CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Elsevier - Publisher Connector

Journal of Traditional and Complementary Medicine Vo1. 3, No. 2, pp. 134-138
Copyright © 2013 Committee on Chinese Medicine and Pharmacy, Taiwan This is an open access article under the CC BY-NC-ND license.

Journal of Traditional and Complementary Medicine
Journal homepage http://www.jtcm.org

Osteoporosis Prevention by Adlay (薏苡 Yì Yǐ: The Seeds of *Coix Lachryma-Jobi* L. var. *ma-yuen* Stapf) in a Mouse Model

Rong-Sen Yang[1], Yi-Hsiang Lu[2], Wenchang Chiang[3], Shing-Hwa Liu[2]

[1]Department of Orthopaedics, College of Medicine, National Taiwan University, Taipei, Taiwan.
[2]Institute of Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan.
[3]Graduate Institute of Food Science and Technology, College of Bioresources and Agriculture, National Taiwan University, Taipei, Taiwan.

ABSTRACT

Osteoporosis is characterized by reduced bone mass and quality due to an imbalanced bone remodeling. A grass crop, adlay (*Coix lachryma-jobi*), is a kind of nourishing food, which has also been used in traditional Chinese medicine. In this study, we investigated the effect of adlay (*C. lachryma-jobi* L. var. *ma-yuen* Stapf) on osteoporosis using an ovariectomized mouse model. The adlay diet (10% and 30% adlay in mouse diet) or water extract of adlay (0.3 g/kg/day) was given to ovariectomized mice for 4 weeks. In some experiments, the primary rat osteoblast cells were used to test the possible mechanism of adlay on osteoporosis. The body weight was slightly increased and uterus weight was markedly decreased in ovariectomized mice, which were not affected by adlay treatment. Adlay diet (30%) and adlay extract feedings significantly reversed the decreased bone alkaline phosphatase activity and calcium contents and bone mineral density in ovariectomized mice. Moreover, adlay extracts increased the osteoblast cell proliferation in a dose-dependent manner. Adlay extracts also increased the protein expressions of proliferating cell nuclear antigen and phosphorylated extracellular signal-regulated kinase (ERK) 1/2 in osteoblast cells. ERK inhibitor PD98059 significantly reversed the increased osteoblast cell proliferation by adlay extracts. Taken together, these findings indicate that adlay effectively alleviates the osteoporotic status in ovariectomized mice. Adlay is capable of increasing the proliferation of osteoblast cells via an ERK-regulated signaling pathway. Adlay may be a helpful healthy food for osteoporosis prevention.

Key words: Adlay, Osteoblast cells, Osteoporosis, Proliferation

INTRODUCTION

Bone remodeling is known as an incorporated interaction between bone resorption and bone formation, which plays an important role in bone homeostasis. Osteoporosis is caused by a dysfunction of endocrine system in bone that results to the inability to maintain the bone homeostasis, leading to a decrease in bone mineral density (BMD), microarchitecture alteration, and eventually skeletal fractures.[1] It has been analyzed that the worldwide annual costs of hip fracture in 1990 were estimated to be about US$ 34.8 billion and are expected to increase over the next 50 years, indicating that osteoporosis is a global public health concern and a great socioeconomic burden.[2] An unbalanced bone remodeling process in which bone resorption is higher than bone formation triggers the decrease in BMD. Osteoporosis might occur

Correspondence to:
Prof. Shing-Hwa Liu, Institute of Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan. Tel: +886-2-23123456, ext. 88605; Fax: +886-2-23410217. E-mail: shinghwaliu@ntu.edu.tw

DOI: 10.4103/2225-4110.110408

in all populations, although it is prevalent in white postmenopausal women.[3] There are several therapeutic strategies for the treatment of osteoporosis, including the reduction of bone loss and the enhancement of bone mass; however, these anti-resorptive drugs (e.g. bisphosphonates) and anabolic drugs (e.g., parathyroid hormone) have their own limitations and safety issues.[1]

A grass crop, adlay (*Coix lachryma-jobi*), is a kind of nourishing food, which has also been used in traditional Chinese medicine for a long time, for example, in the treatment for warts, chapped skin, rheumatism, female endocrine system, and neuralgia. The studies in recent decade have shown that adlay possesses the beneficial effects, including suppression of allergic reactions,[4] influence on the growth of intestinal bacteria,[5] inhibition of lymphoma cell proliferation,[6] inhibition of nitric oxide and superoxide production from macrophages,[7] anti-mutagenic activity,[8] anti-proliferative and chemopreventive effects in lung cancer,[9] hypolipidemic effect,[10] anti-obesity effect,[11] and hypoglycemic effect in streptozotocin-induced diabetic rat model.[12] Our previous study has shown that water extracts of adlay seed effectively enhance the bone formation markers and reduce the bone resorption markers in the bone tissue culture models, suggesting that adlay may be a helpful healthy food for osteoporosis prevention.[13] However, the *in vivo* effect of adlay on the bone system or osteoporosis remains to be clarified. In the present study, we investigated the *in vivo* effect of adlay (*C. lachryma-jobi* L. var. *ma-yuen* Stapf) on osteoporosis using an ovariectomized mouse model. The results showed that adlay effectively alleviates the osteoporotic status in ovariectomized mice.

MATERIALS AND METHODS

Plant materials

Adlay samples, Taichung Shuenyu No. 4 of *C. lachryma-jobi* L. var. *ma-yuen* Stapf, were provided by a farmer (Taichung, Taiwan). The air-dried adlay seeds were dehulled and blended into powder, and then screened through a 20-mesh sieve. The dehulled adaly was added to mouse diet to prepare the adlay diet (10% and 30%). Moreover, the powder of adlay was further extracted as described previously.[13] In brief, after methanolic extraction, the plant materials were continuously extracted with warm water at 50°C for 30 min, and then the plant material was filtered to obtain the warm water extract. The extracts were dried using a freeze-dryer (model SFD-25, Chang Juing Co., Kaohsiung, Taiwan). These extracts of dehulled adaly were stored at −20°C until use.

Ovariectomized mouse model

Adult female Imprinting Control Region (ICR) mice (20-25 g) were obtained from Laboratory Animal Center of the College of Medicine, National Taiwan University, Taipei, Taiwan. All animals were fed with standard Purina Rodent Chow 5001 (Labdiet®, Richmond, IN, USA) and distilled water *ad libitum*. The procedures of the animal study were approved by the Committee of Animal Study in College of Medicine, National Taiwan University (IACUC Approval no.: 20040139). The ovariectomy procedure and sham operation were performed in mice under anesthesia. At 4 weeks after ovariectomy, the mice of adlay treatment groups were fed with diets containing 10% and 30% adlay or adlay water extract (0.3 g/kg/day) for 4 weeks. The other ovariectomized mice and sham control mice were fed with standard diets containing no adlay. At the end of the program, the body weight and BMD were measured and then the mice were sacrificed by decapitation. The weights of the uteri were measured, and the femoral metaphyseal tissues were collected for the measurement of alkaline phosphatase (ALP) and calcium content.

Cell cultures

The primary osteoblast cells were isolated from the calvaria of fetal rats (18 days old) as described previously.[14] In brief, the calvaria of fetal rats were dissected by aseptic technique under anesthesia and divided into small pieces. These pieces were treated with 0.1% collagenase solution for 10 min at 37°C. The collagenase digested pieces were filtered through a 70-μm nylon filter. Cells were grown on plastic cell culture dishes in 95% air –5% CO_2 with Dulbecco's modified Eagle's medium, which was supplemented with 20 mM HEPES and 10% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL) (pH 7.6). The characteristics of osteoblast cells were confirmed by morphology and ALP expression.

Cell proliferation assay

MTS assay

Cell proliferation was detected by a nonradioactive cell proliferation assay kit (CellTiter 9696™ AQueous, Promega, Madison, WI, USA), which contained a tetrazolium compound [3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)] and an electron-coupling reagent [phenazine methosulfate (PMS)]. Osteoblast cells (1×10^5 cells/mL) were incubated for 24 h with or without test materials, and then added at a concentration of 20 μL/well of combined MTS/PMS solution. Absorbance was measured at 490 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader.

Proliferating cell nuclear antigen (PCNA) protein expression

PCNA is a marker for cellular proliferation.[15] Cells were treated with or without test materials for 24 h and analyzed by Western blotting with antibody specific for PCNA.

Measurement of ALP activity

The ALP activity in metaphyseal tissues was measured with a color reaction assay. The tissues were homogenized and centrifuged at 600 × *g* for 5 min, and then the supernatants were used for activity assay. After incubation with *p*-nitrophenylphosphate at 37°C for 10-30 min, the reaction was stopped with 0.1 N NaOH. The absorbance was read at 405 nm. Each value was normalized to the protein concentration.

Measurement of calcium content

The metaphyseal tissues were dissolved in nitric acid overnight, followed by 100 times dilution with distilled water. The calcium levels were determined by Raichem® colorimetric assay (Hemagen Diagnostics, San Diego, CA, USA).

BMD measurement

The BMD of anesthetized mice was measured with a dual-energy X-ray absorptiometer (DEXA, Norland Corporation, Fort Atkinson, WI, USA) using a mode adapted to the measurement of small subject.

Western blot analysis

The samples of cell lysates or nuclear extracts (30-50 μg) were subjected to electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels, and then electroblotted on poly-(vinylidene difluoride) membranes. After blocking, the blots were incubated with antibodies for PCNA (BD-Transduction Laboratories, NJ, USA), extracellular signal-regulated kinase (ERK) 1/2 and phosphorylated ERK, and C23 (Santa Cruz Biotechnology, Dallas, TX, USA) in phosphate-buffered saline within 0.1% Tween 20 for 1 h, followed by two 15-min washes. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min. Enhanced chemiluminescence reagents (Amersham, Piscataway, NJ, USA) were employed to depict the protein bands on membranes.

Statistical analysis

The data are presented as means ± standard error of mean (SEM). All analyses were performed by analysis of variance followed by a Fisher's least significant difference test. A $P < 0.05$ was considered statistically significant.

RESULTS

In vivo effects of adlay diet and adlay extract feedings on osteoporosis in an ovariectomized mouse model

The adlay diet (10% and 30% adlay in mouse diet) or water extract of adlay (0.3 g/kg/day) was given to ovariectomized mice for 4 weeks. As shown in Figure 1, the body weight was slightly increased and uterus weight was markedly decreased ($P < 0.05$) in ovariectomized mice as compared with sham control mice. Both adlay diet and extract treatments did not affect these changes in ovariectomized mice. Moreover, decreases in bone ALP activity and calcium content [Figure 2] and BMD [Figure 3] were also observed in ovariectomized mice as compared with sham control mice ($P < 0.05$). Both adlay diet (30%) and adlay extract feedings significantly reversed the decreased bone ALP activity and calcium content [Figure 2] and bone BMD [Figure 3] in ovariectomized mice ($P < 0.05$).

In vitro effects of adlay extracts on the osteoblast cell proliferation

Next, we tried to investigate the possible mechanism of adlay on osteoporosis prevention using the primary rat osteoblast cells. As shown in Figure 4a, adlay extracts (0.1-1 μg/mL) significantly increased the osteoblast cell proliferation in a dose-dependent manner ($P < 0.05$). Moreover, adlay extracts could also increase the protein expressions of PCNA (a marker for cellular proliferation) and phosphorylated ERK1/2 in osteoblast cells [Figure 4b]. To clarify the role of ERK1/2 in adlay extract-increased osteoblast cell proliferation, the ERK inhibitor PD98059 was used. As shown in Figure 5, PD98059 (10 μM) significantly reversed the increased osteoblast cell proliferation by adlay extracts ($P < 0.05$).

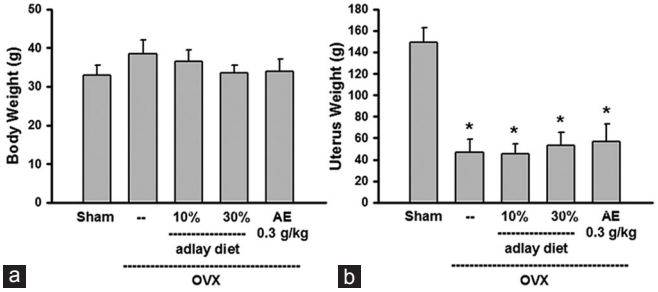

Figure 1. Effects of adlay diet or extract on the body weight and uterus weight in ovariectomized mice. The adlay diet (10% and 30% adlay in mouse diet) or adlay extract (AE, 0.3 g/kg/day) was given to ovariectomized mice for 4 weeks, and then the whole body weight (a) and uterus weight (b) were measured. Data are presented as means ± SE ($n = 6$). *$P < 0.05$ as compared with control

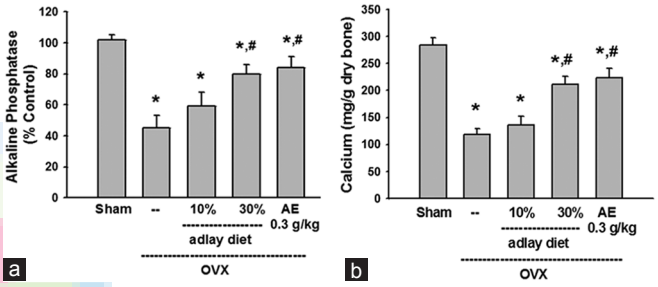

Figure 2. Effects of adlay diet or extract on the ALP and calcium levels in ovariectomized mice. The adlay diet (10% and 30% adlay in mouse diet) or adlay extract (AE, 0.3 g/kg/day) was given to ovariectomized mice for 4 weeks, and then the ALP (a) and calcium (b) levels were measured. Data are presented as means ± SE ($n = 6$). (*$P < 0.05$ as compared with control; #$P < 0.05$ as compared with ovariectomized group)

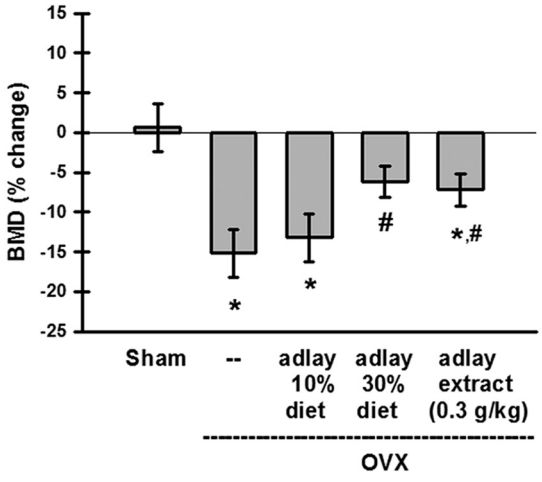

Figure 3. Effects of adlay diet or extract on the BMD levels in metaphyseal tissues isolated from ovariectomized mice. The adlay diet (10% and 30% adlay in mouse diet) or adlay extract (AE, 0.3 g/kg/day) was given to ovariectomized mice for 4 weeks, and then the BMD levels were measured. Data are presented as means ± SE ($n = 6$). (*$P < 0.05$ as compared with control; #$P < 0.05$ as compared with ovariectomized group)

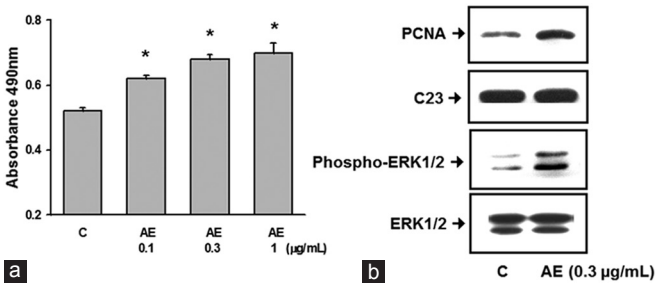

Figure 4. Effects of adlay extracts on the cell proliferation and signaling molecules PCNA and ERK1/2 in rat primary osteoblast cells. (a) Cell proliferation in cultured rat osteoblasts with or without adlay extract treatment (AE, 0.1-1 μg/mL) was determined by MTS assay after 24 h in culture. Data are presented as means ± SE (n = 4). (*P < 0.05 as compared with control. (b) Nuclear fractions or whole cell lysates were immunoblotted with anti-PCNA and anti–phospho-ERK1/2 and anti-ERK1/2 antibodies. C23 (a nuclear protein) or α-tubulin served as control for sample loading and integrity. Results shown are representative of at least three independent experiments with consistent results)

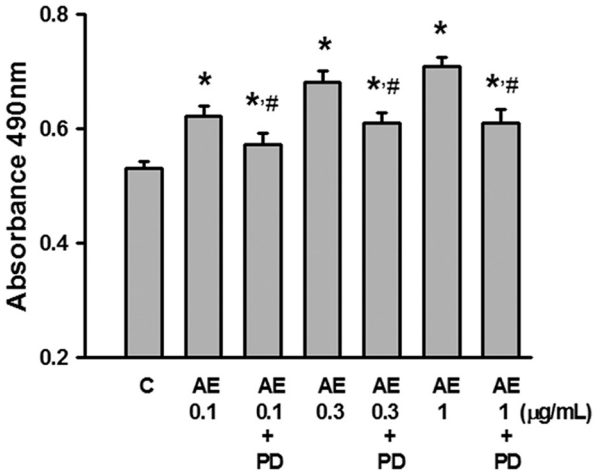

Figure 5. Effect of ERK inhibitor PD98059 on adlay extract-increased cell proliferation in rat primary osteoblast cells. Cells were treated with adlay extracts (AE, 0.1-1 μg/mL) in the presence or absence of PD98059 (10 μM) in cultured rat osteoblasts for 24 h. Cell proliferation was determined by MTS assay. Data are presented as means ± SE (n = 4). *P < 0.05 as compared with control; #P < 0.05 as compared with AE alone

DISCUSSION

The present study shows that adlay diet and adlay water extract feedings effectively alleviated the osteoporotic status in ovariectomized mice. Water extract of adlay seed significantly increased the osteoblast cell proliferation *in vitro*. We further found that ERK signaling is involved in the adlay water extract-increased osteoblast cell proliferation. These findings suggest that adlay is capable of alleviating the estrogen deficiency-induced osteoporosis *in vivo* through the stimulation of osteoblast-mediated bone formation.

Regarding adlay supplementation, previous study showed that feeding the obese rats with crude adlay hull acetone extract could induce the expression of leptin and tumor necrosis factor-α, followed by loss of appetite, body weight, and blood lipids.[16] Adlay oil has been shown to decrease the content of both leptin and low-density lipoprotein in fat tissues.[10] A methanolic extract of adlay seed has also been demonstrated to suppress the gene transcription and expression of cyclooxygenase-2 (*cox-2*) in human lung cancer cells.[17] Additionally, a methanolic extract, but not a water extract, of adlay seed has been found to inhibit the lung cancer cell proliferation *in vitro*.[9] Kuo *et al*., further found that feeding with a diet containing 30% of powdered adlay seed significantly decreased the number of tumors in a lung cancer mouse model.[9] Nevertheless, a water extract of adlay seed, but not a methanolic extract, has been demonstrated to activate the expression of COX-2, ERK1/2, and protein kinase C (PKC)-α in the pregnant female mice, leading to the uterine contraction and increased risk of abortion.[18] In the present study, the ovariectomized mice, an osteoporotic mouse model, were used to test the preventive effect of water extract of adlay seed on osteoporosis. The results showed that the decreased BMD significantly reversed after treatment with adlay diet or adlay extract for 4 weeks, accompanied by recovery of bone ALP activity and bone calcium content. These findings indicate that the water extract of adlay seed is capable of preventing the osteoporosis *in vivo*.

The imbalance in bone remodeling between osteoblasts and osteoclasts with net bone loss is the underlying cause of osteoporosis.[1,19-21] Previous studies have demonstrated that osteoblasts can be activated through the expression of COX-2 to induce the formation of prostaglandins that further modulate various hormones and growth factors to initiate and activate the bone formation process.[22] ERK1/2 are known as the members of the mitogen-activated protein kinase (MAPK) family of cell signaling enzymes regulating many aspects of cellular events, including differentiation, proliferation, and survival.[23] 17β-estradiol has been shown to increase Chinese hamster ovary cell proliferation through the activation of the ERK signaling.[24] It has been shown that an ERK-related signaling pathway is involved in the bone protective effect of sex steroids.[25] Fluid shear stress has also been found to trigger the ERK-regulated *cox-2* gene expression in osteoblast cells.[26] Benzo[a] pyrene, a polycyclic aromatic hydrocarbon, has been shown to increase the osteoblast cell proliferation through an ERK-regulated COX-2 signaling pathway.[27] In this study, we found that the water extract of adlay seed significantly increased cell proliferation and the protein expression of cellular proliferation marker PCNA in primary rat osteoblast cells. We also found that the adlay extract could increase the phosphorylation of ERK1/2 in osteoblast cells and ERK inhibitor PD98059 significantly reversed the adlay extract-increased osteoblast cell proliferation. These findings suggest that ERK signaling is involved in the adlay extract-increased osteoblast cell proliferation, which may contribute to the increased bone formation by adlay observed *in vivo*.

In conclusion, the present findings demonstrated for the first time that adlay possesses protective effect on altered bone metabolism in an ovariectomized mouse model. Adlay is capable of increasing the proliferation of osteoblast cells via an ERK-regulated signaling pathway. Adlay may be a helpful healthy food for osteoporosis prevention.

ACKNOWLEDGMENT

This work was supported by research grants from Council of Agriculture, Executive Yuan, Taiwan (93AS-5.1.3-FDZ1 and 94AS-12.1.3.FD-Z1).

REFERENCES

1. Baron R, Hesse E. Update on bone anabolics in osteoporosis treatment: Rationale, current status, and perspectives. J Clin Endocrinol Metab 2012;97:311-25.
2. Harvey N, Dennison E, Cooper C. Osteoporosis: Impact on health and economics. Nat Rev Rheumatol 2010;6:99-105.
3. NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy. Osteoporosis prevention, diagnosis, and therapy. JAMA 2001;285:785-95.
4. Hsu HY, Lin BF, Lin JY, Kuo CC, Chiang W. Suppression of allergic reactions by dehulled adlay in association with the balance of TH1/TH2 cell responses. J Agric Food Chem 2003;51:3763-9.
5. Chiang W, Cheng C, Chiang M, Chung KT. Effects of dehulled adlay on the culture count of some microbiota and their metabolism in the gastrointestinal tract of rats. J Agric Food Chem 2000;48:829-32.
6. Kuo CC, Shih MC, Kuo YH, Chiang W. Antagonism of free-radical-induced damage of adlay seed and its antiproliferative effect in human histolytic lymphoma U937 monocytic cells. J Agric Food Chem 2001;49:1564-70.
7. Seo WG, Pae HO, Chai KY, Yun YG, Kwon TH, Chung HT. Inhibitory effects of methanol extract of seeds of Job's tears (*Coix lachryma-jobi* L. var. *ma-yuen*) on nitric oxide and superoxide production in RAW 264.7 macrophages. Immunopharmacol Immunotoxicol 2000;22:545-54.
8. Chen HH, Chiang W, Chang JY, Chien YL, Lee CK, Liu KJ, *et al*. Antimutagenic constituents of adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) with potential cancer chemopreventive activity. J Agric Food Chem 2011;59:6444-52.
9. Kuo CC, Chen HH, Chiang W. Adlay (the seeds of *Coix lachryma-jobi* L. var. *ma-yuen* Stapf) is a potential cancer chemopreventive agent toward multistage carcinogenesis processes. J Tradit Complement Med 2012; 2:267-75.
10. Huang BW, Chiang MT, Yao HT, Chiang W. The effect of adlay oil on plasma lipids, insulin and leptin in rat. Phytomedicine 2005;12:433-9.
11. Kim SO, Yun SJ, Lee EH. The water extract of adlay seed (*Coix lachrymajobi* var. *ma-yuen*) exhibits anti-obesity effects through neuroendocrine modulation. Am J Chin Med 2007;35:297-308.
12. Yeh PH, Chiang W, Chiang MT. Effects of dehulled adlay on plasma glucose and lipid concentrations in streptozotocin-induced diabetic rats fed a diet enriched in cholesterol. Int J Vitam Nutr Res 2006;76:299-305.
13. Yang RS, Chiang W, Lu YH, Liu SH. Evaluation of osteoporosis prevention by adlay using a tissue culture model. Asia Pac J Clin Nutr 2008;17 Suppl 1:143-6.
14. Yang RS, Tang CH, Ling QD, Liu SH, Fu WM. Regulation of fibronectin fibrillogenesis by protein kinases in cultured rat osteoblasts. Mol Pharmacol 2002;61:1163-73.
15. Morris GF, Mathews MB. Regulation of proliferating cell nuclear antigen during the cell cycle. J Biol Chem 1989;264:13856-64.
16. Kim SO, Yun SJ, Jung B, Lee EH, Hahm DH, Shim I, *et al*. Hypolipidemic effects of crude extract of adlay seed in obesity rat fed high fat diet: Relations of TNF-α and leptin mRNA expressions and serum lipid levels. Life Sci 2004;75:1391-404.
17. Hung WC, Chang HC. Methanolic extract of adlay seed suppresses COX-2 expression of human lung cancer cells via inhibition of gene expression. J Agric Food Chem 2003;51:7333-7.
18. Tzeng HP, Chiang W, Ueng T, Liu SH. The abortifacient effects from the seeds of *Coix lachrymal-jobi* L. var. *ma-yuen* Stapf. J Toxicol Environ Health A 2005;68:1557-65.
19. Ducy P, Schinke T, Karsenty G. The osteoblast: A sophisticated fibroblast under central surveillance. Science 2000;289:1501-4.
20. Manolagas SC, Jilka RL. Bone marrow, cytokines, and bone remodeling. Emerging insights into the pathophysiology of osteoporosis. N Engl J Med 1995;332:305-11.
21. Teitelbaum SL. Bone resorption by osteoclasts. Science 2000;289:1504-8.
22. Raisz LG. Potential impact of selective cyclooxygenase-2 inhibitors on bone metabolism in health and disease. Am J Med 2001;110 (Suppl 3A):43-8.
23. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, *et al*. Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. Endocr Rev 2001;22:153-83.
24. Razandi M, Pedram A, Greene GL, Levin ER. Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: Studies of ERα and ERβ expressed in Chinese hamster ovary cells. Mol Endocrinol 1999;13:307-19.
25. Kousteni S, Han L, Chen JR, Almeida M, Plotkin LI, Bellido T, *et al*. Kinase-mediated regulation of common transcription factors accounts for the boneprotective effects of sex steroids. J Clin Invest 2003;111:1651-64.
26. Wadhwa S, Godwin SL, Peterson DR, Epstein MA, Raisz LG, Pilbeam CC. Fluid flow induction of cyclooxygenase 2 gene expression in osteoblasts is dependent on an extracellular signal-regulated kinase signaling pathway. J Bone Miner Res 2002;17:266-74.
27. Tsai KS, Yang RS, Liu SH. Benzo[a] pyrene regulates osteoblast proliferation through an estrogen receptor-related cyclooxygenase-2 pathway. Chem Res Toxicol 2004;17:679-84.