

## COUMESTROL AS WELL AS ISOFLAVONES IN SOYBEAN EXTRACT PREVENT BONE RESORPTION IN OVARIECTOMIZED RATS

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**Objective.** Soybeans contain an abundance of phytoestrogens such as genistein and daidzein, and these compounds are thought to protect against bone loss under estrogen deficient conditions. It is possible, however, that phytoestrogens other than isoflavones may suppress bone resorption. The objective of the present study was to determine whether there are any phytoestrogens other than isoflavones in soybeans that can act as antiosteoporotic agents.

**Methods.** The isoflavones genistein and daidzein, and an extract of soybeans that contained very low levels of isoflavones were tested for their ability to reduce bone resorption in ovariectomized rats. The extract of soybeans was further analyzed for its main components by thin layer chromatography and gas chromatography-mass spectrometry.

**Results.** The soybean extract, as well as the two isoflavones, were effective to reduce urinary excretion of deoxypyridinoline and pyridinoline, typical markers of bone resorption in ovariectomized rats. However the extract, unlike the isoflavones, increased the uterine weight of ovariectomized rats significantly. Analysis by thin-layer and gas chromatography revealed that the main constituent of this extract exhibited a chromatographic profile corresponding to coumestrol. This substance was positively identified as coumestrol by gas chromatography-mass spectrometry.

**Conclusion.** These results suggest that the phytoestrogens including coumestrol and isoflavones in soybeans may exert effective prevention against bone resorption in estrogen deficient conditions.

**Key word:** soybean, urinary deoxypyridinoline, coumestrol, genistein, daidzein

Estrogen is effective in suppressing bone resorption (TURNER et al. 1994). Postmenopausal women develop osteoporosis, at least in part, due to estrogen deficiency and estrogen replacement therapy has been shown to reduce postmenopausal osteoporosis (HEIKKINEN et al. 1997). A very recent study, however, has shown that overall health risks from the use of combined estrogen plus progestin therapy exceed benefits among healthy postmenopausal women in the United States (ROSSOUW et al. 2002).

The incidence of hip fracture and of osteoporosis differs considerably between Oriental and Western

Caucasian women, suggesting nutritional factors may be involved (COOPER et al. 1992; ADLERCREUTZ and MAZUR 1997). This may be due to soybeans and vegetables which are consumed in large quantities in the Asiatic diet. Soybeans contain not only estrogenic isoflavones but also other substances such as coumestans and lignans (KURZER and XU 1997; HUTABARAT et al. 2000). All these substances are referred to as phytoestrogens, because they are derived from plants and possess biological activity similar to estrogen. Soybean products are thought to be beneficial in preventing osteoporosis, because they contain estro-

genic isoflavones such as genistein, daidzein and their glycans, each of which has been shown to have bone-sparing effects in the rat, mouse and human (KNIGHT and EDEN 1996; ANDERSON and GARNER 1997; DRAPER et al. 1997; ARJMANDI et al. 1998; THAM et al. 1998; ISHIMI et al. 1999; ALEKEL et al. 2000)

It is possible that phytoestrogens other than isoflavones which are present in soybeans suppress bone resorption under estrogen deficient conditions. The objective of this study is to examine if any substances in soybeans other than isoflavones have antiosteoporotic effects in ovariectomized rats. The present study deals with the antiosteoporotic and uterotrophic effects of a soybean extract devoid of isoflavones on bone resorption and uterine weight.

### Materials and Methods

**Materials.** Genistein, daidzein, genistin, daidzin,  $17\alpha$ -ethinylestradiol and bis(trimethylsilyl) trifluoroacetamide (TMS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Coumestrol was obtained from Fluka Chemika (Buchs, Switzerland). Silica gel of 200~300 mesh was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals were of the purest grades available.

**Extraction and fractionation of phytoestrogens from soybeans.** Dried soy beans (*Glycine max*), cultivated without the use of herbicides or insecticides, were obtained in the City of Koga, Ibaragi. Ten kg of dry soybeans were broken into small pieces using a coffee mill and then extracted with acetone (1.5 l/kg). The extraction was repeated 5 times and the extracts were pooled. The pooled extract was condensed under reduced pressure at  $43\pm 1^\circ\text{C}$  in darkness. The condensed extract was then applied to a silica gel column (300 mm x 55 mm i.d.) and eluted successively with 1) n-hexane; 2) hexane:ethyl acetate (10:1, v/v); 3) hexane:ethyl acetate (5:1, v/v); 4) ethyl acetate and 5) acetone. The volume of each solvent applied was 5 times the column volume. Eluates were evaporated under reduced pressure at  $43\pm 1^\circ\text{C}$  in darkness. Fraction 4 was resuspended in absolute ethanol and then mixed with oleic acid at the rate of 125  $\mu\text{l}/10\text{ml}$ . This fraction was tested for its antiosteoporotic activity in ovariectomized rats, because it contained low levels of isoflavones, if any, when analyzed by thin-layer chromatography.

**Animals.** Animals were treated according to the guidelines for the Animal Care and Use of Laboratory Animals, Dokkyo University School of Medicine. Female rats of the Wistar ST strain were obtained from Charles River Japan Inc. (Kanagawa, Japan). The animals were kept at a temperature of  $23 \pm 2^\circ\text{C}$  with a 12 h light and dark cycle and allowed free access to food and water. The food provided was a proprietary formulation (Rat, Mouse and Hamster pellet chow MF; Oriental Yeast Co. Ltd., Tokyo, Japan) with the following composition (g/100g): crude protein, 23.8 g; fat, 5.1 g; crude fiber, 3.2 g, and water, 7.8 g. The content of daidzin, genistein and coumestrol was below detectable levels. The rats were ovariectomized at the age of 10 weeks and were randomly assigned to treatment groups.

In the first experiment, animals were divided into 4 groups; control group, ovariectomized group, ethinylestradiol-treated group and soybean extract-treated group. Ethinylestradiol was given orally by gastric tube at the daily dose of 0.1 mg/kg body weight. This dose is known to normalize estrogen-deficient conditions (VORE et al. 1983). The estrogen-treated group, therefore, served as positive control. The soybean extract was given intragastrically to ovariectomized rats. The extract given daily to each ovariectomized rat was derived from roughly 60 g of soybeans, equivalent to 250 g soybeans per kg body weight.

In the second experiment, animals were divided into control, ovariectomized, genistein-treated and daidzein-treated groups. The isoflavones were given orally once daily by gastric tube. Genistein or daidzein suspended in 0.5% carboxymethylcellulose was administered intragastrically at the dose of 25 mg per kg body weight. This dose of isoflavones was chosen, because it was estimated to contain comparable amounts of isoflavones to the levels present in the soybeans used for extraction. Control rats received only vehicle.

**Blood sampling and organ preparation.** Rats were sacrificed by decapitation. Blood samples were collected into sampling tubes and sera obtained by centrifugation. The sera thus obtained were used for the assay of osteocalcin and cholesterol. Osteocalcin in the serum was measured by two-site immunoradiometric assay (Mitsubishi Kasei, Tokyo). Serum total cholesterol was measured enzymatically using an L-type assay kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan), according to the method of RICH-

**Table 1**  
**Effects of ovariectomy, estrogen and soybean extract on urinary excretion of deoxy pyridinoline (D-Pyr) and pyridinoline (Pyr).**

Treatment (day after OVX)		0 day	7 day	14 day	21 day
Control (5)	D-Pyr	50.0±2.7	39.7±2.4	37.7±1.3	34.4±2.0
	Pyr	70.6±3.7	67.3 ±3.0	73.0±1.7	65.1±3.4
OVX (5)	D-Pyr		49.1±2.4	48.9±3.1	52.1±2.1
	Pyr		81.9±5.21	87.4±5.20	80.1±3.0
OVX+ethinylestradiol (5)	D-Pyr		43.9±3.7	37.3±1.6	25.5±1.4 **
	Pyr		68.5±4.4	70.1±3.7	52.6±3.9**
OVX+soybean extract (4)	D-Pyr		48.9±3.7	44.5±1.2	39.6±2.9**
	Pyr		80.7±5.7	80.9±2.7	67.4±4.2*

All data are expressed as mean±SEM (nmol/mmol creatinine). Figures in parentheses show the numbers of determinations. OVX, ovariectomy. Asterisks \*, \*\*, significantly different compared with the ovariectomized group at  $p < 0.05$  and  $< 0.02$ , respectively. Blank: not determined.

MOND (1973). Rat uteri were removed and weighed immediately after being dissected free of adjacent fatty tissues.

**Urine sampling.** Each rat was kept in an individual cage for 1 day and the 24 h urine was collected to assay deoxy pyridinoline, pyridinoline and creatinine. Deoxy pyridinoline and pyridinoline in the urine were assayed by high-performance liquid chromatography (HPLC). An ODS column was used to separate deoxy pyridinoline and pyridinoline, which were detected by fluorometric assay with emission at 395 nm and excitation at 296 nm. Creatinine was measured using a creatinine assay kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's instructions. Urinary excretion of both deoxy pyridinoline and pyridinoline were expressed as ng/mmol creatinine.

**Analysis and identification of phytoestrogens in soybean extracts.** Phytoestrogens in the soybean acetone extracts were analyzed by thin-layer chromatography (TLC) and gas chromatography (GC). Briefly, TLC analyses were performed with 5 x 20 cm Silica Gel-G sheets. The solvent used was chloroform: methanol (8:1, v/v). Ten µl of the soybean extracts and standard samples were spotted on the plates and developed for 1 h. Spots on the TLC plates were visualized under ultraviolet (UV) light. Spots were scraped from the plates, pooled and extracted three times with pyridine. The extracts were then evaporated under a flow of nitrogen gas and resuspended in acetonitrile. One hundred µl of bis (trimethylsilyl) trifluoroacetamide (TMS) was heated with

200 µl extract in acetonitrile at 150 °C for 10 min to produce TMS derivatives.

**Gas chromatography-mass spectrometry (GC-MS) analysis.** GC-MS analysis was performed in an AUTOMASS 120 (JEOL Ltd, Tokyo, Japan) equipped with a DB-5 ms fused silica capillary column (30 m x 0.25 mm i.d., film thickness 0.25 µm, J & W Scientific, Folsom, CA, USA). The operating temperatures were as follows: injection port, 270 °C; column, 50-280 °C (20 °C/min); ion source, 180 °C; interface, 230 °C. The flow rate of the carrier gas, helium, was 0.9 ml/min. The mass spectra were obtained during electron impact (EI) running mode. The ionization voltages were 70 eV for EI.

**Statistical analysis.** All the data are expressed as mean ± SEM. Analysis of variance (ANOVA) was performed and Scheffe's multiple comparison test was applied to test the differences between individual groups. A  $p$ -value less than 0.05 was considered statistically significant.

## Results

**Effects of soybean extract on urinary excretion of deoxy pyridinoline and pyridinoline.** Urinary excretion of deoxy pyridinoline (D-Pyr) and pyridinoline (Pyr) in the sham-operated controls decreased gradually over the course of time, while that of ovariectomized rats remained nearly constant for 3 weeks (Table 1). The urinary excretion in the ethinylestradiol-treated group was a little lower than in the control group. The soybean extract significantly

**Table 2**  
Effects of ovariectomy, estrogen and the soybean extract on serum osteocalcin and total cholesterol

Treatment	Serum osteocalcin (ng/ml)	Total cholesterol (mg/dl)	Uterus (mg/100g)
Control (5)	34.0±1.1	74.2±3.2	1.65±0.057
OVX (5)	42.4±3.4	89.4±3.9	0.53±0.031
OVX+ethinyl-estradiol (5)	30.4±1.1*	39.0±2.1*	1.80±0.032
OVX+soybean extract (4)	45.8±3.7	84.8±3.4	0.77±0.008*

All data are expressed as mean ± SEM. Figures in parentheses show the numbers of determinations. OVX, ovariectomy. The asterisk \* shows a statistically significant difference from OVX group at  $p < 0.05$ .

decreased the urinary excretion of both deoxypyridinoline and pyridinoline.

Ethinylestradiol at the dose of 0.1 mg/kg increased the uterine weight of ovariectomized rats to a little above normal (Table 2). Serum levels of osteocalcin, a marker of osteoblast activity, were a little higher in ovariectomized rats than in sham-operated animals, but there was no significant difference among the 4 groups. Ovariectomy slightly elevated serum total cholesterol levels and estradiol supplementation significantly lowered cholesterol levels, while the soybean extract had no effect. The soybean extract slightly but significantly increased the uterine weight of ovariectomized rats.

**Effects of isoflavones.** Both genistein and daidzein (25 mg/kg for each isoflavone) were effective in lowering the urinary excretion of deoxypyridino-

line and pyridinoline (Table 3). However, they showed no effect on the uterine weight of ovariectomized rats (Table 4). Genistein lowered the serum cholesterol levels in ovariectomized rats but daidzein did not. Because these findings suggest the uterotrophic effect of the soybean extract is not due to isoflavones, we further analyzed the soybean extract to identify the substance(s) responsible for the uterotrophic and antiosteoporotic effects.

**Separation and identification of active components in the soybean extract.** The soybean extract tested was eluted by ethyl acetate to identify its active component(s). Analysis performed by thin-layer chromatography revealed a major spot which behaved in the same way as genuine coumestrol, as well as some other minor spots. Upon exposure to UV light this major spot fluoresced in the same way as coumestrol.

After TMS derivatization, the sample from Fraction 4 was further analyzed by gas chromatography (Fig. 1). Three derivatives of genistein were detected (Fig. 1A). The retention time of the main component of the extract exactly corresponded to that of coumestrol. GC-MS confirmed that this main component was actually identical to coumestrol (Fig. 2). The analysis of other minor components revealed that Fraction 4 contained only negligible amounts of daidzein and genistein. A considerable amount of coumestrol was also found in Fraction 3 but not in other fractions (data not shown).

## Discussion

The compounds in soybeans that are beneficial for the prevention of osteoporosis have long been claimed

**Table 3**  
Effects of ovariectomy, genistein and daidzein on urinary excretion of deoxypyridinoline (D-Pyr) and pyridinoline (Pyr).

Treatment (day after OVX)		0 day	7 day	14 day	21 day
Control (5)	D-Pyr	40.5±3.4	40.3±6.6	29.8±2.4	26.3±5.9
	Pyr	66.8±3.8	69.6±6.6	60.8±3.3	50.2±8.2
OVX (5)	D-Pyr		45.1±6.2	51.5±5.0	56.3±3.8
	Pyr		77.5±9.0	87.0±5.8	87.1±3.9
OVX+ genistein (5)	D-Pyr		35.3±3.6	49.1±3.3	44.4±2.2**
	Pyr		62.3±5.9	86.2±4.30	73.2±3.6*
OVX+ daidzein (5)	D-Pyr		49.1±1.8	49.0±4.6	47.6±3.9
	Pyr		77.8±1.6	76.5±5.1	70.5±3.9

All data are expressed as mean±SEM (nmol/mmol creatinine). Figures in parentheses show the numbers of determinations. OVX, ovariectomy. A sterisks \* and \*\* show significant difference from OVX group at  $p < 0.05$  and  $< 0.02$ , respectively. Blank: not determined.

Figure 1.A

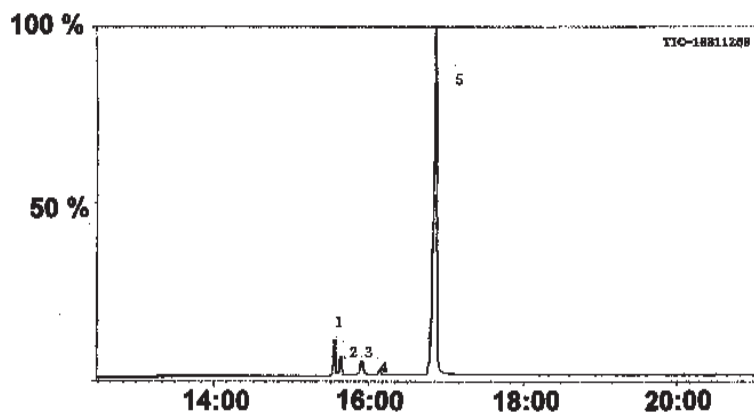


Figure 1.B

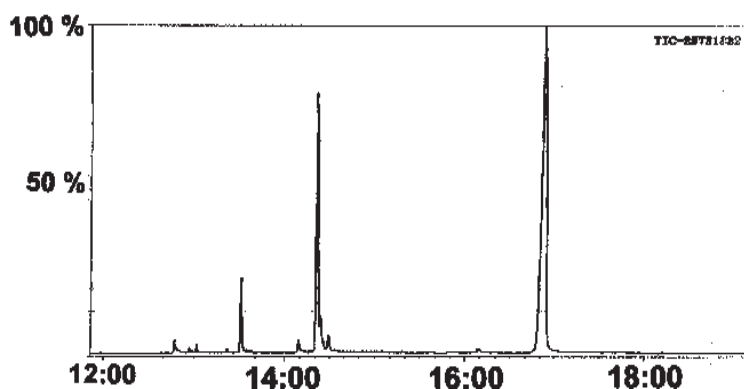


Fig 1 Gas chromatographic analysis of the soybean extract.

A. Separation of authentic samples by gas chromatography; 1, daidzein; 2, genistein derivative 1; 3, genistein derivative 2; 4, genistein derivative 3; 5, coumestrol.

B. Separation of the soybean extract, which was purified by thin layer chromatography.

**Table 4**  
Effects of genistein and daidzein on uterine weight and total cholesterol in ovariectomized rats.

Treatment	Uterine weight (mg/ 100 g body weight)	Total serum cholesterol (mg/ dl serum)
Control (5)	1.85±0.05	80.0±3.9
OVX (5)	0.49±0.03	88.2±2.2
OVX+genistein (5)	0.49±0.02	74.0±3.3*
OVX+daidzein (4)	0.47±0.03	98.6±4.8

All data are expressed as mean ± SEM. Figures in parentheses show the numbers of determinations. OVX, ovariectomy; The asterisk \* shows a statistically significant difference from OVX group at  $p < 0.05$ .

to be isoflavones such as genistein and daidzein, and possibly their glycans (DRAPER et al. 1997; ARJMANDI et al. 1998; SETCHELL 1998; THAM et al. 1998; ISHIMI et al. 1999; ALEKEL et al. 2000). Coumestrol (7,12-dihydroxy-coumestan) has been of minor interest with respect to the human diet. The results of our present study, however, show that the soybean extract containing mainly coumestrol was almost as potent as genistein and daidzein, on the basis of the amount of soybeans consumed, in suppressing urinary excretion of deoxypyridinoline and pyridinoline. These findings suggest that coumestrol, as well as isoflavones found in soybeans, suppress bone resorption in ovariecto-

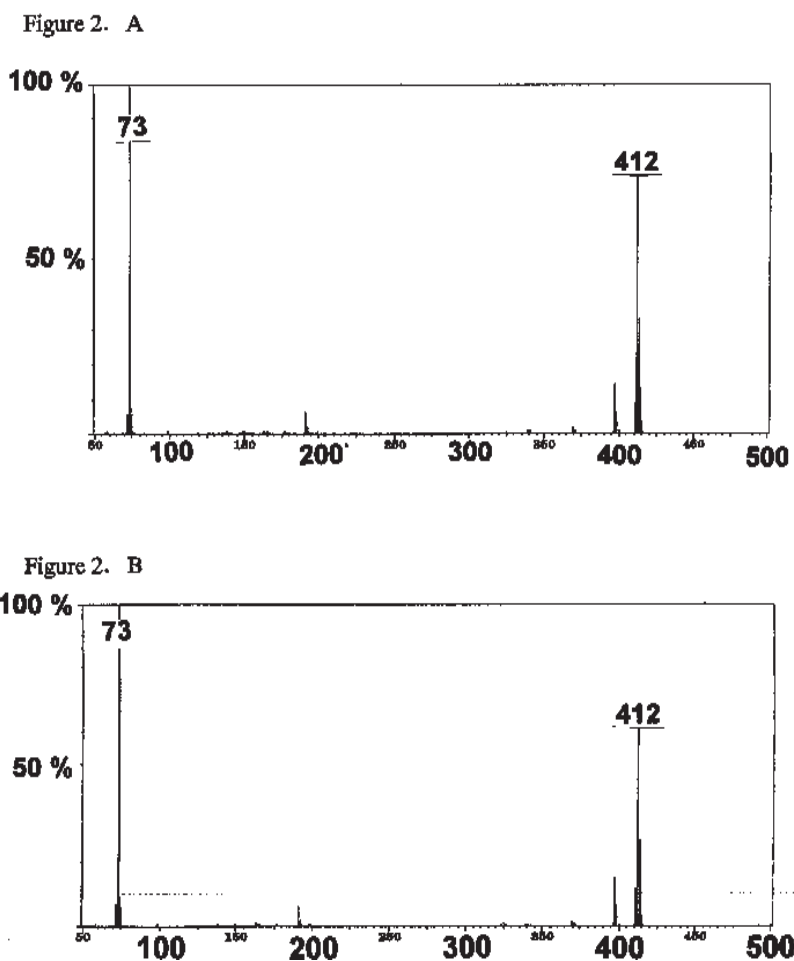


Fig 2 GC-Mass spectrometric analysis of the soybean extract.  
A. Mass spectrogram of authentic coumestrol.  
B. Mass spectrogram of the main peak in Fig.1B.

mized rats. The results further suggest that the bone-protective effect obtained by the consumption of soybeans in humans is not only due to isoflavones but also to coumestrol. Our results, however, do not rule out the possibility that other phytoestrogens, for example 4'-methoxycoumestrol (KURZER and XU 1997), found in soybeans may act as antiosteoporotic agents.

The chromatographic analyses (TLC and GC-MS) of the soybean extract tested in the present study have revealed that it contains mainly coumestrol and only negligible levels of isoflavones. A small amount of coumestrol was also found in Fraction 3 in addition to the extract used (Fraction 4), showing that coumestrol was not completely extracted in Fraction 4.

The maximum levels of daidzein and genistein that could be isolated are 96 and 61 mg/100 g dried soybeans, respectively (VERDEAL and RYAN 1979). In other words, 25 mg of daidzein and genistein can be obtained from 26 and 41 g of soybeans, respectively. The content of coumestrol in soybeans is much less than that of isoflavones; whole soybeans contain only 0.12 mg coumestrol per 100 g (KNUCKLES et al. 1976) or 5 mg/100 g according to LOOKHART et al (1978). In our study, each ovariectomized rat received the extract derived from roughly 60 g of soybeans. If coumestrol was completely extracted in Fraction 4, it could be calculated that 0.07-3 mg of coumestrol per day was given to each ovariecto-



mized rat. However, the actual dose of coumestrol would be somewhat less, as not all coumestrol was extracted in Fraction 4.

The uterotrophic effect of coumestrol, as estimated by the increase in uterine weight of ovariectomized rats, and its antiosteoporotic effect, have been found to be much stronger than those of isoflavones (SOINE 1964; DODGE et al. 1996). The results of our present study also demonstrate that the uterotrophic effect of coumestrol in ovariectomized rats is much stronger than that of the two isoflavones. Although the content of coumestrol in soybeans is much less than that of isoflavones, coumestrol may exert some bone-sparing effect when legumes and their products containing phytoestrogens are consumed.

Coumestrol is a coumarin-like compound with a close structural relationship to stilbestrol as well as to estradiol, and it is found in many different plants (BICKOFF 1960; KURZER and XU 1997; HUTABARAT 2000). It is particularly abundant in sprouted alfalfa and soybeans among vegetables examined (KNUCKLES et al. 1976; REINLI and BLOCK 1996). Japanese people in general consume a fairly large amount of these sprouts in addition to soy products. Populations in the Far East consuming a traditional diet may thus have a significantly higher intake of coumestrol than Westerners. The results of our present study suggest that dietary coumestrol, as well as isoflavones, would be beneficial for the prevention of various diseases caused by estrogen deficiency. A cholesterol-lowering effect of coumestrol has been observed at the dose of 0.1 mg/kg body weight in ovariectomized rats (DODGE et al. 1996) but the soybean extract tested in our study failed to lower serum cholesterol levels.

Coumestrol possesses estrogenic activity approximately 35 times as potent as genistein and has greater estrogenic activity than other derivatives of coumestrol (BICKOFF et al. 1960; SOINE 1964). Coumestrol has a close structural relationship to estradiol and binds to estrogen receptors alpha and beta (SCARLATA and MIKSICEK 1995; HEIKKINEN et al. 1997). It can prevent bone loss in ovariectomized rats with little uterine hypertrophy (DODGE et al. 1996; DRAPER et al. 1997). Not only coumestrol but also its chemical derivative KCA-098 are found to suppress bone resorption both *in vitro* (KAWASHIMA et al. 1996; TSUTSUMI 1995) and *in vivo* (TSUTSUMI et al. 1994).

Rates of osteoporosis differ within populations in different countries, with a lower incidence in Asian women than their Western counterparts. A traditional Oriental diet rich in phytoestrogens is associated with a lower incidence of postmenopausal illness including osteoporosis. For example, Japanese women have a lower risk of hip fracture than Caucasian women (COOPER et al. 1992; ADLERCREUTZ and MAZUR 1997). Recent epidemiological data show that phytoestrogens have a beneficial, rather than a deleterious, effect in humans, e.g. by affording protection against breast and prostate cancer (SETCHELL 1998; STRAUSS et al. 1998; THAM et al. 1998). The uterotrophic effect of coumestrol found in the diet would be minimal, because its content in leguminous plants is much smaller than that of isoflavones. Thus, phytoestrogens including coumestrol and isoflavones may be among the dietary factors affording protective effects against postmenopausal bone loss as well as cancer and cardiovascular disease observed in vegetarians (KNIGHT and EDEN 1996; ADLERCREUTZ and MAZUR 1997).

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