School of Pharmacy, Shenyang Pharmaceutical University, China

Stimulative activity of *Drynaria fortunei* (Kunze) J. Sm. extracts and two of its flavonoids on the proliferation of osteoblastic like cells

F. Li, F. Meng, Z. Xiong, Y. Li, R. Liu, H. Liu

Received January 30, 2006, accepted March 2, 2006

Prof. Famei Li, School of Pharmacy, PO BOX 39, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang, China, 110016
fameili@hotmail.com

Pharmazie 61: 962–965 (2006)

The osteoblastic activity of extracts of *Drynaria fortunei* (Kunze) J. Sm. rhizome was assayed in the UMR106 cell line cultured *in vitro*. An ethanol extract and its fractions were added to the cell culture at different concentrations. Osteoblastic proliferation stimulating activity was determined using the MTT method. The ethanol extract, and its ethyl acetate and *n*-butanol fractions exhibited stimulating activity. Two active constituents were isolated from *n*-butanol fraction by bioassay-directed isolation, and identified as naringin and neoeriocitrin. The latter is reported for the first time from this herbal medicine.

1. Introduction

Drugs of plant origin are a major part of traditional Chinese medicine and other folk medicines. The interest in herbal medicines as an alternative remedy is also steadily increasing in western countries. Plants used in folk medicines have become one of the main sources of drug discovery and development. From ancient times in China, women with low back pain in climacteric and senescent periods have been treated with traditional Chinese medicine. *Drynaria fortunei* (Kunze) J. Sm. (Gusuibu in Chinese) is listed in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission 2005) as a typical "kidney-tonifying" traditional Chinese medicine which has been frequently used clinically for thousands of years to treat bone disorders such as bone fracture, arthritis, osteoporosis and rheumatism. This herbal medicine has been reported to have anti-osteoporotic effects on ovariectomized rats (Ma et al. 1999). Chinese herbal formulations containing *Drynaria fortunei* (Kunze) J. Sm have also been used for osteoporosis in recent years (An et al. 1996; Cui et al. 2001).

The anti-osteoporotic effect of *Drynaria fortunei* (Kunze) J. Sm. has been demonstrated recently using tissue or cell culture assays. By using tissue culture and isotope tracing, it has been found that an injection made from *Drynaria fortunei* (Kunze) J. Sm. significantly promoted calcification of cultivated chick embryo bone primordium, increased alkaline phosphatase (ALP) activity and accelerated synthesis of proteoglycan (Ma et al. 1995). An aqueous extract of *Drynaria fortunei* (Kunze) J. Sm. showed an antioxidant effect on rat osteoblasts from hydrogen peroxide-induced death (Lin et al. 2002). By co-culturing a 70% acetone extract of *Drynaria fortunei* (Kunze) J. Sm. or its immobilized formulation with primary osteoblastic cells or a mixture with alveolar mononuclear cells, Sun et al. (2002, 2003) showed that *Drynaria fortunei* (Kunze) J. Sm. increased intracellular ALP, and secretion of acid phosphatase and prostaglandin E2, down-regulated the expression of osteopontin and osteonectin mRNA, and activated the formation of multi-nucleated osteoclasts without giant osteoclast formation. Jeong et al. (2004a) reported the inhibitory activity of ethanol extracts of *Drynaria fortunei* (Kunze) J. Sm. on bone resorption mediated by cathepsin K in cultured mouse osteoclasts. An aqueous extract of *Drynaria fortunei* (Kunze) J. Sm. has been reported to promote the proliferation, differentiation and mineralization of the osteoblast-like cell line MC3T3-E1 (Jeong et al. 2005a, 2004b). Extracts of *Drynaria fortunei* (Kunze) J. Sm. also stimulated the proliferation, alkaline phosphatase activity, protein secretion and type I collagen synthesis of human osteoprecursor cell line OPC-1 in a dose-dependent manner (Jeong et al. 2005b). As regards to its active constituents, by using a bioassay-guided separation, propelargonidin dimers and trimers were demonstrated to be potential phytoestrogens playing important physiological roles in the prevention of postmenopausal osteoporosis (Chang et al. 2003).

Osteoblast-like UMR 106 cell line has preserved properties of osteoblasts, including cAMP responsive to PTH, high ALP and synthesis of bone specific collagen (Patridge et al. 1983). This cell line has been extensively used as an osteoblast model to study the mechanism of the effect of anti-osteoporotic drugs on osteoblasts (Gray et al. 1987). It has been also used in our laboratory to screen anti-osteoporotic agents from traditional Chinese medicines and plants (Li et al. 2001; Gao et al. 2000; Wang et al. 2001a; Meng et al. 2005). In a previous study (Wang et al. 2001b), the ethanol extract of *Drynaria baronii* (Christ) Diels rhizome, another species of the same family of Polypodiaceae, which has the same application as *Drynaria fortunei* (Kunze) J. Sm. in Chinese folk medicine, showed a promoting effect on the proliferation of UMR 106 cells. Similar activity is reported in this paper for *Drynaria fortunei* (Kunze) J. Sm., and for two active constituents isolated by

using bioassay-directed separation, one of which is obtained from this plant for the first time.

2. Investigations, results and discussion

2.1. Proliferative activity of Drynaria fortunei extract

Drynaria fortunei (Kunze) J. Sm. is effective for the treatment of kidney deficiency manifested by lower back pain, weakness of the legs and osteoporosis, presumably by its function in tonifying the kidney, invigorating the blood, according to traditional Chinese medicinal literature (Geng et al. 1997). To investigate the cellular-level mechanism of *Drynaria fortunei* (Kunze) J. Sm. in the treatment of bone related diseases, particularly osteoporosis, many studies have focused on examining its activity in the *in vitro* cell culture assay. In this study, the ethanol extract from the rhizomes of *Drynaria fortunei* (Kunze) J. Sm. was assayed for proliferative activity on osteoblast-like UMR106 cells. As shown in Fig. 1 the extract stimulated the proliferation of the cells with an increase of 35.2% at a concentration of 10 μg/mL. The maximum activity was similar to that reported by Chang et al. (2003), who found a 36% activity increase from a methanol extract of *Drynaria fortunei* (Kunze) J. Sm. on MCF-7 cells, though the concentration was different in the two experiments. The difference in the effective concentrations may be due to the different cell lines and/or extraction solvents used. An aqueous extract of *Drynaria fortunei* (Kunze) J. Sm. has also been reported to stimulate the proliferation of the MC3T3-E1 pre-osteoblastic cell line and human osteoprecursor cells PPC-1, as well the alkaline phosphatase activity, protein secretion and type I collagen synthesis of cells. All these results together with those presented in this paper demonstrate that *Drynaria fortunei* (Kunze) J. Sm. directly stimulates bone formation.

Compared with the results from our previous study on *Drynaria baronii* (Christ) Diels rhizome, another species of the Polypodiaceae family (Wang et al. 2001b), it was found that both plants showed a similar effect on osteoblastic cell proliferation; however, a higher concentration (0.4 mg/mL) was needed for *Drynaria baronii.* These results indicated that both plants might be used as anti-osteoporosis agents, which has been the case for a thousand years in traditional Chinese medicine.

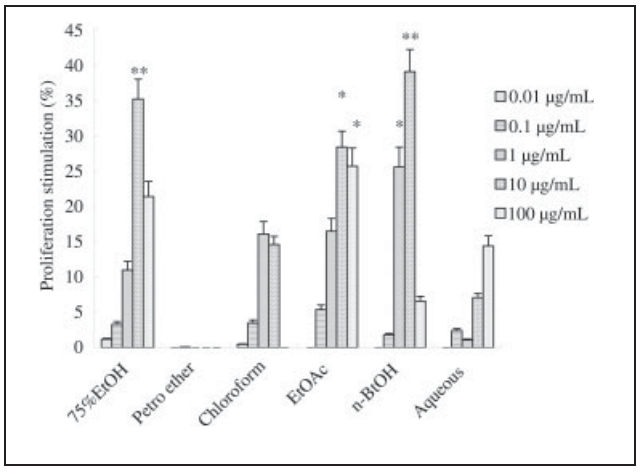


Fig. 1: Effect of extract and fractions from rhizomes of *Drynaria fortunei* (Kunze) J. Sm. on cell proliferation. Concentration of assay solution expressed as weight of raw *Drynaria fortunei* (Kunze) J. Sm. per mL; * $P < 0.05$, ** $P < 0.01$, Significance compared with blank control (MEM) by t-test ($n = 6$)

2.2. Bioassay-directed isolation of constituents

The ethanol extract was fractionated into petroleum ether, chloroform, ethyl acetate, *n*-butanol and aqueous fractions, and the proliferative activity of the fractions on UMR 106 cells was determined. Of these different solvent fractions, the ethyl acetate fraction and *n*-butanol fraction stimulated cell proliferation significantly, with higher activity for *n*-butanol fraction, a growth stimulation ratio of 39.1% at a concentration of 10 μg/mL (Fig. 1). Therfore, the *n*-butanol fraction was also subjected to chromatography on an adsorption resin column to remove some impurities. It was further fractionated on an ODS column, and the combined eluate fractions were examined for activity. Two active constituents were obtained by this bioassay-directed isolation. Other fractions or eluates which did not have significant proliferative activity on osteoblastic cells were not investigated further. More active constituents have been isolated from *Drynaria fortunei* (Kunze) J. Sm. previously using a bioassay-guided separation procedure with an estrogen-sensitive breast cancer cell line as the model (Chang et al. 2003).

2.3. Structural characterization of two active constituents

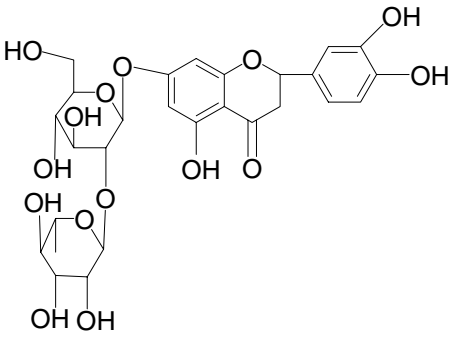
The structures of the two active compounds were characterized on the basis of their physico-chemical properties, UV, IR, MS and NMR spectra, comparing these with those reported in the literature.

Compound **1**: light yellow needle crystals (methanol), m.p.: 191–193 °C. A positive characteristic was observed the in HCl–Mg and Molish reactions. Glucose and rhamnose were identified in its acid-hydrolyzed solution by comparing with reference compounds in TLC. Its UV, IR and MS were consistent with the data on neoeriocitrin in the literature (Kosuge et al. 1994). ^{1}H NMR (DMSO-d_6): 8.5–11.0(brs, O–H), 6.76–6.88(3 H, 2′, 5′, 6′-H), 6.10(2 H, s, 6, 8-H), 5.44(1 H, dd, J = 5.0 Hz, 2-H), 5.12(1 H, d, J = 6.5 Hz, Glc-1-H), 4.65(1 H, m, Rha-1-H), 3.17–3.67(10H, m, Sugar-H), 2.70(2 H, m, 3-H), 1.15(3 H, d, J = 5.0 Hz, Rha-6-H); ^{13}C NMR (DMSO-d_6), 78.8(C-2), 42.3(C-3), 197.4(C-4), 163.1(C-5), 96.4(C-6), 164.9(C-7), 95.3(C-8), 162.9(C-9), 103.5(C-10), 129.3(C-1′), 114.6(C-2′), 145.4(C-3′), 146.1(C-4′), 115.5(C-5′), 118.2(C-6′), 97.5(Glc-C-1), 79.1(Glc-C-2), 77.3(Glc-C-3), 69.7(Glc-C-4), 77.0(Glc-C-5), 60.6(Glc-C-6), 100.5(Rha-C-1), 69.7(Rha-C-2), 69.4(Rha-C-3), 71.9(Rha-C-4), 68.4(Rha-C-5), 18.2(Rha-C-6).

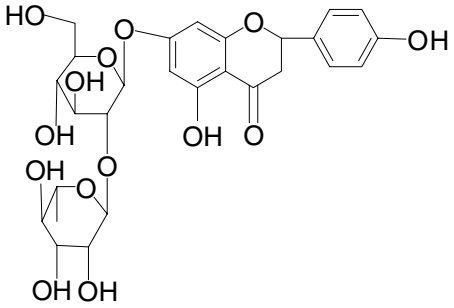
Compound **1** was identified as neoeriocitrin, which has already been isolated from *Ailanthus integrifolia* (Kosuge et al. 1994) and *Citrus* (Nishiura et al. 1971). Moreover, this is the first report on the isolation of neoeriocitrin from *Drynaria fortunei* (Kunze) J. Sm. To our knowledge, its ^{1}H NMR and ^{13}C NMR data have not been reported yet, therefore they are presented in detail in this paper.

Compound **2**: white floccule crystals (methanol), m.p.: 170–172 °C, A positive characteristic was observed in HCl–Mg and Molish reactions. Its UV, IR and MS were consistent with the data of naringin in the literature (Lei et al. 2000). The mixture of compound **2** with naringin (reference compound) exhibited a single spot with identical R_f value on TLC, and a same m.p. as the reference compound.

Compound **2** was characterized as naringin, a known constituent of this herbal drug (Yang et al. 1966) and of several other plants (Lei et al. 2000). Our result supplements the previous report, particularly with regard to for a new compound, neoeriocitrin, found in this herbal medicine.



1



2

2.4. Proliferative activity of two constituents from Drynaria fortunei (Kunze) J. Sm. rhizome

Fig. 2 shows the proliferative activities of the two constituents isolated from *Drynaria fortunei* (Kunze) J. Sm rhizome. Neoeriocitrin stimulated the proliferation of UMR 106 cells in a dose-dependent manner, in the concentration range of 0.01–1 nmol/mL. The maximum activity (33.9% increase) was observed at a concentration of 1 nmol/mL, which is similar to that of sodium fluoride, a positive control which directly stimulates proliferation of bone-forming cells (Fareleg et al. 1983), at a concentration of 10 nmol/mL. Naringin showed significant stimulation of proliferation at a concentration of 10 nmol/mL. This result is considerably different from that found in an assay using MCF-7 and ROS 17/2.8 cells, where much lower effective concentrations were observed (Chang et al. 2003). This difference might result from the different characteristics of cell lines and experimental conditions employed in the two assays. Nevertheless, our study also demonstrated that flavonoids are potentially active constituents which may play important roles in the prevention and treatment of osteoporosis by this herbal medicine. These compounds may be worthy to be developed as drugs as pure compounds.

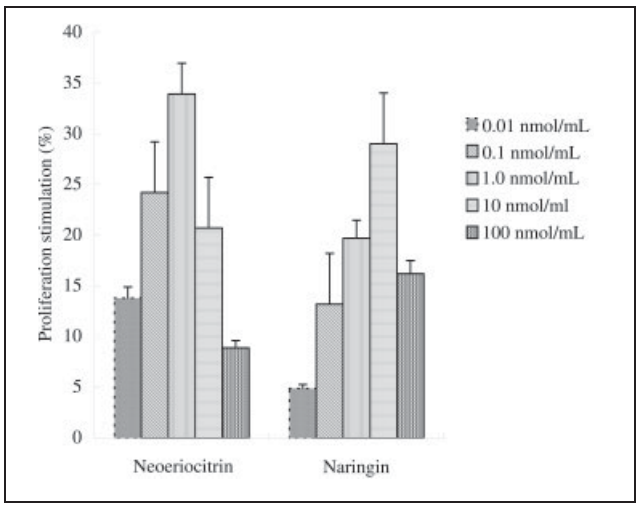


Fig. 2: Activity of neoeriocitrin and naringin on cell proliferation
* $P < 0.05$, ** $P < 0.01$, Significance compared with blank control (MEM) by t-test ($n = 6$)

3. Experimental

3.1. Materials

Raw *Drynaria fortunei* (Kunze) J. Sm. rhizome was purchased from Tianyitang Chinese Drug Store (Shenyang, China) and identified by Professor Qishi Sun (School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, China). A voucher specimen (No. DR9908) was deposited at Shenyang Pharmaceutical University, China. Osteoblast-like UMR 106 cells were described previously (Li et al. 2001). Minimum essential medium (MEM) was obtained from Gibco (Grand Island, NY, USA) and fetal calf serum (FCS) from TBD Bio-engineering Co. (Tianjin, China). Trypsin was supplied by Diffco (Livonia, ML, USA) and tissue culture materials were provided by Nunc (Roskild, Denmark). MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

3.2. Extraction and isolation

Dried rhizomes (500 g) of *Drynaria fortunei* (Kunze) J. Sm. were powdered and refluxed for 2 h with 75% ethanol (5000 mL × 3). The extract was filtered, and the combined filtrate was concentrated to dryness under reduced pressure to give a dark brown mass (210 g). The major part (200 g) of the ethanolic extract was suspended with hot water and successively fractionated with petroleum ether (b.p. 60–90 °C), chloroform, ethyl acetate, and *n*-butanol. The fraction solutions were evaporated yielding the petroleum ether fraction (5 g), chloroform fraction (2 g), ethyl acetate fraction (18 g), *n*-butanol fraction (20 g) and residual aqueous fraction (145 g), respectively. The *n*-butanol fraction (1 g) which retained most activity underwent chromatography on an adsorption resin column followed by an open tubular ODS column (3 × 20 cm, I.D., 10 μm). The ODS column was eluted with water-methanol in a step-gradient of 100 : 0, 100 : 35, 100 : 40, 100 : 42, 100 : 45, 100 : 50 to 1 : 1. One retention volume of the eluate was collected as a fraction. The eluate fractions were monitored by HPLC with UV detection at 283 nm. Fractions with HPLC peak at identified retention time were combined and their activity was determined in the proliferation assay. Two combined fractions (No. 18–22 and No. 31–35) eluted by water-methanol (100 : 40 and 100 : 45) were found to be active in osteoblastic proliferation. This isolation was repeated ten times. A crystalline substance was obtained from each combined fraction and was further purified by recrystallization from methanol. Compounds **1** (110 mg) and **2** (100 mg) with a purity of 99.0% measured by HPLC-DAD then underwent structure identification and cell-bioassay.

3.3. Preparation of test samples

The crude extract of *Drynaria fortunei* (Kunze) J. Sm. and its fractions were dissolved in 75% ethanol yielding stock solutions (10 mg/mL, expressed as the weight of raw material per mL). The concentrations of stock solutions of the two active compounds were 10 mmol/L. The stock solutions were sterilized by filtration with a 0.2 μm aseptic filter (Gelmann Science, Ann Arbor, MI, USA) and stored at 4 °C. All assay samples were prepared by diluting the stock solutions with MEM to the required concentration immediately before use. The blank controls contained MEM and the same proportion of ethanol as the test samples.

3.4. Cell proliferation assay

The activity of the tested extracts, fractions and compounds stimulating proliferation of osteoblast-like UMR106 cells was determined by MTT colorimetric assay using the procedure described previously (Li et al. 2001; Gao et al. 2000; Wang et al. 2001a,b). Cells were trypsinized and plated in 96-well tissue culture plates in serum-containing medium at a concentration of 3×10^4 cells/mL, and allowed to attach for 24 h. The cells were then washed with PBS and cultured with serum-free medium containing various concentrations of each test sample. Sodium fluoride was used as a positive control. After being co-cultured with the sample solutions for 48 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, the medium was removed and MTT solution (1 mg MTT/mL PBS) of 50 μL was added into each well. The incubation was continued for another 4 h to allow MTT to metabolize to formazan. The supernatant MTT was aspirated from the wells and DMSO (150 μL per well) was added to dissolve the formazan

crystals formed. The absorbance was measured using an enzyme immunoassay plate reader (BIO-RAD, Hercules, CA, USA) at a wavelength of 595 nm with a reference at 655 nm.

3.5. Statistical analysis

All data are expressed as mean ± standard deviation. Statistical significance was assessed by using Student's t-test. A p value less than 0.05 was considered significant. Proliferation stimulation (%) was calculated as $(A_{sample} - A_{blank})/A_{blank} \times 100$, where A is the average absorbance of three culture experiments with six replicates each.

Acknowledgement: This study was supported by the National Natural Science Foundation of China (Grant No. 30472168).

References

An L, Wang K, Liu B (1996) 31 Cases of postmenopausal osteoporosis treated with Gubi formula. Xinjiang J Trad Chin Med Pharm 2: 12–13.

Chang EJ, Lee WJ, Cho SH, Choi SW (2003) Proliferative effects of flavan-3-ols and propepargonidins from rhizomes of *Drynaria fortunei* on MCF-7 and osteoblastic cells. Arch Pharm Res 26: 620–630.

Chinese Pharmacopoeia Commission (2005) Herba Epimedii. In: Chinese Pharmacopoeia Commission (ed.), Chinese Pharmacopoeia, Part I. Beijing, p. 229.

Cui SQ, Li SQ, Gang PH, Pei ZG, Liu YM, Jiang, SY (2001) Nourishing kidney prescription gushukang preventing and treating primary osteoporosis. J Chin Med Univ 30: 351–354.

Fareleg JR, Wergedal JE, Baylink DJ (1983) Fluoride directly stimulates proliferation and alkaline phosphatase activity of bone forming cells. Science 222: 330–332.

Gao XY, Wang DW, Li FM, Jiang ZM (2000) The effect of the extracts of Achyranthes bidentata Bl. on proliferation of osteoblast-like UMR 106 cells. J Shenyang Pharm Univ 17: 210–213.

Geng J, Huang W, Ma X (1997) Medicinal Herbs. Beijing, p. 226–227.

Gray TK, Flynn TC, Gray KM, Nabell LM (1987) 17-Beta-estradiol acts directly on the clonal osteoblastic cell line UMR 106. Proc Natl Acad Sci USA 84: 6267–6271.

Jeong JC, Kang SK, Youn CH, Jeong CW, Kim HY, Lee YC, Chang YC, Kim CH (2004a) Inhibition of Drynariae rhizoma extracts on bone resorption mediated by processing of cathepsin K in cultured mouse osteoclasts. Int Immunopharmacol 3: 1685–1697.

Jeong JC, Lee JW, Yoon CH, Kim HM, Kim CH (2004b) Drynariae Rhizoma promotes osteoblast differentiation and mineralization in MC3T3-E1 cells through regulation of bone morphogenetic protein-2, alkaline phosphatase, type I collagen and collagenase-1. Toxicol in Vitro 18: 829–834.

Jeong JC, Lee JW, Yoon CH, Lee YC, Chung KH, Kim MG, Kim CH (2005a) Stimulative effects of Drynariae Rhizoma on proliferation and differentiation of osteoblastic MC3T3-E1 cells. J Ethnopharmacol 96: 489–495.

Jeong JC, Lee BT, Yoon CH, Kim HM, Kim CH (2005b) Effects of Drynariae rhizoma on the proliferation of human cells and the immunomodulatory activity. Pharmacol Res 51: 125–136.

Kosuge K, Mitsunaga K, Koike K, Ohmoto T (1994) Studies on the constituents of Ailanthus integrifolia. Chem Pharm Bull 42: 1669–1671.

Lei HM, Sun WJ (2000) The chemical constituents of *Citrus grandis* Osbecks var *tomentosa* Hort. Northwest Pharm J 15: 203–204.

Li FM, Wang DW, Jiang ZM, Gao XY, Zhao HQ (2001) Activity stimulating osteoblast-like cells proliferation of some traditional Chinese medicinal drugs and other plants. Pharm Biol 39: 351–356.

Lin CY, Sun JS, Sheu SY, Lin FH, Wang YJ, Chen LT (2002) The effect of Chinese medicine on bone cell activities. Am J Chin Med 30: 271–285.

Ma KC, Zhu TY, Liu XR, Liu WZ (1995) Promoting effect of Rhizoma fortunei on the calcification of cultivated chick embryo bone primordium. Chin J Chin Mater Med 20: 178–180.

Ma ZS, Wang R, Qiu MC, Li YK, Zheng FQ, Zhang X (1999) Study of morphologic effects of 4 Chinese herbs by bone histomorphometry in ovariectomized rats. Chin J Obstetrics Gynecol 34: 82–85.

Meng FH, Li YB, Xiong ZL, Jiang ZM, Li FM (2005) Osteoblastic proliferative activity of *Epimedium brevicornum* Maxim. Phytomedicine 12: 189–193.

Nishiura K, Kamiya S, Esaki S, Ito F (1971) Flavonoids in citrus and related genera. Agr Biol Chem 35: 1683–1690.

Patridge NC, Alcorn D, Michelangeli VP, Ryan G., Martin TJ (1983) Morphological and biochemical characterization of four clonal osteogenic sarcoma cell lines of rat origin. Cancer Res 43: 4308–4314.

Sun JS, Lin CY, Dong GC, Sheu SY, Lin FH, Chen LT, Wang YJ (2002) The effect of Gu-Sui-Bu (*Drynaria fortunei* J. Sm) on bone cell activities. Biomaterials 23: 3377–3385.

Sun JS, Dong G.C, Lin CY, Sheu SY, Lin FH, Chen LT, Wang YJ (2003) The effect of Gu-Sui-Bu (*Drynaria fortunei* J. Sm) immobilized modified calcium hydrogenphosphate on bone cell activities. Biomaterials 24: 873–882.

Wang DW, Li FM, Jiang ZM (2001a) Osteoblastic proliferation stimulating activity of *Psoralea corylifolia* extract and two of its flavonoids. Planta Med 67: 748–749.

Wang DW, Li FM, Jiang ZM, Gao XY (2001b) Effect of *Drynaria baronii* rhizome extracts on the proliferation in osteoblast-like UMR 106 cells. Pharm Biol 39: 259–262.

Yang TH, Lee YC, Chung HY (1966) Constituents of *Drynaria fortunei.* Taiwan Pharm J 18: 38–40.