 (169.3%) treatment groups in the concentration of 0.1 μ M as compared with the control group (100%) ($p < 0.05$ or $p < 0.01$) (Figure 2B).

Ratio of RANKL and OPG mRNA expression

The concentration of all compounds was selected as 0.1 μ M. Compared with control group (100%), significantly decreased RANKL/OPG level was found in C1 and C3 treatment groups that were C1 (77.2%) and C3 (81.8%) ($p < 0.05$), while more significantly decreased RANKL/OPG level was found in C4, C5, and C6 treatment groups that were C4 (65.2%), C5 (53.5%), and C6 (61.7%) ($p < 0.01$ or $p < 0.001$).

Body and uterus weight and E₂ assay

Significantly increased body weight was found in the OVX group and decreased uterus weight was found in the OVX group, XEK group, and XEP group compared with that of the sham group ($p < 0.001$, $n = 6$), indicating that the OVX operations was performed successfully in rats (Figure 3A). Compared with the sham group, the significantly lower serum E₂ level in the OVX group was found and shown in Figure 3A that indicated successful depletion of E₂ in OVX rats. E₂ in both XEK and XEP groups were significant higher than that in the OVX group ($p < 0.05$ for XEK and $p < 0.01$ for XEP).

Bone evaluation

The bone mass and bone microarchitecture of L4 vertebrae (Figure 4A) and proximal tibiae (Figure 4B) were measured and analysed using micro-CT. The results of Figure 4A showed significantly lower relative bone volume and BMD in

L4 vertebrae in OVX group ($p < 0.01$) compared with that of sham group. As compared with OVX group, significantly higher values (higher percentages) were shown both in XEK and XEP groups: BV/TV (53.5% and 63.7%), BMD (mg HA/cm³) (15.2% and 22.9%), Conn.D. (1/mm³) (17.9% and 40.9%), Tb.N (1/mm) (11.5% and 25.4%), and Tb.Th (mm) (18.8% and 23.6%), respectively ($p < 0.05$ for all). There was no significant difference in above parameters found between the two treatment groups ($p > 0.05$). In Figure 4B, as compared with the sham group, the significant lower values measured at proximal tibiae in OVX group indicated OVX-induced reduction in both bone mass and BMD ($p < 0.01$). In the treatment groups, XEP group showed significant higher values compared with the OVX group ($p < 0.05$), while XEK group only showed marginal increase ($p > 0.05$). The results presented the higher percentages than that in OVX group that were BV/TV (29.2% vs. 53.6%), BMD (mg HA/ccm) (18.9% vs. 25.3%), Conn.D. (1/mm³) (54.9% vs. 96.3%), Tb.N (1/mm) (29.9% vs. 46.3%), and Tb.Th (mm) (30.9% vs. 30.8%), respectively (Figure 4B). However, there was no difference found between the XEP group and XEK group ($p > 0.05$).

Bone mechanical properties

Figure 3C and D present the mechanical properties of L4 vertebra and the tibia midshaft. Compression test on L4 vertebra (Figure 3C) showed that compared with the significantly lower values in OVX group, the values in XEK and XEP groups were all significantly higher in E-modulus (Mpa) (81.8% and 72.1%), compressive strength (Mpa) (74.6% and 87.3%), and energy (J) (123% and 199%), respectively ($p < 0.05$ for all). However, the three-point bending test on tibiae (Figure 3D) showed no significant differences in the tested mechanical parameters between OVX group and XEK or XEP groups ($p > 0.05$ for both).

isolated from XEP, six compounds (peaks were indicated with the red 1–6 numbers in E) were identified as C1: hexandraside F, C2: epimedin A, C3: epimedin B, C4: epimedin C, C5: icariin, and C6: 2''-O-rhamnosylcariside II. There were higher contents of C1, C2, C3, and C5 in XEK and higher contents of C4 and C6 in XEP. The peak at 19.18 minutes was speculated to be icariitin-3-O- α -L-rhamnoside, which was identified using high performance liquid chromatography–mass spectrometry.

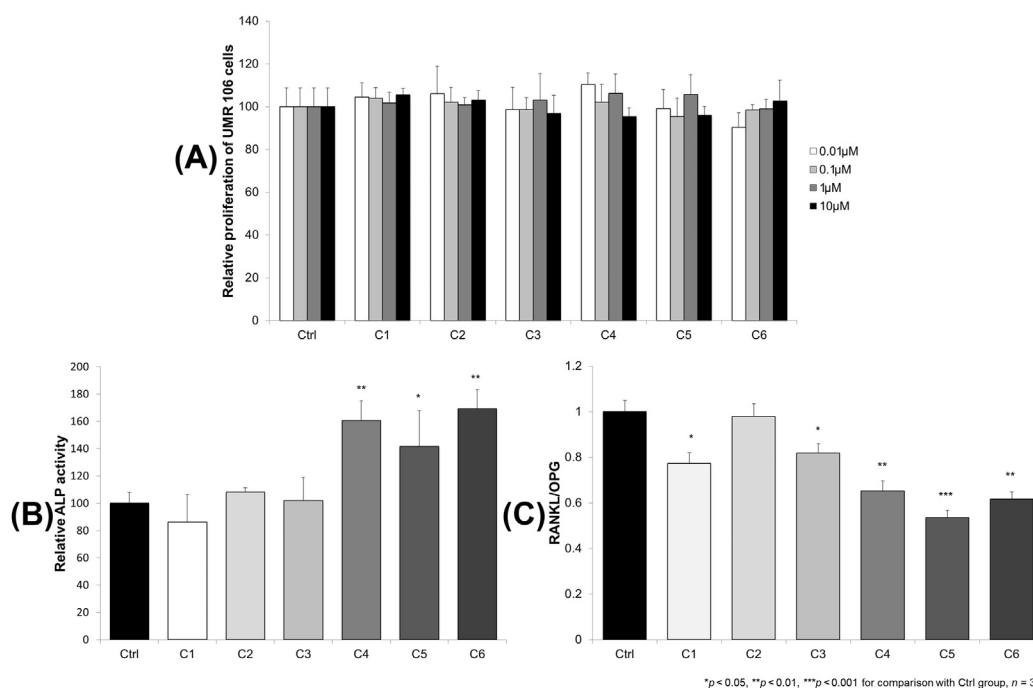


Figure 2 *In vitro* evaluation of six identified compounds. (A) Cell proliferation assay: for test cells proliferation, four concentrations of all six compounds were used for assay, including 0.01 μM, 0.1 μM, 1 μM, and 10 μM. No significant difference was found between the treatment group and the control group at three different concentrations ($p > 0.05$, $n = 3$); (B) alkaline phosphatase activity assay: the results presented the increased alkaline phosphatase activity in C4, C5, and C6 treatment groups in the concentration of 0.1 μM in contrast with the control group ($*p < 0.05$, $**p < 0.01$, $n = 3$); (C) the ratio of receptor activator of nuclear factor kappa-B and osteoprotegerin mRNA expression: The results showed significantly decreased receptor activator of nuclear factor kappa-B/osteoprotegerin level in compounds treatment groups that C1, C3, C4, C5, and C6 ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $n = 3$). ALP = alkaline phosphatase; C1 = hexandraside F; C2 = epimedin A; C3 = epimedin B; C4 = epimedin C; C5 = icariin; C6 = 2''-O-rhamnosylcariside II; Cntrl = control; RANKL/OPG = receptor activator of nuclear factor kappa-B/osteoprotegerin.

Bone turnover markers assay

Compared with the sham group, significantly higher levels of serum PINP and CTX were found in the OVX group (Figure 3B), indicating increased bone turnover in OVX rats. There was no significant difference found between OVX group and XEK group or XEP group in PINP ($p > 0.05$ for both), while CTX was found significantly lower only in the XEP group but not in the XEK group compared with the OVX group ($p < 0.05$).

Bone histomorphometry

In histological evaluation, Goldner's trichrome staining and fluorescent images of MMA sections for trabecular bone presented the cytological significant difference between the OVX group and XEK group or XEP group (Figures 5 and 6). The OVX group showed significantly lower values than the sham group. As compared with the OVX group, a significantly higher percentage of values were found in the XEK group and XEP group respectively, including Ob.S/BS (91.5% and 121.9%), Os.S/BS (125.4% and 131.8%), Os.Ar (271.9% and 349.7%), MS (49.7% and 73.4%), and MAR (113.7% and 134.2%), while significantly lower levels in Fc.Ar (37.2% and 49.2%), ES/BS (23.6% and 29.9%), BFR/BS (35.2% and 31.9%), and BFR/BV (61.2% and 63.1%) ($p < 0.05$ for all). The results

indicated the dual functions of XLGB Fufang with both bone anabolism enhancement and bone erosion inhibition for two different species of *Epimedium* in terms of the enhanced osteoblastic activities and inhibition of osteoclastic function.

Discussion

Our chemical analysis demonstrated that the content of icariin in XEK, an index for the quality control of XLGB Fufang, is comparable to XEP that also met the regular requirements in the Chinese Pharmacopoeia, in spite of some degree differences in several flavonoid contents between XEK and XEP [11,13]. The general objective of our comparable study was however designed to prove if XEK was able to substitute XEP in traditional XLGB formula with regards to its treatment efficacy, including mechanical properties and histomorphometry [28] in addition to their associated biological and biochemical mechanisms using both *in vitro* and *ex vivo* assays.

Micro-CT analysis demonstrated the efficiency in the prevention of OVX-induced bone loss at fourth lumbar vertebrae and proximal tibiae in both the XEK group and XEP group as compared with the OVX group. Clinical relevant characterisation of aging skeleton is the loss of a substantial fraction of its mechanical competence,

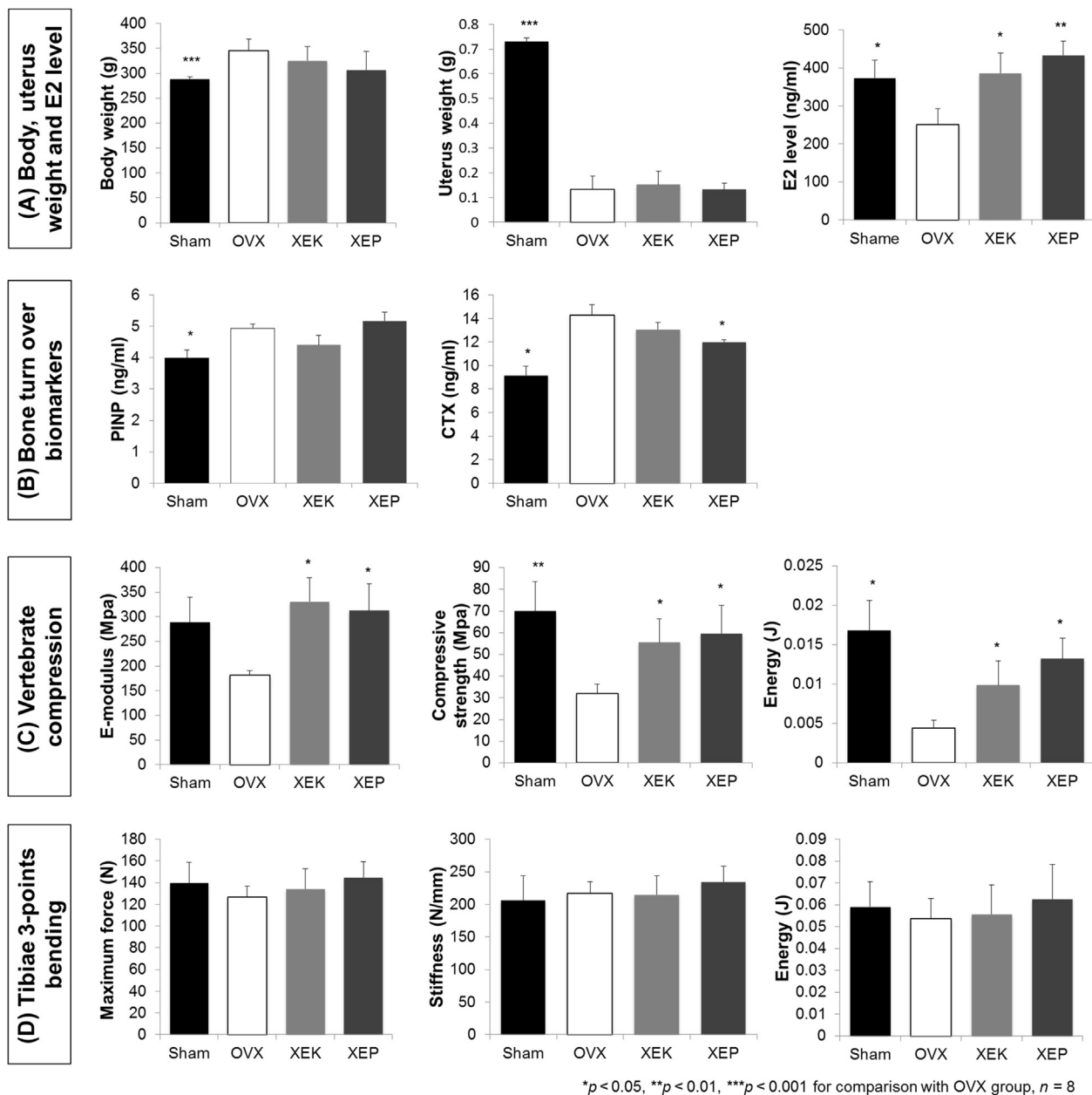


Figure 3 Quantitative analysis of anthropometric, biochemical and biomechanical tests. (A) Body and uterus weight and estradiol (E_2) assay: significantly increased body weight and decreased uterus weight of rats were found in ovariectomised (OVX), *Epimedium koreanum* (XEK), and *Epimedium pubescens* (XEP) groups compared with that in sham group (** $p < 0.001$, $n = 6$); and compared with the sham group, significantly lower levels of serum E_2 were found in OVX group. E_2 in both XEK and XEP groups were significantly higher than that in the OVX group (* $p < 0.05$ for XEK and ** $p < 0.01$ for XEP, $n = 4$). (B) Enzyme-linked immunosorbent assays of biochemical markers in serum. Compared with the sham group, significantly higher levels of serum amino-terminal propeptide and carboxy-terminal telopeptide were found in OVX group, yet without significant difference among OVX group and XEK or XEP groups in amino-terminal propeptide ($p > 0.05$, $n = 8$), while carboxy-terminal telopeptide was found to be significantly lower in XEP group compared with OVX group (* $p < 0.05$, $n = 8$). (C) Compression test on lumbar vertebra showed no significant difference between the XEK group and XEP group, but all showed significantly higher E-modulus, compressive strength, and energy as compared with that of OVX group (* $p < 0.05$, ** $p < 0.01$, $n = 6$). (D) Three-point bending test on the midshaft of the tibiae showed no significant difference in maximum force, energy, and stiffness among OVX and the two treatment groups ($p > 0.05$, $n = 6$). CTX = carboxy-terminal telopeptide; E_2 = estradiol; PINP = amino-terminal propeptide.

especially in metabolically more active trabecular bone compartments. Compression test of the current study on trabecular bone of fourth lumbar vertebrae presented the remarkable higher bone strength properties in both the XEK

group and XEP group as compared with the OVX group. However, the tibiae cortical bone in the OVX group did not show significant lower bone strength than that in the sham group, and that also did not present significant better

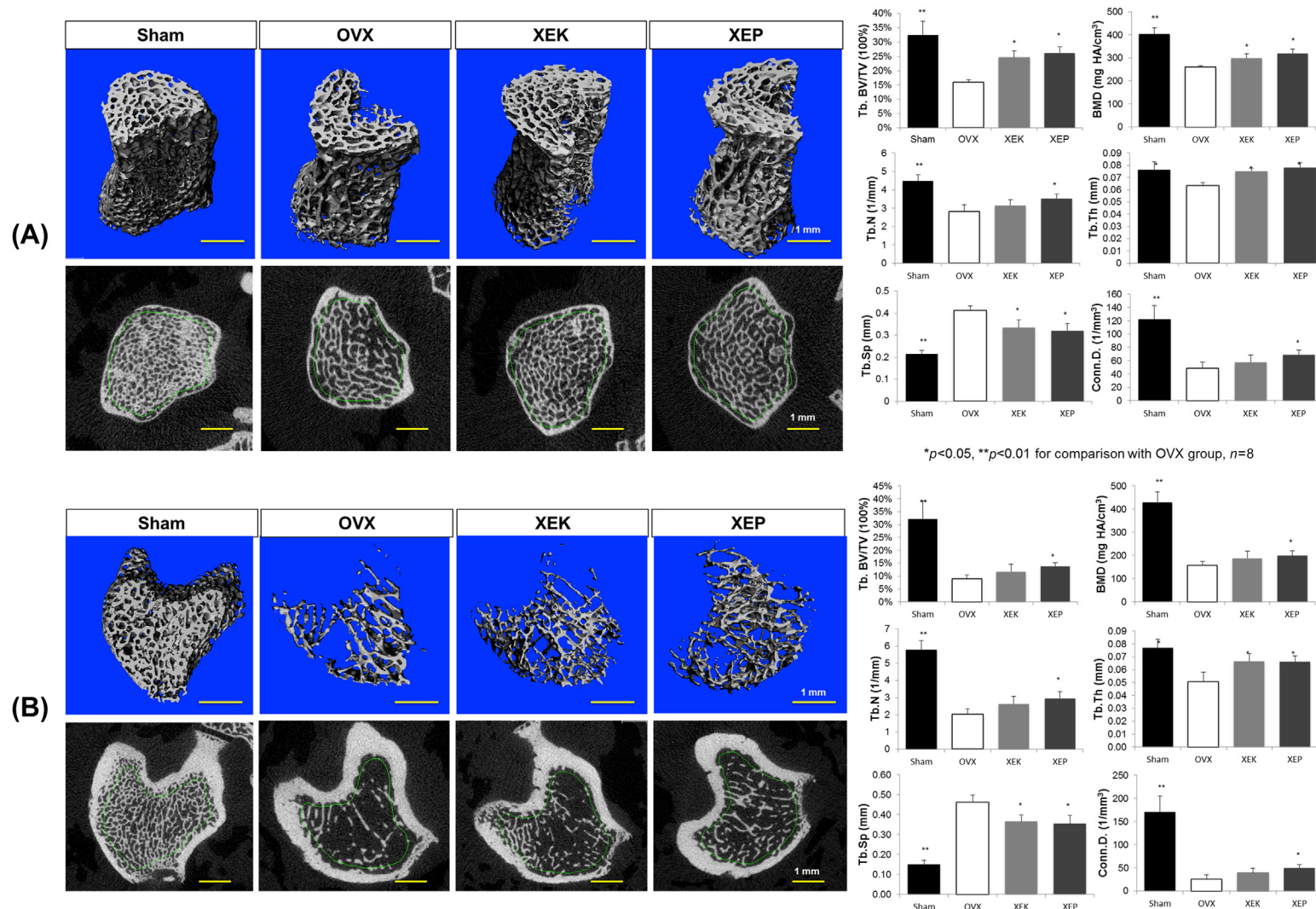


Figure 4 (A) Representative micro-computed tomography two-dimensional and three-dimensional images and quantitative analysis of the fourth lumbar vertebrae (L4) 3 months after treatment showed no differences between *Epimedium koreanum* (XEK) and *Epimedium pubescens* (XEP) groups, and both with significantly higher bone tissue volume fraction, bone mineral density, trabecular number and trabecular thickness, but lower trabecular separation as compared with that of the ovariectomised (OVX) group (* $p < 0.05$, $n = 8$). (B) Representative micro-computed tomography two-dimensional and three-dimensional images and quantitative analysis of proximal tibiae 3 months after treatment revealed no difference between the XEK group and XEP group. XEP showed significantly higher bone tissue volume fraction, bone mineral density, connective density, trabecular number, and trabecular thickness, and lower trabecular separation as compared with the OVX group (* $p < 0.05$, $n = 8$), and XEK showed significantly higher trabecular thickness and lower Tb.Sp compared with the OVX group ($p > 0.05$, $n = 8$). BMD = bone mineral density; BV/TV = bone tissue volume fraction; Conn.d. = connective density; OVX = ovariectomised; Tb.N. = trabecular number; Tb.Sp = trabecular separation; Tb.Th = trabecular thickness; XEK = *Epimedium koreanum*; XEP = *Epimedium pubescens*.

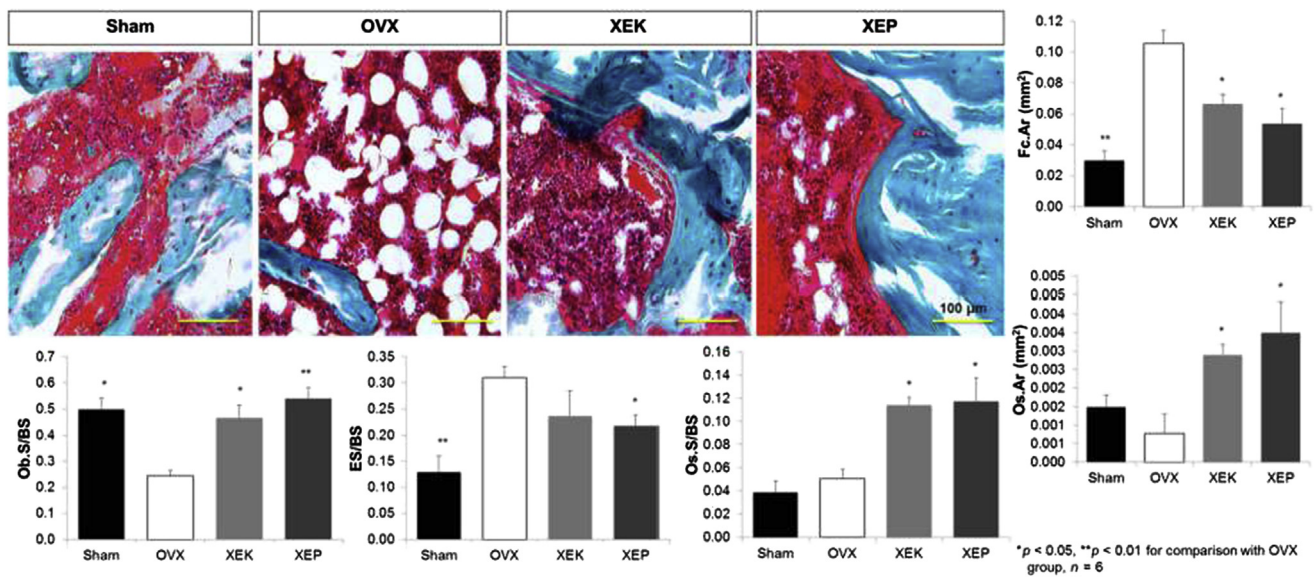


Figure 5 Representative Goldner's trichrome staining and histomorphometry in undecalcified MMA sections. Significantly higher osteoblasts surface and bone surface, osteoid surface and bone surface, and osteoid area, and significantly lower fat cells area and erosion surface and bone surface were found in *Epimedium koreanum* and *Epimedium pubescens* groups as compared with the ovariectomised group ($*p < 0.05$ or $**p < 0.001$, $n = 6$), yet without difference found between *Epimedium koreanum* group and *Epimedium pubescens* group. ES/BS = erosion surface and bone surface; Fc.Ar = fat cells area; Ob.S/BS = osteoblasts surface and bone surface; Ob.S/BS = osteoid surface and bone surface; Os.Ar = osteoid area; OVX = ovariectomised; XEK = *Epimedium koreanum*; XEP = *Epimedium pubescens*.

mechanical properties in XEK and XEP groups compared with OVX group. This result implied the tibiae cortical bone was not remarkably impaired by OVX itself or at least in early stage of oestrogen depletion designed for the current study. The fact that most osteoporotic fractures occur at locations rich in trabecular bone, that explained the lower trabecular bone strength in vertebral bone and/or early sensitive changes of trabecular bone in bone strength, than that of the cortical bone dominant tibial shaft where it showed slow and less sensitive changes in bone strength [29,30]. In the present study, we did not find differences in structural and mechanical differences between the XEK group and XEP group, implying similar antiosteoporotic efficacy of two different species of *Epimedium*. The *in vitro* results in Figure 2 showed higher ALP activity (Figure 2B) and lower RANKL/OPG ratio (Figure 2C), especially in the three kinds of compounds – epimedin C, icariin, and 2''-O-rhamnosylcariside II. The inverted relationship found between ALP activity and RANKL/OPG ratio suggested the osteogenic and antiosteoclastic potentials in the two treatment groups due to multiple herbal compounds [31]. It is known that higher ALP activity indicates upregulated osteogenesis, and higher RANKL/OPG ratio secreted from osteoblasts implies inhibition to osteoclast maturation and function, i.e., less activated osteoclasts and weaker bone resorption activity. This was supported by histomorphometric data in Figure 5 where the lower ES/BS ratio was measured in the two treatment groups as compared with the OVX group. For the comparative analysis between the two *Epimedium* species, higher levels of icariin in the XEK group, and also higher levels of epimedin-C and 2''-O-rhamnosylcariside II in the XEP group could present a slight difference and similar osteogenesis abilities that result in

ultimately similar effects of bone mass, bone microstructure, and biomechanical properties.

For investigation of further underlying mechanisms of XLGB formulae on the prevention of oestrogen-depletion induced bone loss, histomorphometry was performed for quantitative comparison. The results showed higher bone turnover (high ES/BS in Figure 5 and high BFR/BS and BFR/BV in Figure 6) in the OVX group as compared with that of the sham group. Postmenopausal osteoporosis is a bone disease in which the bone resorption rate is increased because of oestrogen deficiency, and BFR/BS or BFR/BV is also increased to compensate for the high bone resorption. The common form of high turnover (high BFR/BS and BFR/BV) is deleterious to the skeleton because there is net loss of bone. Especially in the early stages, the tested bone turnover dynamic parameters are always higher, such as MS/BS, MAR, BFR/BS, and BFR/BV in histomorphometry. This could be consistent with the balanced and coupled bone formation and resorption that did not result in detectable changes in bone volume. However, for the relative late stage of osteoporosis, such as more than 3 months post-OVX in our current experimental study, the bone formation rate was decreased so that it seemed contradictory to the finding on the resorption rate and the net bone loss. Su et al [32] reported a decreased MAR and BFR/BS 35 days post-OVX operation in mice models compared with that 14 days post-OVX operation. In the presented study (Figure 6), although a lower MAR was observed in the OVX group that used to stand by the less new bone formation, the higher BFR/BS and BFR/BV were still found in the OVX group compared with the sham group that was supported by higher MS/BS, i.e., with more mineralising surface. The effective treatment in the XEK group or XEP group could supplement the deficiency of bone formation in OVX rats via overall enhanced osteogenic activity.

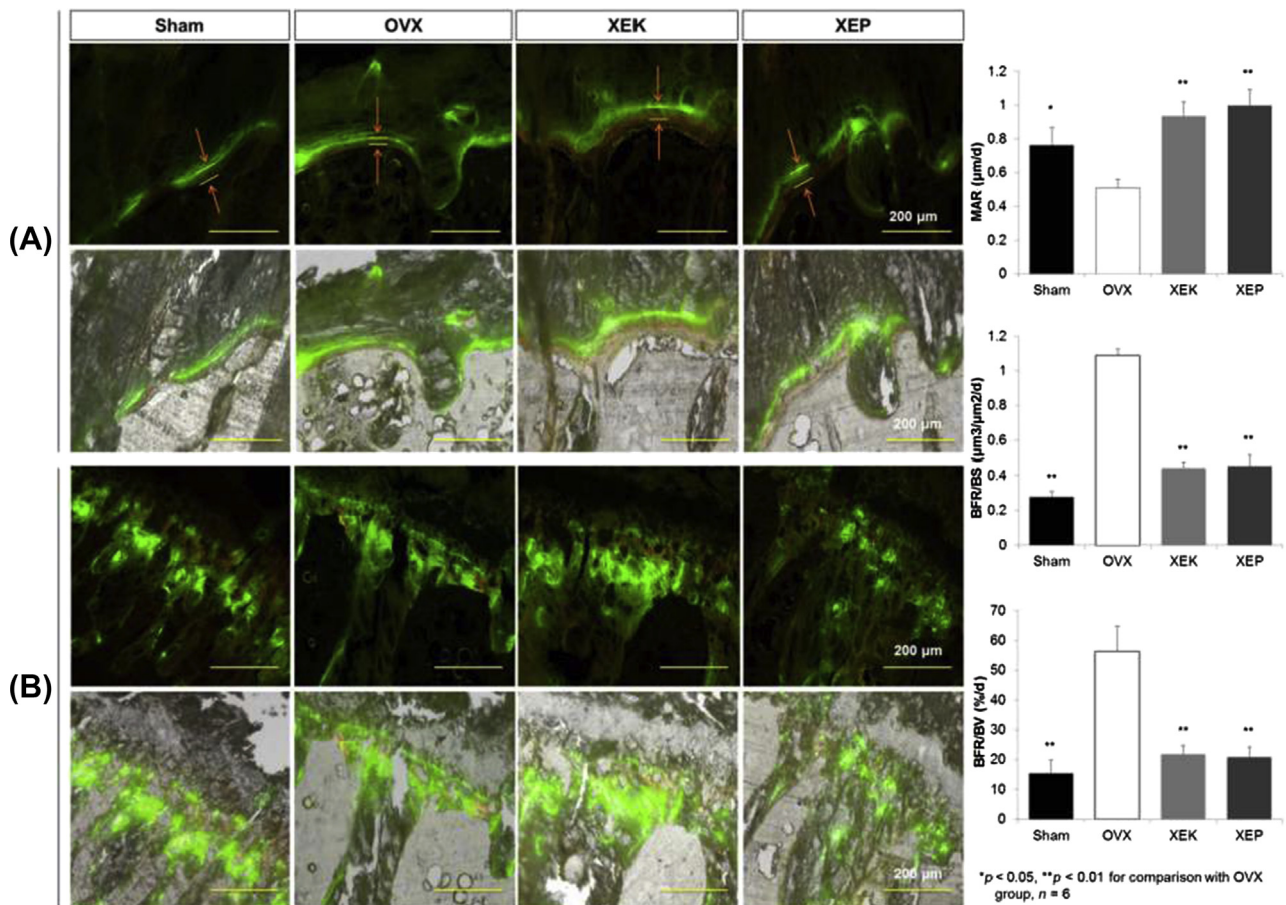


Figure 6 Representative fluorescence imaging and histomorphometry in undecalcified MMA sections. (A) Distance between the two-labelled lines in fluorescence images for calculating bone mineral apposition rate (MAR), and superimposed fluorescence and optical view in trabecular bone site of proximal tibiae among different groups. The MAR in ovariectomised (OVX) group was significantly reduced as compared with that of Sham group. However, the distance in *Epimedium koreanum* (XEK) and *Epimedium pubescens* (XEP) groups showed significantly larger width in contrast to OVX group (* $p < 0.05$ or ** $p < 0.001$, $n = 6$). (B) Mineralised matrix properties between OVX and two treatment groups. No difference was found between XEK and XEP groups, but significantly higher levels of mineralising surface and MAR were found in both XEK and XEP groups as compared with that of OVX group, while lower levels of bone formation rate per unit of bone surface and bone formation rate per unit of bone volume were found in both XEK and XEP groups in contrast to OVX group (* $p < 0.05$ or ** $p < 0.001$, $n = 6$). BFR/BS = bone formation rate per unit of bone surface; BFR/BV = bone formation rate per unit of bone volume; MAR = bone mineral apposition rate; OVX = ovariectomised; XEK = *Epimedium koreanum*; XEP = *Epimedium pubescens*.

Our previous studies showed that *Epimedium*-derived flavonoids, especially flavonoid aglycone icaritin, possess great potential in increasing osteoblast-like cell proliferation, osteoid formation, and mineralisation [33,34]. As compared with the OVX group, the treatment groups of our current study showed less adipogenesis and more osteoid formation adjacent to the mineralised bone with even enhanced osteoblasts and suppressed osteoclasts activities based on the results of Goldner's Trichrome staining. It implied that herbal Fufang XLGB was formulated with the key composition of *Epimedium* that showed inherent osteogenic activity in terms of promoting osteoid formation and mineralisation, as well as inhibitory effects in the suppression of osteoclasts activity as evidenced with reduced bone erosion surface. *Epimedium*-derived flavonoid icaritin alone could enhance bone mesenchymal stem cell (MSC) homing and differentiation *in vitro*, which might be directly involved in the bone morphogenetic protein

signalling pathway and associated with the Wnt signalling pathway [33,34]. In addition, the role of adipogenic reduction could be another regulation of pathologic mechanisms. During osteoporosis development, the enhanced differentiation of adipocytes from bone MSCs inhibit the osteoblast differentiation. Icaritin has effects on the inhibition of adipogenic differentiation of MSCs [34]. As one of the bioactive molecules of *Epimedium*, icaritin or desmethylcaritin may present similar mechanisms or signalling pathways in osteogenesis and adipogenesis with XEK and XEP. The exact difference in mechanism or signalling pathway between the two species of *Epimedium* could not be delineated in the current study, and therefore we used bone structure and mechanical properties as the end-point of intervention effects.

As expected, the serum E_2 significantly decreased in the OVX rats as compared with that of the sham group. Meanwhile, serum E_2 in the XEP group and XEK group were both

significantly higher than that in the OVX animals. ELISA was used for the current study, which is a test that uses antibodies and colour change to identify a substance. ELISA for testing E_2 uses an E_2 antibody as the solid-phase in this enzyme immunoassay to detect the presence of E_2 in the serum due to its affinity to the E_2 antibody. In this immunoassay, other substance such as the phytoestrogens or their metabolites of XLGB in serum, which are able to bind to the E_2 antibody, would also be tested as E_2 . Previous studies found that serum E_2 levels were much higher when measured by immunoassay compared with that determined by mass spectrometry in patients treated with exemestane, which is steroidal with a similar structure with E_2 [35,36]. Furthermore, it was reported that some compounds in XLGB were able to bind to ER [37], so we postulated that the higher " E_2 level" in XLGB groups was not the real serum E_2 . This was further indicated by the results that E_2 was able to significantly increase the uterus weight in OVX animals as reported before. Unlike E_2 , XEP or XEK was a phytoestrogen-rich formula that did not exert similar stimulating effect on uterus. This also hinted that MS would be more favourable to test serum E_2 when treated with oestradiols or phytoestrogens. It is known that oestrogen expresses its activities by binding to different oestrogen receptors (ERs), including ER α and ER β . ER β is more abundant than ER α in bone tissue, while ER α it is mainly distributed in reproductive cells and is the dominant receptor mediating the effects of E_2 in the breast and uterus [25,38]. Therefore, it is postulated that XEP or XEK may have potential antiosteoporotic effects on OVX mice via ER β in bone or membrane/cytoplasm-initiated oestrogen receptor signalling pathway.

The biochemical markers served as an important modulator of bone turnover always indicate excessive resorption by osteoclasts or inadequate bone formation by osteoblasts due to the depletion of oestrogen in women [29]. An increase of bone turnover has been documented at the time of menopause that is 37–52% and 79–97% increases in the bone formation and bone resorption marker levels, respectively, and this high bone turnover is just associated with a low bone mass. PINP and CTX are relevant bone formation and resorption markers that reflect and determine biological response of skeleton tissues during drug treatment for osteoporosis. Based on the results, there were significantly higher levels of PINP and CTX in OVX group than that in sham group. As compared with the OVX group, no significantly higher PINP level detected both in XEK and XEP groups, which could be explained as that a short detectable time window used to reflect the actual levels of serum biomarkers that should be followed by a stable platform of bone formation during osteoporosis treatment. In addition, significantly higher CTX level was found in OVX group compared with the sham group that implied a high bone turnover activity. Our *in vitro* tests showed a significant decrease in RANKL/OPG level in epimedin C, icariin, and 2''-O-rhamnosylcariside II compounds, so both XEK and XEP should play an antiresorption role as supported by significantly lower serum CTX level in XEP group and less prominently lower CTX in XEK group compared with the OVX group, an index of suppression of osteoclastic activities during bone remodelling. However, there was no significantly decreased CTX level found in XEK

group in contrast to OVX group, and this might be explained by a mechanism of 'uncoupling' that the deterioration of microstructure often requires a longer period of time, and the alterations of biochemical markers on bone turnover in serum become detectable shortly after pharmacological intervention in osteoporosis therapy [39]. It might still have certain differences between XEK and XEP in terms of effective time window although at the time of study completion it did not show significant difference in CTX level between XEP and XEK groups. As the current study just used one time point, i.e., end-point to measure the treatment efficacy of XEK and XEP to osteoporosis, future studies should be designed to detect temporal changes of serum biomarkers.

The above results indicated treatment efficacy of herbal Fufang XLGB containing either XEK or XEP for the prevention of OVX-induced bone loss and deterioration of its mechanical properties. No significant difference was found between the XEK group and XEP group, yet with the XEP group showing slightly higher mean values, implying XEP used for formulating herbal Fufang XLGB might even possess better potentials for prevention of OVX-induced bone loss. Based on the chemical analysis of XEP and XEK, seven flavonoids have different contents in two formulae. There is more epimedin C and 2''-O-rhamnosylcariside II in XPE than that in XEK, and more hexandraside F, epimedin A, epimedin B, icariin, and icaritin-3-O- α -L-rhamnoside in XEK than that in XEP. Our *in vitro* evaluation showed that epimedin A, epimedin C, icariin, and 2''-O-rhamnosylcariside II significantly promoted ALP activity in osteoblast-like cells, while hexandraside F, epimedin B, epimedin C, icariin, and 2''-O-rhamnosylcariside II all decreased RANKL/OPG, indicating that they were able to inhibit osteoclastogenesis at a 10 μ M concentration. The *in vitro* results suggested that all of the six compounds might contribute to the antiosteoporotic effects of two XLGB formulae and resulting in the comparable efficacy of XEP and XEK. As the efficacy evaluation was based on systemic biological effects, multifunctional composite of herbal Fufang XLGB could facilitate comprehensive effects in antiosteoporosis based on total composed compounds. In addition, the chemical analysis results are in line with our previous published report that epimedin C is the main compound of *E. pubescens*, while icariin is the main flavonoid in *E. koreanum*. As the main components in XEP and XEK, respectively, the similar bioactivity of epimedin C and icariin may contribute greatly to prevention of OVX-induced bone loss with comparable effects from different XLGB formulae. Various studies were performed to investigate the efficacy [31], pharmacokinetics [40], and metabolism [41] of icariin. Recently, epimedin C had received more and more attention [42]. Both epimedin C [42] and icariin [31] exerted antiosteoporosis effects in OVX rats or mice. Further, our recent metabolic study of XEP found that both epimedin C and icariin were able to be absorbed into blood, moreover there was more icariin in the plasma than epimedin C in the metabolites although it was not the case in the original XEP herb [15]. Moreover, only the absorbed chemical components or the related metabolites in blood, which maintained a certain concentration in the target organ(s) for a period of time, such as for the early time period, are essential to realize their therapeutic effects. Thus, we should not only

compare the chemical constituents of compounds in the XLGB formula, but also investigate the metabolic profiles of XEP and XEK in the future.

As a candidate for the selection of effective ingredient to formulate antiosteoporosis herbal Fufang, the anti-osteoporosis effect of *E. koreanum* could be further investigated for exploring its dosing effects in the future that should supplement low or high dosage groups of XEK and XEP to investigate the optimal dose-effect dependent manner for further reference to formulae selection and evaluation.

Conclusion

The formulation of commercially available antiosteoporosis XLGB Fufang with either XEK or XEP showed similar efficacy in the prevention and treatment of OVX-induced osteoporosis, as measured for both bone mass and bone strength. These findings implied that both XEK and XEP *Epimedium* species could be potentially developed for formulation of current herbal Fufang XLGB capsules for established clinical applications.

Conflicts of interest

All the authors declare no conflicts of interest.

Funding/support

This study was supported by “12.5 Major New Drug Creating Special Projects from the Ministry of Science and Technology of China (2011ZX09201-201-01)”, “Shenzhen government project (GJHS20130402135334943)”, and “the National Natural Science Foundation of China (81220108028, 81302782), as well as the National Major Scientific and Program of Introducing Talents of Discipline to Universities (B13038)”.

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