

Herbal Medicine for Osteoporosis and Related Conditions

Background of the Invention

5 The present invention relates to a herbal medicine useful for the treatment of
osteoporosis and other conditions. It may be of use in the prevention or treatment of
obesity and diabetes. The medicine is derived from a number of plant materials used
in traditional Chinese medicine. More specifically, we have found that a mixture of
six herbal materials has desirable activities. Furthermore, the activities of the mixture
may be enhanced by fractionation/purification. Desirably the individual herbal
10 materials are fractionated/purified in individually appropriate ways prior to mixing
with the other materials.

Osteoporosis is a disease of the skeleton in which the bone loses density.
Bones become brittle and prone to fracture. Osteoporosis is diagnosed when bone
15 density has decreased to the point where fractures will happen with mild stress.
Bones most likely to break from osteoporosis are in the hip, wrist, and spine. Women
past menopause are the group most likely to develop osteoporosis, but it is not
uncommon in men over 65.

20 Bone remodelling is the process in which bones are broken down through
resorption and then built back up again through bone formation. This is a natural
process that takes place all the time on different parts of skeletal bone. Usually these
two processes balance each other out and a stable level of bone mass is maintained.
Until a healthy person is around 40, the process of breaking down and building up
25 bone by cells called osteoclasts and osteoblasts is a nearly perfectly coupled system,
with one phase stimulating the other. As a person ages, however, or in the presence of
certain conditions, this system breaks down and the two processes become
unbalanced.

30 Osteoclasts are specialized bone cells that break down bone mass through a
process often called resorption of bone. The action of osteoclasts is balanced by the
activity of osteoblasts that build up bone mass. In the bones of healthy adults, bone

mass remains relatively stable because osteoblasts and osteoclasts are balancing each other and working together to maintain the bone mass. This allows bones to continuously remodel and to heal when damaged. The function of osteoclasts can be affected by various drugs and key nutrients.

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In a recent publication, Lee and colleagues (2007) make the surprising observation that bone, a tissue not previously thought to impact bioenergetics, can affect adiposity, glucose metabolism, and insulin sensitivity. People with obesity and diabetes are known to have abnormalities of bone metabolism. Increased adiposity is a risk factor for fracture in children, and diabetics are prone to fracture, although there is little evidence that they are predisposed to osteoporosis (Schwartz et al., 2001).

However, the rate of bone turnover is decreased in diabetics as reflected by diminished expression of biomarkers of bone resorption and formation, including osteocalcin, an osteoblast specific protein (Gerdhem et al., 2005, Osteoporosis Int., 16, (506-1512). Common thinking holds that the decrease in osteoblast activity (the cells that build bone) results from the consequences of diabetes including hyperglycemia, and decreased levels or effectiveness of insulin and IGF-1 (Inaba, 2004). Lee et al. (2007) now present data to challenge this notion and raise the provocative concept that the reduction in osteoblast function may contribute to obesity and glucose intolerance. In this scenario, a vicious cycle would exist in which the metabolic sequelae of diabetes suppress osteoblast function, leading to diminished osteocalcin bioactivity, which in turn further aggravates obesity and insulin resistance.

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Summary of the Invention

We have developed a medicine that can alleviate or prevent (or at least delay the onset of) osteoporosis and related conditions by a double-pronged attack, in that it can both increase the bone-building activity (due to osteoblasts) and decrease the bone resorption activity (due to osteoclasts). Thus the medicine can be used for one or more of the following:

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1. Treating osteoporosis
2. Reducing rate of bone fractures
3. Increasing bone mineral density
4. Restoring bone structure
5. Alleviation of menopausal symptoms such as dryness and hot flushes

Furthermore the medicine may be used in the treatment of other conditions linked to the activities of osteoblasts and/or osteoclasts. These may include obesity, glucose intolerance and/or diabetes.

Our medicine has been demonstrated to promote the proliferation of osteoblasts. Therefore, it is expected to reduce the body weight through the activation mechanism of osteoblasts.

In further embodiments, the disclosure relates to methods of treatment of mammals (human and non-human); methods of manufacturing medicines (which may comprise steps of fractionating components); and use of herbal components in the manufacture of compositions for use in therapy.

The present disclosure relates to a composition derived from six plant materials:

- (i) *Herba epimedii* 淫羊藿 (Yin Yang Huo)
- (ii) *Fructus Psoraleae* (*Psoralea coryfolia*) 补骨脂 (Bu Gu Zhi)
- (iii) *Radix Rehmanniae preparatae* (*Rehmannia glutinosa*) 熟地黄 (Shu Di)
- (iv) *Cortex Eucommiae* 杜仲 (Du Zhong)
- (v) *Fructus Cnidii* 蛇床子 (She Chuang Zhi)
- (vi) *Radix Astragali* (*Astragalus Membranaceus*) 黄芪 (Huang Qi)

Some components of these plant materials have been reported in the literature. For example, geniposidic acid from Du Zhong (Kim et al 2003), osthole from She Chuang Zi (Kuo et al. 2005), corylin and bavachin from Bu Gu Zhi (Wang et al.

2001) (Xiong et al, 2003), icarrin from Yin Yang Huo (Xie et al. 2005) and polysaccharides from Shu Di (Oha et al. 2003) and Huang Qi were reported.

Preferred compositions of the disclosure contain extracts of the 6 herbal materials in the ratio (in parts by weight):

- (iv) Du Zhong: 2.5-10, preferably 4-8, most preferably 5 parts
- (ii) Bu Gu Zi: 1.5-6, preferably 2-4, most preferably 3 parts
- (iii) Shu Di: 1.2-5.0, preferably 1.5-3, most preferably 2.5 parts
- (v) She Chuang Zhi: 1-4, preferably 1.2-2.8, most preferably 2 parts
- (vi) Huang Qi: 0.3-1.5, preferably 0.5-1.0, most preferably 0.75 parts
- (i) Yin Yang Huo: 0.15-0.75, preferably 0.25-0.5, most preferably 0.375 parts

The extracts are preferably produced by ethanol extraction for (i), (ii), (iv) and (v), and water-extraction for (iii) and (vi). These initial extracts may be partially purified, suitable techniques being illustrated herein.

Compositions disclosed herein may contain further components, e.g. conventional adjuvants and/or excipients. They may contain further active ingredients, e.g. a source of calcium such as calcium carbonate which may be provided by oyster shell or pearl powder. Suitably the composition, which may be in a unit dosage form, provides 0-1400mg of calcium daily.

Excipients may include lactose or starch. Other common excipients include magnesium stearate, dextrin, povidone, celluloses, talc and sucrose. One function may be to counteract a tendency of the herbal materials to absorb moisture and become lumpy. Other possible functions of excipients include flavouring (e.g. sweeteners) and colouring. Excipients may comprise binders, disintegrants, fillers (diluent) and lubricants. Liquid formulations may include liquid vehicles, e.g. water.

In a first aspect, there is provided a pharmaceutical composition comprising the following 6 herbal materials or extracts thereof: (i) Yin Yang Huo; (ii) Bu Gu Zhi; (iii) Shu Di; (iv) Du Zhong; (v) She Chuang Zhi; and (vi) Huang Qi.

In a second aspect, there is provided a method of producing a pharmaceutical composition as defined above including a step of preparing extracts of said herbal materials.

In a third aspect, there is provided the use of a pharmaceutical composition as defined above in the manufacture of a medicament for treating a condition linked to activities of osteoblasts and/or osteoclasts.

In a fourth aspect, there is provided the use of a pharmaceutical composition as defined above in the manufacture of a medicament for treating a mammal for one or more of: reducing the rate of bone fracture; increasing bone mineral density; and restoring bone structure.

Brief Description of the Drawings

Fig 1(i) to (vi) is a set of graphs illustrating the activity of 6 individual herbal extracts on osteoclasts.

Fig 2 is a graph like those of Fig 1 but illustrating the activity of mixtures of
5 extracts of all 6 herbal materials.

Fig 3(i) to (vi) is a set of graphs illustrating the activity of 6 individual herbal extracts ((i) Yin Yang Huo; (ii) Bu Gu Zhi; (iii) Shu Di; (iv) Du Zhong; (v) She Chuang Zhi; and (vi) Huang Qi) on osteoblasts.

Fig 4a and Fig 4b are graphs like those of Fig 3 but illustrating the activity of
10 mixtures of extracts of all 6 herbal materials, after 24hrs (Fig 4a) and 48hrs (Fig 4b).

Fig 4c is a graph showing best fitting curves for activity of the mixtures on proliferation activity of osteoblasts.

Fig 5 is a graph illustrating the activities of the mixture (with and without partial purification) on the femoral bone mass index.

Fig 6 and 7 are dose response curves for the mixture (with and without partial
15 purification) on bone mineral density in the femur (Fig 6) and the lumbar spine (Fig 7).

Fig. 8 is a graph relating dosage with the partially purified structure to change in body weight.

Description of the Preferred Embodiments

Extraction Procedures

We extracted the six herbs, individually, with 70% aqueous ethanol and with water, and compared the results. For each herb, 3 different samples from different
25 sources were used.

(a) Extraction of raw herb (i) (Yin Yang Huo):

- (a) using 70% EtOH: 500 g of dried leaves of Ying Yang Huo were ground and extracted with 4 L of 70% EtOH (ACS grade ethanol obtained from Megachem) for one hour, three times, under reflux. The combined extract was filtered and the filtrate was concentrated and dried under vacuum.
- 5 (b) using water: 500 g of dried leaves of Yin Yang Huo were ground and extracted with 5 L of water for two hours, two times, under reflux. The combined extract was filtered and the filtrate was concentrated and dried under vacuum.
- 10 (b) Extraction of raw herb (ii) (Bu Gu Zhi):
- (a) using 70% EtOH: 500 g of dried fruit of Bu Gu Zhi was ground and extracted with 4 L of 70% EtOH for one hour, three times, under reflux. The combined extract was filtered and the filtrate was concentrated and dried under vacuum.
- 15 (b) using water: 500 g of dried fruit of Bu Gu Zhi was grinded and extracted with 5 L of water for two hours, two times, under reflux. The combined extract was filtered and the filtrate was concentrated and dried under vacuum.
- 20 (c) Extraction of raw herb (iii) (Shu Di):
- (a) using 70% EtOH: Dried herb of Shu Di (500 g) was extracted with 4 L of 70% EtOH for one hour, three times, under reflux. The extracts were filtered and the filtrate was concentrated and dried under vacuum.
- (b) using water: Dried herb of Shu Di (500 g) was extracted with 5 L
- 25 of water for two hours, two times, under reflux. The extracts were filtered and the filtrate was concentrated and dried under vacuum.
- (d) Extraction of raw herb (iv) (Du Zhong):
- (a) using 70% EtOH: Air dried bark of Du Zhong (500 g) was chopped
- 30 and extracted with 4 L of 70% EtOH for one hour, three times, under reflux. The combined extract was filtered and the filtrate was concentrated and dried under vacuum.

(b) using water: 500 g of dried bark of Du Zhong was chopped and extracted with 5 L of water for two hours, two times, under reflux. The combined extract was filtered and the filtrate was concentrated and dried under vacuum.

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(e) Extraction of raw herb (v) (She Chuang Zhi):

(a) using 70% EtOH: 500 g of dried fruit of She Chuang Zhi was chopped and extracted with 4 L of 70% EtOH for one hour, three times, under reflux. The combined extract was filtered and the filtrate was concentrated and dried under vacuum.

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(b) using water: 500 g of dried fruit of She Chuang Zhi was chopped and extracted with 5 L of water for two hours, two times, under reflux. The combined extract was filtered and the filtrate was concentrated and dried under vacuum.

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(f) Extraction of raw herb (vi) (Huang Qi):

(a) using 70% EtOH: 500 g of dried root of Huang Qi was extracted with 4 L of 70% EtOH for one hour, three times, under reflux. The combined extract was filtered and the filtrate was concentrated and dried under vacuum.

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(b) using water: 500 g of dried root of Huang Qi was extracted with 5 L of water for two hours, two times, under reflux. The combined extract was filtered and the filtrate was concentrated and dried under vacuum.

HPLC Analysis of the crude extracts:

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HPLC analyses of the extracts were carried out using a Waters 2695 separation module coupled with Waters 2487 Dual λ Absorbance Detector and Empower Pro software. The HPLC system was equipped with a C18 Column (250 x 4.6 mm, 5 μ m), C18 Guard Cartridge, 3 mm. The analyses were performed at room temperature. Each sample was prepared at 1mg/ml concentration using 60% EtOH as solvent. The injection volume of the sample was 20 μ l. The mobile phase was a mixture of acetonitrile and 0.1% formic acid at a flow rate of 1.0 ml/min and method

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used was fullscan_2b. HPLC chromatograms were recorded at 254 nm and 315 nm of wavelength. Details of the HPLC method are shown in Table 1.

Table 1: HPLC method

1% formic acid in H₂O %	ACN %	Time (min)
96	5	0
50	50	30
20	80	31
20	80	34
96	4	35
96	4	40

HPLC grade solvents were purchased from Fisher Scientific and Standard
5 samples Osthole and Icarrin were purchased from China Pharmaceutical and
Biological manufacturer and the standard Geniposidic acid was obtained from
Chroma Dex.

Results of the HPLC analysis are included in Table 2.

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**Table 2: Extraction Yields and Active Components from Three Different Sources
of China**

Raw Herb	Extraction Yield		Active component %	
	Water	70% EtOH	Water Extraction	70% EtOH extraction
Bu Gu Zhi (ii)	28.55 %	30 %	-	-
	39.33 %	29.16 %	-	-
	31.09 %	27.90 %	-	-
She Chuang Zhi (v)	20.07 %	21 %	No peak	0.91 % (Osthole)
	24.68 %	18.48 %	No peak	1.56 % (Osthole)
	16.86 %	22.47 %	No peak	1.90 % (Osthole)
Du Zhong (iv)	14 %	15 %	0.12 % (GPA)	0.27 % (GPA)
	14.03 %	12.14 %	0.90 % (GPA)	2.33 % (GPA)
	12.80 %	15.63 %	0.17 % (GPA)	1.22 % (GPA)
Yin Yang Huo (i)	22 %	17.30 %	0.28 % (Icarrin)	0.75 % (Icarrin)
	11.53 %	11.62 %	0.25 % (Icarrin)	2.86 % (Icarrin)
	15.72 %	12.95 %	0.59 % (Icarrin)	0.85 % (Icarrin)
Shu Di (iii)	76 %	67.65 %	-	-
	68.10 %	61.87 %	-	-
	70.31 %	65.61 %	-	-
Huang Qi (vi)	54 %	34 %	-	-
	36.35 %	29.40 %	-	-
	36.20 %	33.24 %		-

From the results, it was concluded to use extraction with 70% EtOH for herbs (i), (ii), (iv) and (v); and extraction with water for herbs (iii) and (vi).

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Preparation of extracts (larger scale):

Extraction:

Extraction of raw herb (i) (Yin Yang Huo) using 70% EtOH: 2 kg of dried leaves of Ying Yang Huo were ground and extracted with 16 L of 70% EtOH for one hour, three times, under reflux. The combined extract was filtered and the filtrate was concentrated and dried under vacuum.

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Yield: 12.98%

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Extraction of raw herb (ii) (Bu Gu Zhi) using 70% EtOH: 750 g of dried fruit of Bu Gu Zhi (obtained from He Bei) was ground and extracted with 6 L of 70% EtOH for one hour, three times, under reflux. The combined extract was filtered and the filtrate was concentrated and dried under vacuum.

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Yield: 29.3%

Extraction of raw herb (iv) (Du Zhong) using 70% EtOH: Air Dried bark of Du Zhong (1.5 kg) was chopped and extracted with 12 L of 70% EtOH for one hour, three times, under reflux. The combined extract was filtered and the filtrate was concentrated and dried under vacuum.

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Yield: 12.14%

Extraction of raw herb (v) (She Chuang Zhi) using 70% EtOH: 700 g of dried fruit of She Chuang Zhi was chopped and extracted with 5.6 L of 70% EtOH for one hour, three times, under reflux. The combined extract was filtered and the filtrate was concentrated and dried under vacuum.

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Yield: 22.74%

5 Extraction of raw herb of (iii) (Shu Di) using water: Dried herb of Shu Di (300 g) was extracted with 3 L of water for two hours, two times, under reflux. The combined extract was filtered and the filtrate was concentrated and dried under vacuum.

Yield: 55.8%

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Extraction of raw herb (vi) (Huang Qi) using water: 600 g of dried root of Huang Qi was extracted with 6 L of water for two hours, two times, under reflux. The combined extract was filtered and the filtrate was concentrated and dried in vacuum.

15 Yield: 29.8%

Preparation of composition (unrefined)

20 The extracts of the raw herbs obtained above, were mixed in the ratio (Bu Gu Zhi: She Chuang Zi: Yin Yang Huo: Shu Di: Du Zhong: Huang Qi 1.5: 1: 1.5: 1.25: 1.25: 1.5) to get 1 kg of the composition.

Name of herb		Quantity of crude extract
Bu Gu Zhi	(ii)	187.5 g
She Chuang Zi	(v)	125 g
Yin Yang Huo	(i)	187.5 g
Shu Di	(iii)	156.25 g
Du Zhong	(iv)	156.25 g
Huang Qi	(vi)	187.5 g

25 Procedures for partial purification (or “fractionation”) of the extracts were developed:

Partial purification of crude extract of (i) (Yin Yang Huo): a column (55 x 700 mm) was packed with Silica gel (60-230 mesh) using ethyl acetate. A slurry of 35 g of the crude extract (70% EtOH extract) of Yin Yang Huo was prepared using 350 g of Silica gel in MeOH. Active fraction was eluted using 20% MeOH in ethyl acetate (5 litres) as eluant. Fraction was concentrated and dried in vacuum. Same procedure repeated for 10 times.

Yield: 29.4%

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Partial purification of crude extract of (ii) (Bu Gu Zhi): we used a lipophilic crosslinked dextran-based resin (in this example, Sephadex LH-20) This was pre-swelled in MeOH overnight and then packed in a Column (55 x 700 mm). 35 g of the crude extract (70% EtOH extract) of Bu Gu Zhi was dissolved in 100 ml of MeOH using sonication and loaded onto the column. Approximately 4 litres of MeOH was used as eluant. MeOH fraction was concentrated and evaporated to dryness under vacuum. The same procedure was repeated 4 times.

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Yield: 70.1%

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Partial purification of crude extract of (iii) (Shu Di): 358 g of the crude extract (water extract) of Shu Di was taken and dissolved in 350 ml of water, and then 100% EtOH was added slowly in order to form a precipitate. The total amount of 100% EtOH added was 2.8 L. The mixture was kept in a refrigerator overnight for complete precipitation, then filtered. The residue obtained was vacuum dried for 24 hours.

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Yield: 29.32%

Partial purification of crude extract of (iv) (Du Zhong): a column (55 x 700 mm) was packed with Silica gel (60-230 mesh) using acetone. A slurry of 35 g of the crude extract (70% EtOH extract) of Du Zhong was prepared using 350 g of Silica gel

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in MeOH. Active fraction was eluted using acetone (10 litres) as eluant. Fraction was concentrated and dried under vacuum. Same procedure repeated for 6 times.

Yield: 29.4%

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Partial purification of crude extract of (v) (She Chuang Zhi): 200 g of the crude extract (70% EtOH extract) of She Chuang Zhi was placed in a 5 L round bottom flask. Acetone (4l) was added and stirred for 1 hour, then filtered. The residue was stirred for 1 hour with a further 4l of acetone. The procedure was repeated 6 times. Finally all the eight filtrates were combined and concentrated and dried overnight under vacuum.

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Yield: 41.41%

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Partial purification of crude extract of (iv) (Huang Qi): 158 g of the crude extract (water extract) of Huang Qi was taken and dissolved in 150 ml of water, then 100% EtOH was added slowly in order to form a precipitate. The total amount of 100% EtOH added was 1.2 L. The mixture was kept in a refrigerator overnight for complete precipitation, then filtered. The residue obtained was vacuum dried for 24 hours.

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Yield: 64.5%

The partially purified or fractionated samples of the crude extracts of the raw herbs obtained above were mixed in the ratio (Bu Gu Zhi: She Chuang Zi: Yin Yang Huo: Shu Di: Du Zhong: Huang Qi 1.5: 1: 1.5: 1.25: 1.25: 1.5) to get 500 g of GMA partial purified sample.

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Name of herb	Quantity of partially purified samples
Bu Gu Zhi	93.75 g
She Chuang Zi	62.5 g
Yin Yang Huo	93.75 g

Shu Di	78.125 g
Du Zhong	78.125 g
Huang Qi	93.75 g

Action of crude and partially purified herb extracts on osteoclasts

A study was made to elucidate the effectiveness of the herbs on anti-proliferation of rat osteoclast cells *in vitro*.

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Materials

All media components were purchased from Sigma-Aldrich (USA) unless otherwise stated. Fetus bovine serum was purchased from Hyclone, USA. TRAP staining kit, 0.25% Trysin-EDTA solution, MTT (Thiazolyl Blue Tetrazolium Bromide) and DMSO were purchased from Sigma-Aldrich, USA. All chemicals were the highest grade available from Sigma Chemical Co. 96-wells plates were bought from NUNC, Denmark.

15 Methods

Preparation of Osteoclast Cultures

Femurs were obtained, from 3-4 weeks old young female wistar rats (100-125 g), following sacrifice. Femurs were washed through 4 washes (30 ml each time) with femur wash medium, α -minimum essential medium (α -MEM) containing 1 mg/ml Penicillin G, 0.5 mg/ml gentamycin and 3 mg/ml fungizone. Epiphyses were removed from rat femurs. Bone marrow cells were obtained by using a syringe for flushing femurs from both ends of the shaft with α -MEM containing 15% (v/v) fetal bovine serum (Hyclone), 0.1 mg/ml penicillin G, 0.05 mg/ml gentamycin, 300 ng/ml fungizone, 0.28 mM L-Ascorbic Acid 2-Phosphate, 10 nM dexamethasone, 10 ng/ml M-CSF, 10 ng/ml RANKL and 10 ng/ml TNF- α . Medium was collected from both end of femurs and centrifuged at 300 g for 3 minutes. After centrifugation, two thirds of the supernatant from the top was removed. The remaining fractions were used as a source of osteoclast precursors. Cell suspensions were mixed and inoculates 0.1 ml

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(1×10^5 bone marrow cells) onto each well in 96 wells-plate (NUNC). The cells were incubated in 37°C, 5% CO₂, 100% humidity. Medium was changed after 24 hours with osteoclast medium and cells were refeed 3 times weekly.

5 TRAP-positive cell staining

Osteoclast cells were suspended in phenol α -MEM containing 15% FBS and plated at a concentration of 5×10^5 cells/well into a 24-well culture dish in the presence of 10 ng/ml RANKL and incubated for 24 h. The medium and factors were replaced after 3 days. After 5 days of culture, the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP), a typical marker enzyme of osteoclast using the commercial TRAP staining kit (Sigma, 387-A) according to the manufacturer's instructions. TRAP+ cells with more than three nuclei were counted as TRAP positive MNCs.

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Proliferation Assay

Dose-response curves for the crude and partially purified extracts, individually and as the combination of 6 herbs, were determined by the anti-proliferation assay determined by MTT (Thiazolyl Blue Tetrazolium Bromide) on the percent survival of the rat osteoclast cells on a 96-well microtitre plate. 10,000 viable cancer cells at exponential growth phase in 100 μ l were seeded into each well of a 96-well microtitre plate. The cells were cultured overnight for cell attachment at 37°C in 5 % carbon dioxide atmosphere. The used medium from each well was discarded the next morning and 200 μ l of fresh media were added. 2 μ l of herb samples at various concentrations were added into each well and the plate was incubated at 37°C in 5 % carbon dioxide atmosphere for 24 hours.

The appropriate amount of MTT was dissolved in serum-free medium to obtain a concentration of 1 mg/ml medium. 50 μ l of the MTT solution was transferred into each well. Cells were incubated for 4 hrs at 37°C in 5 % carbon dioxide atmosphere. Medium was aspirated from the well and 150 μ l DMSO was added into

each well to dissolve the blue formazan precipitate. Optical absorbance of the reduced MTT was then measured at 550 nm using a microtiter plate reader. The percent viable cells were calculated with the following: Sample OD / Control OD x 100%.

5 Results

Fig 1 contains graphs showing the anti-proliferation activity of the various materials on osteoclasts. All values are expressed as mean $\pm$ standard deviation.

10 Triangles relate to crude extracts; squares relate to partially purified materials.

Fig 1 (i) shows that the crude extract of Yin Yang Huo inhibited the proliferation of osteoclast cells (37%) at 500 $\mu\text{g/ml}$, whereas the partially purified fraction that contained icarrin inhibited 54% of osteoclast cells proliferation at 500 $\mu\text{g/ml}$. Yin Yang Huo modulated osteoclastogenesis by inhibiting the proliferation of osteoclast cells (Xie et al. 2005). Besides that, Yin Yang Huo treatment can effectively suppress the OVX-induced increase in bone turnover possibly by both an increase in osteoblastic activities and a decrease in osteoclastogenesis (Xie et al. 2005).

20 Fig 1 (ii) shows that the crude extract of Bu Gu Zhi inhibited proliferation (33%) of osteoclast cells at 500 $\mu\text{g/ml}$. But the partial purified fraction that contained corylin and bavachin inhibited the proliferation of osteoclast cells effectively (50%) at 500 $\mu\text{g/ml}$. Concentration that needed to inhibited the proliferation of osteoclast cells is lesser in the partial purified fraction compare with the crude extract of Bu Gu Zhi.

Fig 1 (iii) shows that crude extract of Shu Di inhibited the proliferation of osteoclast (37 %) cells at 500 $\mu\text{g/ml}$. The partially purified fraction from Shu Di that contained polysaccharides inhibited 48 % of the proliferation of osteoclast cells, which was 11 % more compared with the crude extract. Du Zhong, Shu Di inhibited the proliferation of osteoclast cells in a dose-dependent manner.

Fig 1 (iv) shows that Du Zhong inhibited the proliferation of osteoclast cells in a dose-dependent manner. Crude extract of Du Zhong inhibited the proliferation (30%) of osteoclast cells at 500 µg/ml, whereas partial purified fraction from crude extract that contained geniposidic acid (GPA) inhibited effectively the proliferation as 47% of osteoclast cells at 500 µg/ml.

Fig 1 (v) shows that crude extract of She Chuang Zhi inhibited proliferation of osteoclast cells at 500 µg/ml (32%). Partially purified fraction that contained osthole inhibited proliferation of osteoclast cells at 500 µg/ml as much as 54%. She Chuang Zhi inhibited osteoclast formation and differentiation also reported by Qin et al. 2003. She Chuang Zi inhibited osteoclast formation and differentiation by decreased and inhibited TRAP activity of osteoclasts. Besides that, it also decreased the osteoclastic bone resorption pit area and Ca^{2+} release from bone slices (Qin et al. 2003).

Fig 1 (vi) shows that crude extract of Huang Qi inhibited the proliferation of osteoclast cells at 500 µg/ml (31%). The partially purified fraction that contained polysaccharides showed the enhancement of the anti-proliferation effects on osteoclast cells at 50 µg/ml (48 %). Concentration needed to inhibit the osteoclast cells is lesser in the partial purified compared with the crude extract of Huang Qi.

For all the 6 herbs, the partially purified fraction showed greater positive results compared with the crude extract. The anti-proliferation effects on osteoclast cells are summarized in **Table 3**.

Potency of the fractionated components can also be expressed as ED_{50} (the concentration of crude extracted or partially fractionated component to have 50% inhibition). The results are summarized in **Table 4**.

Table 3: Summary of the anti-proliferation activity on osteoclast cells of individual herb extracts before and after partial purification.

Name	Origin	Activity of crude extract at 500 $\mu\text{g/ml}$ on osteoclast cells	Active component	Activity of active fraction at 500 $\mu\text{g/ml}$ on osteoclast cells
Shu Di	He Bei	Inhibited 37% proliferation	Polysaccharide	Inhibited 48% proliferation
Huang Qi	He Bei	Inhibited 31% proliferation	Polysaccharide	Inhibited 48% proliferation
She Chuang Zi	He Bei	Inhibited 33% proliferation	Osthole	Inhibited 54% proliferation
Yin Yang Huo	Shi Chuan	Inhibited 37% proliferation	Icarrin	Inhibited 54% proliferation
Du Zhong	Bo Chou	Inhibited 30% proliferation	GPA	Inhibited 47% proliferation
Bu Gu Zhi	He Bei	Inhibited 33% proliferation	Corylin & Bavachin	Inhibited 50% proliferation

Table 4: ED₅₀ of the anti-proliferation activity on osteoclast cells of individual herb extracts before and after partial purification.

		EC ₅₀ (g/ml)	Rel Potency
Shu Di	Partially purified	4.83E-04	4.22
	Crude Extract	2.04E-03	
Huang Qi	Partially purified	4.53E-04	14.97
	Crude Extract	6.78E-03	
She Chuang Zi	Partially purified	3.03E-04	15.63
	Crude Extract	4.73E-03	
Du Zhong	Partially purified	4.95E-04	5.85
	Crude Extract	2.90E-03	
Bu Gu Zhi	Partially purified	4.06E-04	15.92
	Crude Extract	6.46E-03	
Ying Yang Huo	Partially purified	2.70E-04	4.38
	Crude Extract	1.18E-03	

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Similar tests were carried out using compositions of 6 crude extracts (2 versions) and 6 partially purified extracts. The results are displayed in Fig 2, where solid circles represent the first crude composition; triangles represent the second crude composition; and open circles represent the partially purified composition.

The second version of the crude extract gave a better activity in inhibiting proliferation of osteoclast cells compared with the first version at the same concentration. Moreover, the partially purified fraction (which was derived from the extracts used in the second crude extract) inhibited 46% growth of osteoclast cells at 500 µg/ml compared with the second version of the crude extract which inhibited 39 % growth at 500 µg/ml.

The EC₅₀ values were:

20	1 st crude	:	2.71 x 10 ⁻³ g/ml
	2 nd crude	:	2.94 x 10 ⁻³ g/ml
	Partially purified	:	5.27 x 10 ⁻⁴ g/ml

Action of crude and partially purified herb extracts on osteoblasts

A study was made to elucidate the effectiveness of the herbs on the proliferation of hFOB human osteoblast cells.

Materials

The conditionally immortalized human fetal osteoblastic cell line hFOB was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, <http://www.atcc.org>). hFOB was developed by conditionally immortalizing human fetal osteoblasts with a temperature-sensitive mutant of the SV40 large T antigen (*ts-SV40LTA*) gene. At the permissive temperature of 33.5°C, the *ts-SV40LTA* is active and the hFOB cells proliferate rapidly, whereas at the nonpermissive temperature of 39.5°C, the *ts-SV40LTA* is inactive, and the cells differentiate and display the phenotype of mature osteoblasts. Fetus bovine serum was purchased Hyclone, USA. G418, Ham's 12:DMEM (1:1), 0.25% Trypsin-EDTA solution, MTT (Thiazolyl Blue Tetrazolium Bromide) and DMSO were purchased from Sigma-Aldrich, USA. 96-well plate was bought from NUNC, Denmark.

Methods

Cell Lines

The hFOB cells were cultured according to ATCC protocol in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 0.3 mg/ml G418, hereafter called complete medium. Cells were cultured at 34°C in a humidified atmosphere of 5% CO₂/95% air.

Proliferation Assay

Dose-response curves of the six individual herb crude extracts, the six partially purified fractions, and compositions containing material from all six herbs were

determined by proliferation assay determined by MTT (Thiazolyl Blue Tetrazolium Bromide) on the percent survival of the osteoblast cells, hFOB on a 96-well microtitre plate.

5 10,000 viable cancer cells at exponential growth phase in 100 μ l were seeded into each well of a 96-well microtitre plate. The cells were cultured overnight for cell attachment at 37°C in 5 % carbon dioxide atmosphere. The used medium from each well was discarded in the next morning and 200 μ l of fresh media were added. 2 μ l of herbal samples at various concentrations were added into each well and the plate was
10 incubated at 37°C in 5 % carbon dioxide atmosphere for 24 hours.

MTT was dissolved in serum-free medium to obtain a concentration of 1 mg MTT /ml medium. 50 μ l of MTT solution was transferred into each well. Cells were incubated for 4 hrs at 37°C in 5 % carbon dioxide atmosphere. Medium was aspirated
15 from the well and 150 μ l DMSO was added into each well to dissolve the blue formazan precipitate. Optical absorbance of the reduced MTT was then measured at 550 nm using a microtiter plate reader. The percent viable cells were calculated with the following: Sample OD / Control OD x 100%

20 **Results**

Graphs of the results are shown in Fig 3. Diamonds represent crude extracts; squares represent partially purified materials. All values are expressed as mean $\pm$ standard deviation.

25 Fig. 3 (i) shows that crude extract of Yin Yang Huo induced proliferation of hFOB osteoblast cells (8%) at 200 μ g/ml, whereas partially purified fraction that contained icarrin induced proliferation of hFOB osteoblast cells (20%) at 100 μ g/ml. Concentration needed to induce the osteoblast cells is lesser and the proliferation
30 activity is greater in partially purified fraction compared with the crude extract. Yin Yang Huo increased bone turnover rate to restore the loss of trabecular bone

architecture in rats by its direct action of stimulating bone formation and inhibiting bone resorption (Xie et al. 2005).

Fig 3 (ii) shows that crude extract of Bu Gu Zhi induced proliferation (20%) of hFOB osteoblast cells at 50 µg/ml. But the partially purified fraction that contained corylin and bavachin induced proliferation (20%) of hFOB osteoblast cells at 25 µg/ml. Concentration needed to induce the osteoblast cells is lesser in the partially purified fraction compare with the crude extract of Bu Gu Zhi (50 µg/ml).

Corylin from *Psoralea coryfolia* (Bu Gu Zhi) exhibited osteoblastic proliferation stimulating activity in UMR 106 cell line cultured *in vitro*. The flavanoids corylin and bavachin were isolated and identified as active principles by activity-guided fractionation (Wang et al. 2001). The results suggested that *Psoralea coryfolia* fruit extracts and corylin and bavachin might stimulate bone formation or have potential activity against osteoporosis. Xiong et al. (2003) also observed the effect of extract and corylin fraction of *Psoralea corylifolia* L. extract on osteoblastic activity. These effects suggest that *Psoralea corylifolia* L. extract and corylin would stimulate bone formation (Xiong et al. 2003).

Fig 3 (iii) shows that crude extract of Shu Di induced proliferation of hFOB osteoblast (7%) cells at 50 µg/ml. Partially purified fraction from Shu Di that contained polysaccharides did not induce proliferation of hFOB osteoblast cells. There was no improvement in concentration as well as the proliferation activity on osteoblast in partial purified fraction from the crude extract of Shu Di.

Fig 3 (iv) shows that crude extract of Du Zhong induced little proliferation (5%) of hFOB osteoblast cells at 100 µg/ml, whereas partial purified fraction from crude extract that contained geniposidic acid (GPA) induced more proliferation as 13% of hFOB osteoblast cells at 100 µg/ml.

GPA from *Cortex Eucommiae* (Du Zhong) increases the proliferation of osteoblast-like cells and augments bone growth and suppresses bone loss also, as

reported by Kim et al. (2003). Apart from this, Ha (2003) also reported that the Eucommia bark extract and its constituents, geniposidic acid, geniposide and aucubin increased the proliferation of osteoblast-like cells to stimulate osteogenesis. In addition, using the coculture of mouse bone marrow cells and ST-2 cells, these extracts and compounds inhibited osteoclastic activities (Ha 2003).

Fig 3 (v) shows that crude extract of She Chuang Zhi induced proliferation of hFOB osteoblast cells at 100 µg/ml (7%). Partial purified fraction that contained osthole induced proliferation of hFOB osteoblast cells at 12.5 µg/ml (12%). The concentration needed to induce the osteoblast cells is lesser in the partial purified compare with the crude extract of She Chuang Zhi. Kuo et al. (2005) reported that osthole from *Fructus Cnidii* (She Chuang Zi) significantly induced the differentiation in two human osteoblast-like cell lines, MG-63 and hFOB.

As reported by Meng et al. (2004), extracts from fruits of *Cnidium monnieri* (L.) *Cuss (umbelifera)* were screened using the osteoblast-like UMR106 cells *in vitro*. Three coumarins (osthole, bergapten and imperatorin) were isolated and their effects on osteoblastic proliferation were investigated. Osthole, a major pharmacologically active constituent, significantly promoted the cells' activity. Bergapten and imperatorin were less effective than osthole. These results suggested *Cnidium monnieri* (L.) *Cuss* extracts might have potential activity against osteoporosis, and its partial purified fraction might contain active constituents stimulating osteoblasts. (Meng et al. 2004).

As shown in Fig. 3 (vi), crude extract of Huang Qi induced proliferation of hFOB osteoblast cells at 100 µg/ml (17%). The partially purified fraction that contained polysaccharides induced proliferation of hFOB osteoblast cells at 50 µg/ml (15 %). The concentration needed to induce the osteoblast cells is lesser in the partially purified compared with the crude extract of Huang Qi.

These results are summarised in Table 5.

Table 5: activity of single herb extracts on osteoblasts

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Name	Activity of Crude extract	Active component	Activity of active fraction
Shu Di	Induced proliferation (10 %) at 50 µg/ml	Polysaccharide	No significant improvement
Huang Qi	Induced proliferation (17 %) at 100 µg/ml	polysaccharide	Induced proliferation (15 %) at 50 µg/ml
She Chuang Zhi	Induced proliferation (7 %) at 100 µg/ml	Osthole	Induced proliferation (12 %) at 12.5 µg/ml
Yin Yang Huo	Induced proliferation (8 %) at 200 µg/ml	Icarrin	Induced proliferation (20 %) at 100 µg/ml
Du Zhong	Induced proliferation (5 %) at 100 µg/ml	GPA	Induced proliferation (13 %) at 100 µg/ml
Bu Gu Zhi	Induced proliferation (20 %) at 50 µg/ml	Corylin & Bavachin	Induced proliferation (20 %) at 25 µg/ml

Similar tests were carried out using compositions containing all 6 crude extracts (2 versions) and 6 partially purified extracts. The results are displayed in Fig 4a (activity after 24 hours) and Fig 4b (activity after 48 hours). Diamonds and squares represent the first and second versions, respectively, of the “crude” compositions while triangles represent the “purified” compositions. It can be seen that the second version is somewhat more active than the first “crude” version, while the “purified” composition is much more active: after 24 hours (Fig 4a) the second “crude” version stimulated 17% growth at 200 µg/ml. After 48 hours (Fig 4b), the proliferation activities on osteoblast cells are not promising after 48 hrs. Only 6 % of the proliferation of osteoblast cells can be noted at the 50 µg/ml concentration in 48 hrs partial purified fraction treatment compared with 18 % at 50 µg/ml after 24 hrs

partial purified fraction treatment. Osteoblast cells (hFOB) died after 48 hrs of treatment with partial purified fraction and crude extract compared to the control cells.

5 The best curve fitting of the first and second “crude” versions and the “purified” composition on the proliferation activity of osteoblasts (hFOB) is shown in Fig 4c. The squares and triangles represent the first and second “crude” versions while the circles represent the “purified” composition.

Animal Models

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Tests have been carried out to measure the effects of the “crude extract” (second “crude” versions) and the “purified” composition.

15 Materials have been tested in ovariectomized rats, a popular and generally accepted osteoporosis animal model.

20 After orally dosing continuously for three months at 0.12, 0.25, 0.5 and 0.8 g/kg/day, twice a day, the bone mass index, wet weight and dry weight of femur, bone mineral densities of femur and lumbar were measured and calculated. Body weights of rats were measured weekly. The results are summarized in Tables 6 and 7.

Table 6: Bone mass index and wet weight of the femur (mg/cm) from rats of all experimental groups

Groups	Rat #	Bone Mass Index ($\bar{x} \pm s$)	Wet Weight (mg/cm) ($\bar{x} \pm s$)
Sham-operated Normal Control	12	4.1404±0.36233	198.3774±12.47043
Ovariectomized Model	12	3.4909±0.47565 ^{##}	190.7690±8.2086
Fosamax[®]	10	3.4202±0.24757	205.8757±18.31985 ^{**}
Crude Extract			
1.4 g/kg	11	3.6026±0.49182	193.8621±7.94898

0.5 g/kg	10	3.4736±0.18521	192.6831±13.58409
Partially Purified			
0.12 g/Kg	11	3.4200±0.29461	188.8743±9.34391
0.25 g/Kg	11	3.5885±0.24125	197.8749±11.43143
0.5 g/Kg	11	3.7375±0.32996	195.5449±23.14863
0.8 g/Kg	6	4.1492±0.34708**	197.7648±12.56049

#: P<0.05 compared to sham-operated normal control

##: P<0.01 compared to sham-operated normal control

*: P<0.05 compared to ovariectomized model

**: P<0.01 compared to ovariectomized model

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Based on the data of femoral bone mass index, the dose-response curve can be best fitted with an ED50 value of 0.474 g/kg/day, dosed twice a day, as shown in Figure 5. Compared to crude extract prepared as described in the document (GuMiAn Patent File A), the partially purified active components were about 4 times more potent.

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Figure 5 shows dose-response curves of partially purified active components (solid points) and crude extract (open circles) on the femoral bone mass index in ovariectomized osteoporosis animal model. All the values are expressed as mean ± standard deviation. The data points were fitted with a non-linear regression program, GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

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We measured the bone mineral densities of femur and lumbar spine. The values were tabulated in Table 7. On BMD of femur, Fosamax® and partially purified active components at all dosages were statistically significant from the value of ovariectomized model group. However, on BMD values of lumbar spine, only groups dosed with Fosamax®, GuMiAn crude extract at 1.4 g/kg/day, and partially purified GuMiAn at 0.5 g/kg/day showed statistically difference as compared to the ovariectomized model group.

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Dose response curves of GuMiAn crude extract and partially purified GuMiAn can be best fitted with a non-linear regression software program (Figure 6 for femur and Figure 7 for lumbar spine). Improvement in the potency in vivo is clearly indicated.

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Table 7: Bone mineral densities of femur and lumbar spine from each experimental group.

Groups	Rat #	Femur ($\bar{x} \pm s$)	Lumbar Spine ($\bar{x} \pm s$)
Sham-operated Normal Control	12	0.1373±0.00349	0.1497±0.0544
Ovariectomized Model	12	0.1309±0.00452#	0.1415±0.00425#
Fosamax [®]	10	0.1436±0.00483**	0.1610±0.00774**
Crude Extract			
1.4 g/kg	11	0.1322±0.00602	0.1581±0.00627**
0.5 g/kg	10	0.1307±0.00732	0.1456±0.01165
Partially Purified			
0.12 g/Kg	11	0.1358±0.00536*	0.1451±0.00633
0.25 g/Kg	11	0.1362±0.00522*	0.1470±0.00897
0.5 g/Kg	11	0.1382±0.00477**	0.1517±0.01008**
0.8 g/Kg	6	0.1365±0.00460*	0.1462±0.00939

#: P<0.05 compared to sham-operated normal control

5 ##: P<0.01 compared to sham-operated normal control

*: P<0.05 compared to ovariectomized model

** : P<0.01 compared to ovariectomized model

10 Fig 6 shows dose-response curves of partially purified GuMiAn (solid points) and GuMiAn crude extract on the bone mineral density of femur in ovariectomized osteoporosis animal model. All the values are expressed as mean ± standard deviation. The data points were fitted with a non-linear regression program, GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

15

Fig 7 shows dose-response curves of partially purified GuMiAn (solid points) and GuMiAn crude extract on the bone mineral density of lumbar spine in

ovariectomized osteoporosis animal model. All the values are expressed as mean $\pm$ standard deviation. The data points were fitted with a non-linear regression program, GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

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Obesity

The effects of the purified composition on the body weight of the rats in the groups described above were determined.

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We measured the body weight of each experimental animal group weekly. The ovariectomized rats had been noticed to have a pronounced body weight increase compared to the sham-operated normal control group. Treatment of the ovariectomized animals with the partially purified composition showed a significant decrease in the body weight increment. Figure 8 shows a plot of the average body weight increment of the treated animals with an ED50 of 0.27 g/kg/day.

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The data points were fitted with a non-linear regression program, GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

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Human Studies

A pilot study has been carried out of the safety and efficacy of a composition according to the invention when administered to postmenopausal osteoporotic women.

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For this study, the “crude extract” (“GZS”) was formulated into capsules.

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Methods

Women with osteoporosis as defined by the bone mineral density (BMD) score of <2.5 SD peak bone mass in either the hip and/or spine were enrolled in the study. All subjects are >5 years post menopause, up to the age of 70 years. The subjects gave informed consent. Subjects with renal, thyroid, parathyroid dysfunction, major disease, diabetic requiring insulin, and taking steroids, bisphosphonates, hormone replacement therapy, or calcitonin were excluded from the study.

The study is a randomized open-labeled trial, 18 weeks treatment, with a 9-month follow-up. Patients received either Ca et Vit D 2 tablets a day (control arm) or Ca et Vit D + GZS capsules (treatment arm).

Markers of bone formation: bone-specific alkaline phosphatase, and markers of bone resorption: urinary deoxy-pyridiolone, and urinary cross-linked N-telopeptide were assessed. BMD was assessed at baseline and at the end of the study.

Results

22 subjects were enrolled into the study, with 4 randomized into the control arm and 18 in the treatment arm. 18 subjects completed the study. 3 subjects in the active arm and 1 subject in the control arm withdrew respectively. There were no meaningful changes in the bone formation and resorption markers. The mean ($\pm$ SD) changes in BMD in the control and treatment groups are as follows: hip -11.26 and -2.51 percent; and spine -3.80 and -3.82 percent respectively. There were no fractures in both groups. 7 patients complained of minor side effects with GZS capsules, like "heatiness", headache and bloatedness.

Conclusions

In postmenopausal osteoporotic women, the composition according to the invention appears to reduce the bone loss measured in the hips but not the spine in the 9-month study period. It appears to be safe with only minor side effects. Larger and longer-term studies are needed to confirm its efficacy in ameliorating bone loss.

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 While the invention has been described above by reference to preferred
embodiments, it will be understood by those skilled in the art that various changes and
modifications may be made without departing from the spirit and scope of the
20 invention. It is intended to cover all such changes and modifications by the appended
claims.

CLAIMS:

1. A pharmaceutical composition comprising the following 6 herbal materials or extracts thereof: (i) Yin Yang Huo; (ii) Bu Gu Zhi; (iii) Shu Di; (iv) Du Zhong; (v) She Chuang Zhi; and (vi) Huang Qi.
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2. A pharmaceutical composition according to claim 1 which comprises extracts of the six herbal materials produced by a process comprising extraction with water or an alcoholic solvent.
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3. A pharmaceutical composition according to claim 1 or claim 2 which contains extracts of (i) Yin Yang Huo, (ii) Bu Gu Zhi, (iv) Du Zhong and (v) She Chuang Zhi produced by extraction with an alcoholic solvent.
- 15 4. A pharmaceutical composition according to claim 3 wherein the alcoholic solvent is aqueous ethanol.
5. A pharmaceutical composition according to claim 4 wherein the alcoholic solvent is 70% aqueous ethanol.
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6. A pharmaceutical composition according to any preceding claim which contains extracts of (iii) Shu Di, and (vi) Huang Qi produced by extraction with water.
7. A pharmaceutical composition according to any preceding claim wherein component (i) is produced by fractionating an extract of Yin Yang Huo by chromatography on silica gel.
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8. A pharmaceutical composition according to any preceding claim wherein component (ii) is produced by fractionating an extract of Bu Gu Zhi by chromatography on a lipophilic crosslinked dextran-based resin.
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9. A pharmaceutical composition according to any preceding claim wherein component (iii) is produced by fractionating an extract of Shu Di by precipitating it from an aqueous solution by the addition of ethanol.
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10. A pharmaceutical composition according to any preceding claim wherein component (iv) is produced by fractionating an extract of Du Zhong by chromatography on silica gel.
- 5 11. A pharmaceutical composition according to any preceding claim wherein component (v) is produced by fractionating an extract of She Chuang Zhi by extracting a dried extract with acetone, followed by evaporation to dryness of the acetone extract.
- 10 12. A pharmaceutical composition according to any preceding claim wherein component (vi) is produced by fractionating an extract of Huang Qi by precipitating it from an aqueous solution by the addition of ethanol.
- 15 13. A pharmaceutical composition according to any preceding claim which contains extracts of said herbal materials in the proportion, in parts by weight:
 - (i) Yin Yang Huo: 0.15 - 0.75 parts;
 - (vi) Huang Qi: 0.3 - 1.5 parts;
 - (v) She Chuang Zhi: 1-4 parts;
 - (iii) Shu Di: 1.2-5.0 parts;
 - 20 (ii) Bu Gu Zhi: 1.5 - 6 parts; and
 - (iv) Du Zhong: 2.5-10 parts.
- 25 14. A pharmaceutical composition according to claim 13 wherein the proportions are:
 - (i) 0.25 - 0.5 parts;
 - (vi) 0.5 - 1.0 parts;
 - (v) 1.2 - 2.8 parts;
 - (iii) 1.5 - 3.0 parts;
 - (ii) 2 - 4 parts; and
 - 30 (iv) 4 - 8 parts.
15. A pharmaceutical composition according to claim 14 wherein the proportions are:
 - (i) 0.375 parts of Yin Yang Huo; (vi) 0.75 parts of Huang Qi;
 - 35 (v) 2 parts of She Chuang Zhi; (iii) 2.5 parts of Shu Di
 - (ii) 3 parts of Bu Gu Zhi; and (iv) 5 parts of Du Zhong.

16. A pharmaceutical composition according to any preceding claim including one or more excipients or adjuvants.
- 5 17. A method of producing a pharmaceutical composition according to any preceding claim including a step of preparing extracts of said herbal materials.
- 10 18. Use of a pharmaceutical composition according to any of claims 1-16 in the manufacture of a medicament for treating a condition linked to activities of osteoblasts and/or osteoclasts.
- 15 19. Use according to claim 18 wherein the condition is selected from osteoporosis, menopausal symptoms, obesity, glucose intolerance and diabetes.
20. Use of a pharmaceutical composition according to any of claims 1-16 in the manufacture of a medicament for treating a mammal for one or more of: reducing the rate of bone fracture; increasing bone mineral density; and restoring bone structure.