

Biological Activity of Phenolic Compounds in Seeds and Leaves of Safflower (*Carthamus tinctorius* L.)

Won-Jung Lee¹, Sung-Hee Cho, Jun-Young Lee and Sang-Won Choi[†]

Department of Food Science and Nutrition, Catholic Univeristy of Daegu, Hayang, Gyeongbuk 712-702, Korea

¹*Department of Physiology, School of Medicine, Kyungpook National University, Daegu 700-422, Korea*

Abstract

Biological activity of phenolic compounds in seeds and leaves of safflower (*Carthamus tinctorius* L.) were evaluated using several *in vitro* and *in vivo* assays. Six phenolic constituents were isolated from the seeds and identified as *N*-feruloylserotonin, *N*-(*p*-coumaroyl)serotonin, matairesinol, 8'-hydroxyarctigenin, acacetin 7-*O*- β -D-glucoside (tilianine) and acacetin. Six phenolic compounds exhibited considerable antioxidative activity, and especially two serotonins showed potent DPPH radical scavenging activity and antiperoxidative activity against rat liver microsomal lipid peroxidation induced by the hydroxyl radical generated *via* a Fenton-type reaction. Additionally, six phenolic compounds possessed comparable cytotoxicity against three cancer cells, Hela cell, MCF-7 and HepG2 cell, and particularly acacetin and its glycosides had the most potent cytotoxicity. Moreover, we found that feeding safflower seeds attenuated bone loss, and lowered levels of plasma and liver lipids in ovariectomized rats. Serotonins, lignans and flavones stimulated proliferation of the osteoblast-like cells in a dose-dependent manner (10^{-15} - 10^{-6} M), as potently as E₂ (17 β -estradiol). Particularly, serotonins were mainly responsible for bone-protecting and lipid lowering effects in ovariectomized rats. Meanwhile, eight flavonoids, including a novel quercetin-7-*O*-(6''-*O*-acetyl)- β -D-glucopyranoside and seven known flavonoids, luteolin, quercetin, luteolin 7-*O*- β -D-glucopyranoside, luteolin-7-*O*-(6''-*O*-acetyl)- β -D-glucopyranoside, quercetin 7-*O*- β -D-glucopyranoside, acacetin 7-*O*- β -D-glucuronide and apigenin-6-*C*- β -D-glucopyranosyl-8-*C*- β -D-glucopyranoside were first isolated and identified from safflower leaf. Among these flavonoids, luteolin-acetyl-glucoside and quercetin-acetyl-glucoside showed potent antioxidative activities against 2-deoxyribose degradation and lipid peroxidation in rat liver microsomes. Luteolin, quercetin, and their corresponding glycosides also exhibited strong antioxidative activity, while acacetin glucuronide and apigenin-6,8-di-*C*-glucoside were relatively less active. Finally, changes in phenolic compositions were also determined by HPLC in the safflower seed and leaf during growth stages and roasting process to produce standardized supplement powders. These results suggest that phenolic compounds in the roasted safflower seed and leaf may be useful as potential sources of therapeutic agents against several pathological disorders such as carcinogenesis, atherosclerosis and osteoporosis.

Key words: Safflower(*Carthamus tinctorius* L.) seed and leaf, phenolic constituents, antioxidant, cytotoxicity, antiosteoporosis, lipid lowering activity

INTRODUCTION

Antioxidants play an important role in protection cell membranes against damages caused by active oxygen species, which are reportedly associated with several pathologic conditions, including carcinogenesis, mutagenesis, atherosclerosis, and aging (1,2). Apart from synthetic antioxidants which are suspected to toxic effects, dietary natural antioxidants such as L-ascorbic acid, α -tocopherol, and several phenolic compounds have been found to play considerable roles in prevention above pathological diseases as scavenger or quencher of active oxygen radicals (3,4).

Recently, much attention has been received on the development of naturally occurring phytoestrogens (PEs) whose structures and molecular weights are similar to steroid estrogens and that possess some estrogenic and/or anti-estrogenic effects either inherently or after conversion by intestinal flora (5,6). Isoflavones in soybeans and lignans in flaxseeds are well-known as phytoestrogens that played important physiological roles in prevention of several cancers, osteoporosis and coronary heart disease (7,8). At present, synthetic estrogen (17β - estradiol, ethinylestradiol) replacement therapy has been widely used for the prevention and treatments of hypertension and osteoporosis of postmenopausal women caused by estrogen deficiency. However, some women are reluctant to take synthetic estrogen replacements because of potentially increased cancer risks (9,10). For these reasons, much extensive search for safe and efficacious novel antioxidants and phytoestrogens has been undertaken.

Safflower (*Carthamus tinctorius* L. Compositae) seed, which is rich source of linoleic acid, has been currently used for the treatment of cataclasis, osteoporosis and rheumatism in Korea. However, their effects has not been scientifically evaluated. Recently, safflower seed powder and extract were found to heal bone fracture (11-13) and improve lipid metabolism in high fat and high cholesterol-fed rats (14-16). Several phenolic compounds, including serotonins, lignans and flavones have been isolated and identified from the seeds (17,18) and flower petals (19). Among phenolic compounds, serotonin derivatives were shown to possess strong antioxidative (20-22), antiinflammatory (23) and growth-promoting activities (24). Additionally, lignans and flavone were also known to exert significant antioxidative and cytotoxic activities (22,25-27). However, few phytochemical studies on antiosteoporosis and antiatherosclerotic actions of the seeds have not been reported. Recently, we found that the defatted safflower seeds markedly attenuated bone loss in ovariectomized rats (28), and phenolic compounds played major roles in prevention of osteoporosis and atherosclerosis in ovariectomized rats (29,30). Thus, safflower seeds are renewed interest as potential sources of therapeutic agents against pathological diseases. Apart from beneficial effects of the seed, utilization of safflower meal as feed and food has been partially limited by some factors such as bitter taste (31) and cathartic effect (32,33). However, recent studies revealed that roasting and steaming process could be mitigated the deleterious effects in fresh safflower seeds and also enhanced antioxidative activity of the seed extract (18,25). Until now, despite many studies have

been reported on the chemistry and biological actions of the safflower seeds and flowers, few phytochemical studies on the safflower leaf, which is used as wild edible green, are available. As a part of a continuing search for natural antioxidants, we previously found that the methanolic extract of the safflower leaf had strong antioxidative activity, and the activity was increased in relation to the roasting and steamming process (34). Eight flavonoids have been isolated and identified from safflower leaf as active principles of antioxidative activity (35). In this study, we summarized several previous studies on biological actions such as antioxidative, cytotoxic, antiosteoporotic and lipid lowering activities of phenolic constituents isolated from safflower seed and leaf. Additionally, changes in phenolic compounds were analysed by HPLC during growth stage and roasting process of safflower seeds.

MATERIALS AND METHODS

Materials and reagents

Safflower seeds and leaves were harvested from the farm in Uisong, Gyeongbuk, Korea. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), 2-deoxyribose, H₂O₂, 17 β -estradiol, genistein and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). FeSO₄ · 7H₂O, L-ascorbic acid, α -tocopherol, and butylated hydroxyanisole (BHA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Several tissue culture media and reagents were purchased from Gibco BRL, Life Technologies (Gland island, NY, USA). All other reagents used for this study were of analytical grade.

Extraction, isolation and identification of phenolic compounds

Phenolic compounds including serotonins, lignans and flavonoids were isolated and identified from safflower seed and leaf, as described previously (21,35).

HPLC analysis

HPLC for analysis of phenolic compounds in safflower seed and leaf was performed on a Gilson 506B HPLC System coupled with Gilson 170 UV-vis detector, and Gilson 231 XL autosampler with a 10 μ l loop. HPLC analysis was carried out using a YMC Pack Pro C18 column (5 μ m, 46 \times 250 mm, YMC Inc., USA) with Guard-Pak HPLC precolumn inserts. The separation was conducted using a linear gradient from 0.1% v/v H₃PO₄ in 20% MeOH to 80% MeOH for 40 min at a flow rate of 1.0 ml/min with detection at UV_{280,305,350nm}. Peaks were identified by co-chromatography with authentic samples isolated previously.

Antioxidative activity

(1) DPPH radical scavenging activity

DPPH radical scavenging activity was determined by slightly modified method of Brand-Williams *et al.* (36).

(2) 2-Deoxyribose degradation and lipid peroxidation assay

Two *in vitro* assays for evaluation of antioxidative activity of isolated phenolic compounds were performed by method described previously (37). All experiments were performed in triplicates.

Cell lines

HepG2 human hepatocellular carcinoma cell, MCF7 human breast adenocarcinoma cell, and Hela human cervix adenocarcinoma cell lines, as well as human normal liver cell WRL68 were kindly provided by Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea), and Korea Cell Line Bank, respectively (Seoul, Korea).

Cytotoxicity assay

Cytotoxicity assay was carried out according to slightly modified original MTT colorimetric assay described previously (26). Four replicate wells were used to obtain data, and all experiments were performed in triplicates. A curve plotting concentration against percentage proliferation was used to calculate half-maximal proliferation concentration (IC_{50}).

Animals and Diets

Female Sprague-Dawley rats weighing 165 ± 10 g were obtained from the Korean Center for Experimental Animals and maintained with standard chow in a facility equipped with stainless steel cages having wire mesh bottoms and constant temperature ($22 \pm 1^\circ\text{C}$) and lighting (light on, 08:00-20:00 h) for 2 weeks. Sixty rats were bilaterally ovariectomized and ten rats were sham operated under pentobarbital anesthesia (40 mg/kg, ip). After the surgery, ovariectomized rats were then randomly allocated to six groups (each $n=10$); ovariectomized control (OVX), 17-estradiol-treated (E_2), 15% (w/w) (SP15) and 30% (w/w) defatted safflower seed powder treated (SP30), safflower seed extract I (SE I, crude EtOH ext.) and II treated (SE II, EtOAc fr.) groups. All seven groups of rats including the sham group were fed experimental diets (Table 1) and had free access to tap water for 4 weeks. Four ovariectomized groups treated with safflower seed powder or extracts were pair-fed to the OVX group. In the experimental diets, the defatted safflower seed powder was added at a level of 15 or 30% (w/w) and contents of starch and casein were adjusted as in the Table 1, considering contents of starch, protein and fiber as 43, 24 and 33%, respectively (11). SE I and SE II were added to diets at the level of 1% (w/w). The E_2 group was subcutaneously injected with 50 μg of E_2 dissolved in soybean oil twice per week. Food intake was measured daily and body weight every three days. All animal procedures were reviewed and approved by the Catholic University of Daegu Intramural Animal Care and Use Committee.

Table 1. Composition of experimental diets

(g/kg diet)

Ingredient	Group			
	Sham OVX, E2 ^a	SP15	SP30	SE I SE II
Casein	150	114	78	150
Corn starch	485	436	387	485
Sucrose	150	150	150	150
Lard	40	40	40	40
Soybean oil	75	75	75	75
Mineral mix ^b	40	40	40	40
Vitamin mix ^c	10	10	10	10
Cellulose	50	-	-	50
SP	-	150	300	-
SE I, SE II				10
Kcal/kg	4175	4113	3867	4134

^aFifty μ g of 17-estradiol in soybean oil was injected subcutaneously twice a week for 4 weeks.

^bAIN-76 mineral mix.

^cAIN-76 vitamin mix.

SP, powder of defatted safflower seeds.

SE I & SE II, methanol and ethylacetate extract of defatted safflower seeds, respectively, as described in Materials and Methods.

Preparation of sample for lipid analysis and determination

Preparation procedure of sample for lipid analysis and levels of total cholesterol, HDL-cholesterol, triglyceride level were performed as described in the previous report (15).

Scanning electron microscopy

To evaluate three dimensional changes in the amount and structure of the trabecular bone, the proximal portion of the right tibias of three rats in each experimental group was processed for scanning electron microscopy, as described previously (28).

Tissue preparation and histomorphometry

The histomorphometric analysis of trabecular bone amount and adipocyte content were carried out by previous method (28).

Statistical analysis

Some data were represented as mean \pm SE. The data were analyzed statistically using the SPSS for Windows statistical program (SPSS, Chicago, IL). One-way ANOVA with Tukey's multiple test was used

for the animal study and Student's *t*-test was used for the cell study. A probability of less than 5% was required for significance.

RESULTS AND DISCUSSION

Identification of phenolic compounds in safflower seed and leaf

The ethylacetate soluble material from the defatted methanol extract of roasted safflower seeds was subjected to repeated chromatographic separation on silica gel, Sephadex LH-20 and reverse-phase HPLC to yield six phenolic compounds, *N*-feruloylserotonin, *N*-(*p*-coumaroyl)-serotonin], matairesinol, 8'-hydroxyarctigenin, acacetin and acacetin 7-*O*- β -D-glucoside (tilianine), in the pure state (22). The structures of the known compounds have been assigned on the basis of their mass, UV and NMR spectral data in comparison with data reported in the literature. In particular, acacetin 7-*O*- β -D-glucoside was first isolated from the seeds, although five phenolic compounds have already been reported in the roasted safflower seeds.

Meanwhile, the *n*-butanol fraction of the boiling water extract from the safflower leaf was chromatographed successively on silica gel, Sephadex LH-20 and reverse-phase silica gel to give pure eight flavonoids (35). The known seven flavonoids, quercetin, luteolin, quercetin 7-*O*- β -D-glucopyranoside, luteolin 7-*O*- β -D-glucopyranoside, luteolin-7-*O*-(6''-*O*-acetyl)- β -D-glucopyranoside, acacetin-7-*O*- β -D-glucuronide and apigenin-6,8-*di*-*C*-glucopyranoside, were identified by comparison of their ¹H- and ¹³C-NMR spectra with literature data. In particular, quercetin- 7-*O*-(6''-*O*-acetyl)- β -D-glucopyranoside was first isolated and identified from plant sources.

Antioxidative activity of phenolic compounds

The DPPH radical scavenging activity of six phenolic compounds isolated from the roasted safflower seeds is shown in Table 2. Among six phenolic compounds, two serotonins, the predominant components of the seeds, possessed more potent radical scavenging activity than that of α -tocopherol, BHA and L-ascorbic acid, well-known reference antioxidants. Two lignans also exhibited stronger activity, while two flavonoids were less considerable activity. This observation supports previous reports that phenolic compounds containing NH and OH functional group have strong hydrogen donating or radical scavenging ability, and the methoxylation and glycosylation of the hydroxyl group in polyphenols causes a decrease in activity.

Table 2. DPPH radical scavenging activity of six phenolic compounds isolated from the roasted safflower seeds

Compound	IC ₅₀ (µM)
<i>N</i> -Feruoylserotonin	6.4
<i>N</i> -(<i>p</i> -Coumaroyl)serotonin	9.2
Matairesinol	52.6
8'-Hydroxyarctigenin	71.4
Acacetin 7- <i>O</i> -β-D-glucoside	102.1
Acacetin	95.8
L-Ascorbic acid	34.3
α-Tocopherol	27.5
BHA	38.4

IC₅₀ represents the sample concentration at which the absorbance shows 50% of control.

Values are shown as the mean of *n*=3.

Standard deviation is omitted for simplicity.

L-Ascorbic acid, α-tocopherol and BHA are used as positive references.

Moreover, the hydroxyl radical of several active oxygen radicals has a high and indiscriminate activity and can slowly cause severe damage to susceptible biomolecules. However, this damage can be modulated by dietary antioxidant phenolic compounds. Although numerous studies have been reported on hydroxyl radical ($\cdot\text{OH}$) scavenging activity of phenolic compounds including flavonoids, information on the $\cdot\text{OH}$ scavenging activity of serotonins, lignans and acetyl flavonoids remains very limited. Antioxidative activity of six phenolic compounds in rat liver microsome system is shown in Table 3. Two serotonins and acacetin exhibited much stronger antioxidative activity than that of α-tocopherol, a reference compound. Matairesinol also showed significant activity comparable to that of α-tocopherol. However, 2-hydroxyarctigenin and tilianine showed weaker activity than their corresponding matairesinol and acacetin, indicating that addition of hydroxyl and glucosyl group to matairesinol and acacetin decreased antioxidative activity due to low affinity of them for the lipid membranes. It is well known that hydroxyl radical is formed by the addition of H₂O₂ and Fe²⁺ in biological systems. Therefore, these findings suggest that the potent inhibitory effects of two serotonins, acacetin and matairesinol on lipid peroxidation induced by H₂O₂ and Fe²⁺ in microsomes might be partly due to the action of hydroxyl radical scavengers. Recently, Roh et al. (25) reported that two serotonins in the roasted safflower seeds markedly inhibited the lipid peroxidation induced by ascorbic acid/Fe²⁺ and ADP/Fe³⁺/NADPH in rat liver microsomes. Based on these results, the strong antioxidative activity of serotonin derivatives, lignans and flavones has been suggested to explain the claimed beneficial effects of safflower seeds against degenerative diseases induced by active oxygen radicals.

Table 3. Inhibitory effects of six phenolic compounds isolated from the roasted safflower seeds on lipid peroxidation induced by H₂O₂ and FeSO₄ in rat liver microsome

Compound	IC ₅₀ (μM)
N-Feruoylserotonin	1.3
N-(<i>p</i> -Coumaroyl)serotonin	1.5
Matairesinol	2.3
8'-Hydroxyarctigenin	7.6
Acacetin 7- <i>O</i> -β-D-glucoside	10.8
Acacetin	1.2
α-Tocopherol	4.4

IC₅₀ represents the sample concentration required for 50% inhibition of lipid peroxidation.

Values are shown as the mean of *n*=3.

Standard deviation is omitted for simplicity.

α-Tocopherol is used as a positive reference.

Meanwhile, eight flavonoids including acetyl flavonoids isolated from safflower leaf were tested for their antioxidant properties using hydroxyl radical-induced 2-deoxyribose degradation and lipid peroxidation of rat liver microsomes, and the results are shown in Table 4. All flavonoids acted as a hydroxyl radical scavenger, and significantly inhibited 2-deoxyribose degradation and lipid peroxidation in a dose-dependent manner. Among these flavonoids, quercetin-acetyl- glucoside and luteolin-acetyl- glucoside exhibited the most potent antioxidative activities in two *in vitro* antioxidative model systems; in particular, their activities were comparable to that of α- tocopherol, a reference antioxidant against the lipid peroxidation in rat liver microsomes. In addition, luteolin, quercetin and their corresponding glycosides showed a strong hydroxy radical scavenging activity without big differences in scavenging efficiencies among them. However, acacetin glucuronide and apigenin glycoside had a weaker activity than those of the six other flavonoids. Thus, it is interesting to note that the presence of the 6''-acetyl group increases the hydroxy radical scavenging activity in the lipid membrane model, although the participation of the 6''-acetyl group in the antiperoxidative and superoxide scavenging activities of flavonoids has already been established in previous work. Moreover, it was found that the glycosylation of the hydroxyl group in flavonoids decreased antioxidative activity due to a low affinity for biological lipid membranes. From above data, a comparison of hydroxyl radical scavenging activity between flavones and flavonols demonstrated the importance of the presence of the acetyl group and the *ortho*-catechol moiety in ring B, irrespective of the addition of a hydroxyl group at the C-3 position. Thus, these results suggest that the safflower leaf containing peculiar acetyl flavonoid glycosides, together with well-known antioxidative flavonoids, luteolin and quercetin, may be useful as a potential source of natural antioxidant capable of inhibiting a variety of pathological diseases induced by peroxidation of membrane lipids. Further *in vivo* study on antioxidative activity of phenolic compounds isolated from safflower seed and leaf is needed.

Table 4. Inhibitory activity of flavonoids 1-8 from safflower leaf against 2-deoxyribose degradation and rat liver microsomal lipid peroxidation induced by hydroxyl radical generated via a Fenton-type reaction

Compound	IC ₅₀ (μM)	
	2-Deoxyribose	Lipid peroxidation
Comp. 1	3.02 ^c	1.23 ^c
Comp. 2	3.77 ^b	1.11 ^c
Comp. 3	2.98 ^c	2.87 ^b
Comp. 4	2.91 ^c	0.95 ^c
Comp. 5	3.81 ^b	2.66 ^b
Comp. 6	3.73 ^b	0.85 ^c
Comp. 7	8.42 ^a	5.64 ^a
Comp. 8	8.67 ^a	6.43 ^a
α-Tocopherol		0.98 ^c

IC₅₀ values represent mean of three independent determinations.

Standard deviation is omitted for simplicity.

Values with the different superscript letter in each column are significantly different at $P < 0.05$.

α-Tocopherol is used as a positive reference.

Comp. 1, luteolin; Comp. 2, quercetin; Comp. 3, luteolin 7-*O*-β-D-glucopyranoside; Comp. 4, luteolin-7-*O*-(6''-*O*-acetyl)-β-D-glucopyranoside; Comp. 5, quercetin 7-*O*-β-D-glucopyranoside; Comp. 6, quercetin-7-*O*-(6''-*O*-acetyl)-β-D-glucopyranoside; Comp. 7, acacetin 7-*O*-β-D-glucuronide; Comp. 8, apigenin-6-*C*-β-D-glucopyranosyl-8-*C*-β-D-glucopyranoside.

Cytotoxicity of phenolic compounds

Cytotoxicities of phenolic compounds isolated from safflower seed and leaf against three cancer and normal cell lines are shown in Table 5. All compounds exerted considerable cytotoxicity against cancer cell lines in a dose-dependent manner. Among phenolic compounds, quercetin, luteolin and acacetin exhibited the most potent cytotoxicity against three cancer cells with IC₅₀ values of 56.6 & 58.4 μg/mL for Hela cell, 37.7 & 38.2 μg/mL for MCF-7 cell and 62.1 & 67.5 μg/mL for HepG2 cell, respectively. In addition, two lignans and two serotoninins showed strong cytotoxicity, although their activity was lower than that of three flavonoids. This findings indicated that the strong cytotoxicity of the above EtOAc fraction against three cancer cell lines was mainly responsible for flavonoids, and other serotoninins and lignans to some extent. Meanwhile, two serotoninins and lignans did not have nearly cytotoxicity, and two flavonoids except for quercetin showed only about 50% cytotoxicity against a normal human liver cell (WRL68) at concentration of 100 μg/mL (26). Thus, phenolic compounds exhibited much higher selective cytotoxicity toward cancer cell lines than normal cell line. Serotoninins are known to act as a messenger in the brain and also as a hormone in the intestine. However, the cytotoxic effect of serotoninins against cancer cell lines is still very limited. This is the first report on cytotoxicity of serotonin derivatives against Hela, MCF-7 and HepG2. In particular, matairesinol, which is a diphenolic compound found in flaxseed, is well-known as phytoestrogen which play important physiological roles in inhibition of hormone-dependant several cancers (8). In addition, quercetin, luteolin and acacetin also have been

reported to have several biological effects, such as anti-carcinogenic, anti-inflammatory and antioxidative activity.

Table 5. Cytotoxicity of six phenolic compounds isolated from safflower seeds on three different cancer cell lines

Compound	IC ₅₀ (µg/ml) [*]		
	HepG2	Hela	MCF-7
S1	89.4	58.0	51.8
S2	90.7	56.4	52.1
L1	65.5	55.5	52.1
L2	74.5	56.0	55.2
F1	67.5	58.4	38.2
F2	62.1	56.6	37.7

*IC₅₀ represents the concentration of a compound required for 50% inhibition of growth of cancer cell.

Values are shown as the mean of *n*=3..

Standard deviation is omitted for simplicity.

S1, *N*-Feruoylserotonin; **S2**, *N*-(*p*-coumaroyl)serotonin; **L1**, matairesinol; **L2**, 8'-hydroxy- arctigenin; **F1**, acacetin 7-*O*-β-D-glucoside; **F2**, acacetin.

Lipid lowering effects of phenolic compounds

Previously, the defatted methanol extract and ethylacetate soluble fraction from safflower seeds were found to have lipid lowering effects through reducing plasma and liver cholesterol and triglyceride levels (15). Furthermore, effects of phenolic compounds isolated from safflower seed on the levels of plasma and liver lipids in ovariectomized female rats fed high cholesterol diets for 4 weeks are investigated and presented in **Table 6**. Plasma levels of total cholesterol were not different among ovx-control (114.8±8.2 mg/dL) and sham (106.3±4.7 mg/dL) but that lower in ovx-lignans (85.3±4.7 mg/dL), ovx-flavones (89.7±4.9 mg/dL) and ovx-serotonins (80.1±3.2 mg/dL). HDL-cholesterol levels of ovx-safflower component groups had a tendency to increase compared to sham and ovx-control groups, but were not significantly different. However, the ratio of HDL to total cholesterol in the ovx-lignans and the ovx-flavones groups was increased by 30~40%, and especially that of the ovx-serotonins group was increased by 58% compared to ovx-control and sham groups. Additionally, plasma triglyceride levels in the ovx-safflower component groups were reduced by 10~17% compared to the ovx-control group. Meanwhile, safflower component groups had little effect on levels of liver cholesterol and triglyceride, but the ovx-flavonoids group, and the ovx-safflower lignans and flavonoids groups showed tendency to reduce levels of liver cholesterol and triglyceride, respectively.

Table 6. Effects of phenolic compounds isolated from safflower seeds on the levels of plasma and liver lipids in ovariectomized female rats fed high cholesterol diets for 4 weeks

	Sham	OVX-control	OVX + lignans	OVX + flavonoids	OVX + serotoninins
Plasma lipid (mg/dL)					
Total cholesterol	106.3±4.7 ^{ab}	114.8±8.2 ^a	85.3±4.7 ^b	89.7±4.9 ^b	80.1±3.2 ^b
HDL-cholesterol	27.2±6.8	30.1±2.7	32.2±2.4	35.2±3.8	35.1±2.3
Triglyceride	60.5±5.4 ^b	62.2±5.4	56.2±2.8 ^b	56.1±6.8	70.1±6.3
HDL-/total cholesterol	26.4±8.6 ^b	27.6±9.4 ^b	37.8±9.1 ^{ab}	39.6±13.3 ^{ab}	43.7±14.0 ^a
Liver lipid (mg/g)					
Cholesterol	31.7±2.6 ^b	39.5±2.8 ^a	41.4±2.1 ^a	34.9±2.9 ^{ab}	38.5±2.3 ^a
Triglyceride	38.5±4.1 ^b	54.5±6.3 ^a	47.8±2.6 ^b	45.2±5.6 ^{ab}	61.7±4.5 ^a

OVX-control: Phenolic compound-free group in ovariectomized rats.

OVX+lignans: Lignans (L1 + L2) treated group.

OVX+flavonoids: Flavonoids (F1 + F2) treated group.

OVX+serotoninins: Serotoninins (S1 + S2) treated group.

Values are mean ±SD (*n*=10) and those in the same column not sharing common superscript letters are significantly different at *p*<0.05 by Tukey's test.

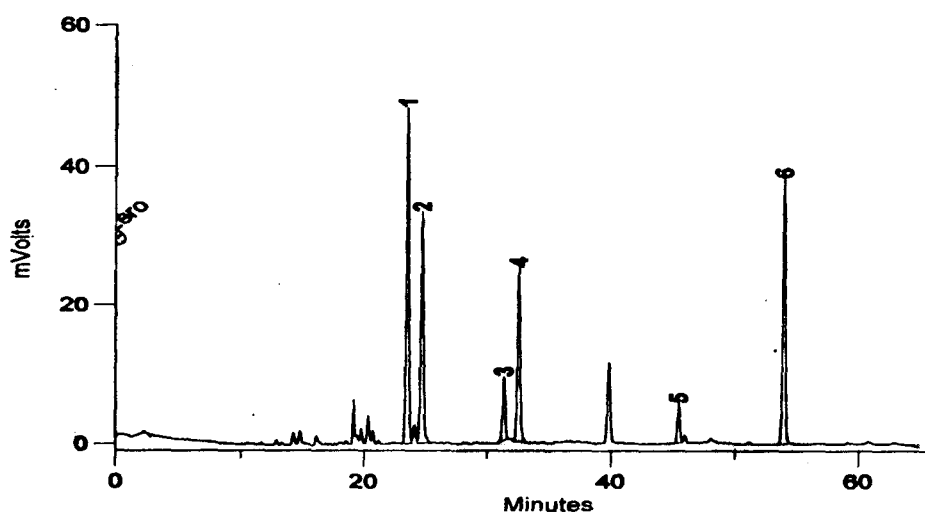


Fig. 1. HPLC chromatogram of ethylacetate fraction from the defatted methanol extracts of roasted safflower seed.

1, *N*-feruoylserotonin; 2, *N*-(*p*-coumaroyl)serotonin; 3, matairesinol; 4, 8'-hydroxyarctigenin; 5, tilianine; 6, acacetin.

HPLC condition: column, YMC Pack Pro C18 column (5µm, 46 × 250 mm, YMC Inc., USA); linear gradient elution from solvent A (0.1% H₃PO₄ in 20% MeOH) to solvent B (80% MeOH) for 60 min; flow rate, 1.0 ml/min; detection, UV_{280nm}.

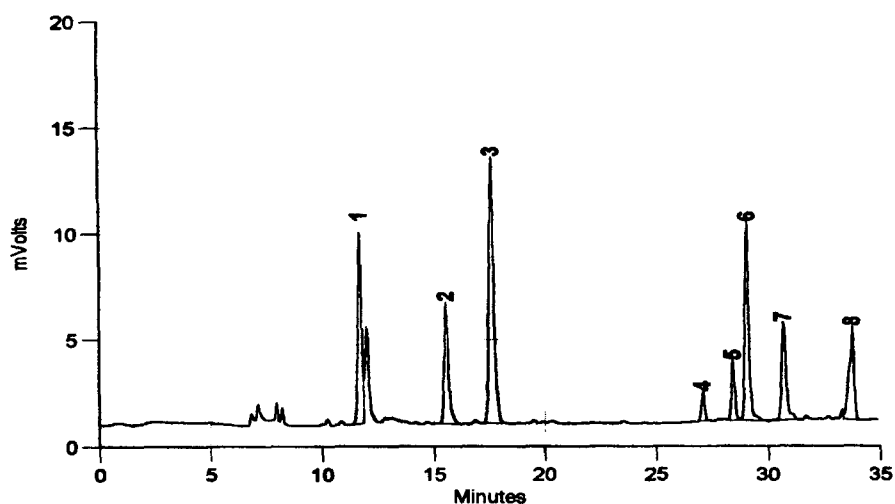


Fig. 2. HPLC chromatogram of *n*-butanol fraction from the boiling water extracts of safflower leaf.

1, apigenin-6-*C*- β -D-glucopyranosyl-8-*C*- β -D-glucopyranoside; 2, quercetin 7-*O*- β -D-glucopyranoside; 3, luteolin 7-*O*- β -D-glucopyranoside; 4, quercetin-7-*O*-(6''-*O*-acetyl)- β -D-glucopyranoside; 5, luteolin-7-*O*-(6''-*O*-acetyl)- β -D-glucopyranoside; 6, quercetin; 7, luteolin; 8, acacetin 7-*O*- β -D-glucuronide. HPLC condition: column, YMC Pack Pro C18 column (5 μ m, 46 \times 250 mm, YMC Inc., USA); linear gradient elution from solvent A (0.1% H₃PO₄ in 20% MeOH) to solvent B (80% MeOH) for 60 min; flow rate, 1.0 ml/min; detection, UV_{340nm}.

Histological evaluation: Scanning electron microscopy and H-E staining

Photographs of SEM and H-E staining of representative tibia are shown in Fig. 3 and 4, respectively. The amount and structure of the secondary spongiosa area of the metaphysis was markedly different between the sham and the Ovx-C groups. In contrast to dense and well-formed trabecular bone of the sham group, Ovx caused a dramatic reduction in the trabecular bone in the central metaphyseal region below the primary spongiosa. Trabecular bone remaining in the central metaphyseal region of the Ovx-C group exhibited thin rod-like structure while more plate-like trabecular bone remained in peripheral region near the endocortical surface. Administration of E₂ to the Ovx animals completely prevented the trabecular bone loss, whereas feeding defatted safflower seed powders (SP15, SP30) or extracts (SE1, SE2) partially prevented bone loss. Although the amount of the trabecular bone in the central metaphyseal region was less in the four safflower seed groups than in the Ovx+E₂ or sham group, it was much greater than in the Ovx group. The amount and structure of the trabecular bone were similar among the four safflower seed groups.

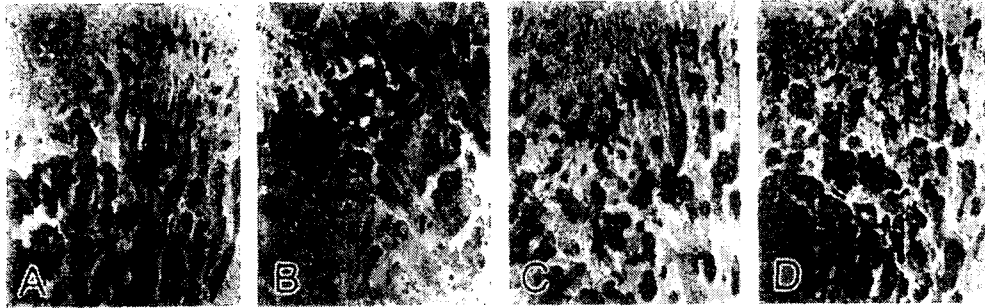


Fig. 3. Scanning electron micrographs of the proximal tibial hemisections from sham-operated rats (A) and ovariectomized (OVX) rats untreated (B) or treated with 17 β -estradiol (C), and defatted safflower seed powder (D) for 4 weeks. Original magnification $\times 20$.

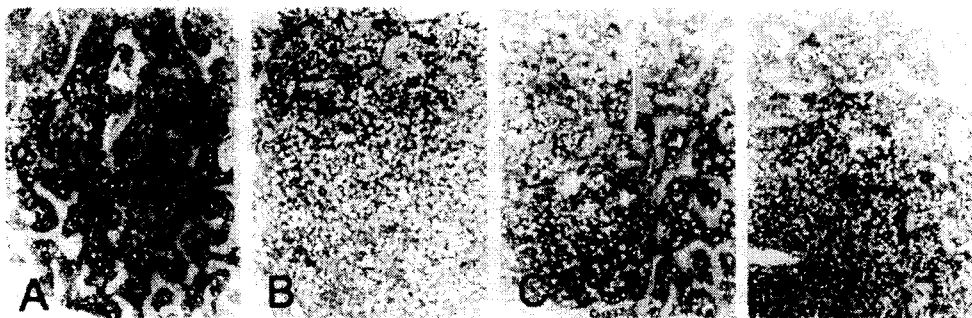


Fig. 4. Photomicrographs of proximal tibia metaphysis from sham-operated rats (A), and ovariectomized (OVX) rats untreated (B) or treated with 17 β -estradiol (C) and defatted safflower seed powder (D) for 4 weeks. Sections are stained with Hematoxylin-Eosin. Original magnification $\times 58$.

Histomorphometry

Histomorphometrical analysis showed that the trabecular bone density of the proximal metaphysis of the tibia in the OVX group and the four safflower seed groups were 41% and 75-87% of the sham group, respectively (Fig. 5-A). Trabecular bone density levels of four safflower seed and the E₂ groups were nearly 2- and 3-fold higher than that of the OVX group, respectively. The four safflower seed groups exhibited very similar trabecular bone density values, and the SP30 group showed the highest level among them. The amount of osteoid bone in the OVX and E₂ groups was markedly reduced to one third of the sham level (Fig. 5-C). In contrast, the amount of osteoid bone was maintained at the sham level in the SP15 and SP30 groups and was about 75% of the sham level in the SE I and SE II groups. Adipocyte density in the bone marrow increased over 60% in the OVX group, but decreased 30% in the E₂ group when compared with the sham group (Fig. 5-D). However, the four safflower seed groups showed similar marrow adiposity to that of the sham group.

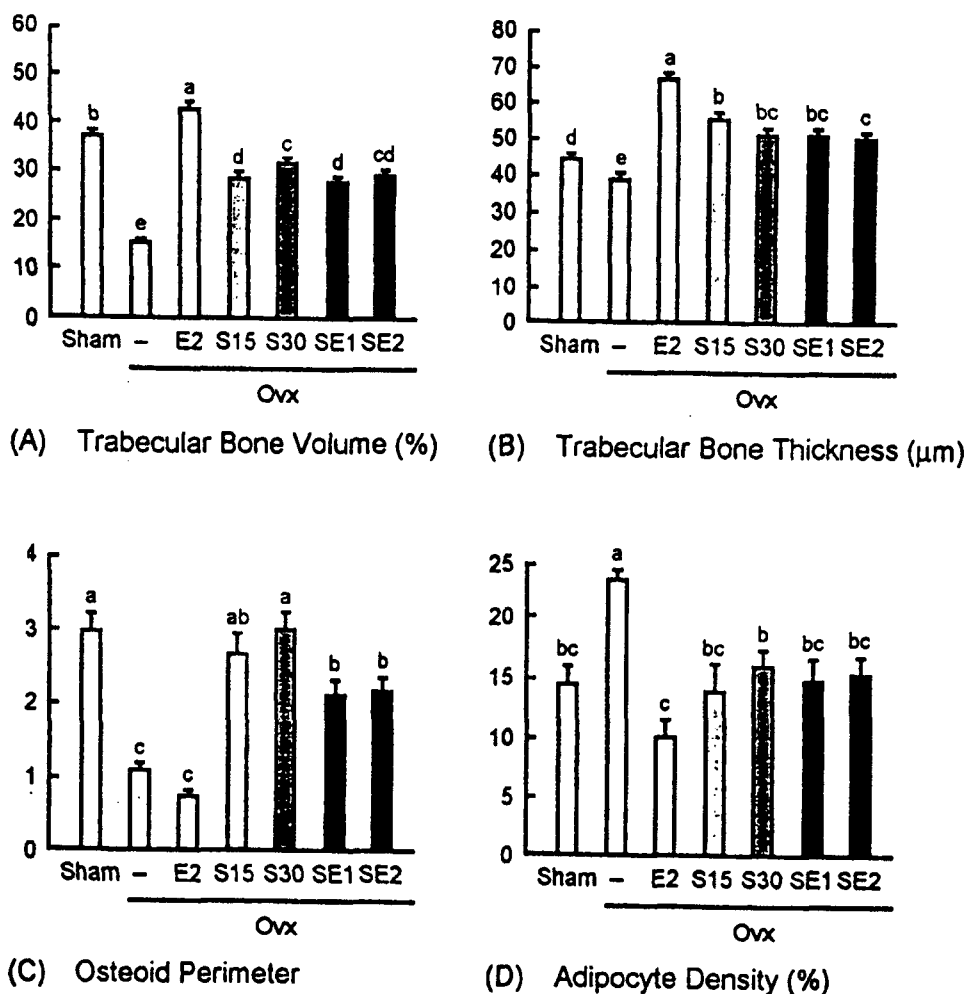


Fig. 5. Histomorphometrical analysis of trabecular bone density (A), bone thickness (B), osteoid perimeters (C) and adipose tissue content (D) of the tibia of sham-operated and ovariectomized (OVX) rats untreated or treated with 17β -estradiol (E2) and defatted safflower powder (S15, S30, SE1, SE2) for 4 weeks. Different superscript letters show significantly differences at $P < 0.05$ by Tukey's test.

Effect of phenolic compounds on osteoblasts proliferation

To elucidate the underlying mechanisms of the beneficial effects of safflower seeds on the bone, effects of six phenolic compounds isolated from the defatted safflower seeds on the proliferation of ROS 17/2.8 osteoblast-like cells were investigated. Six phenolic compounds, lignans, flavones, and serotonin derivatives, stimulated proliferation of ROS 17/2.8 cells in a dose-dependent manner (Fig. 6). All tested phenolic compounds profoundly increased proliferation of the cells even at a very low concentration of 10^{-15} M. Particularly, lignans and flavones showed slightly higher effect than serotonin derivatives, but the effects of all three compounds were comparable with the E_2 effect. We recently demonstrated that phenolic compounds in safflower seeds could be acted as phytoestrogens which produced a rapid activation of mitogen activated protein (MAP) kinase, at least partially via membrane estrogen receptor of the cultured osteoblastic cells (38).

In summary, phenolic compounds such as serotonins, lignans and flavones isolated from safflower seeds possessed considerable antioxidative and lipid lowering effects, as well as prevented bone loss in

ovariectomized rats. Particularly, *in vitro* study with separated three phenolic compounds showed that lignans and flavones, two main kinds of phytoestrogens, as well as serotonin derivatives stimulated proliferation of osteoblast-like cells even at a very low concentration. Therefore, the bone-protecting and lipid lowering effects of safflower seeds seem to be attributed to their phenolic compounds, and intake of safflower seeds may be useful in preventing bone loss and coronary heart disease caused by estrogen deficiency.

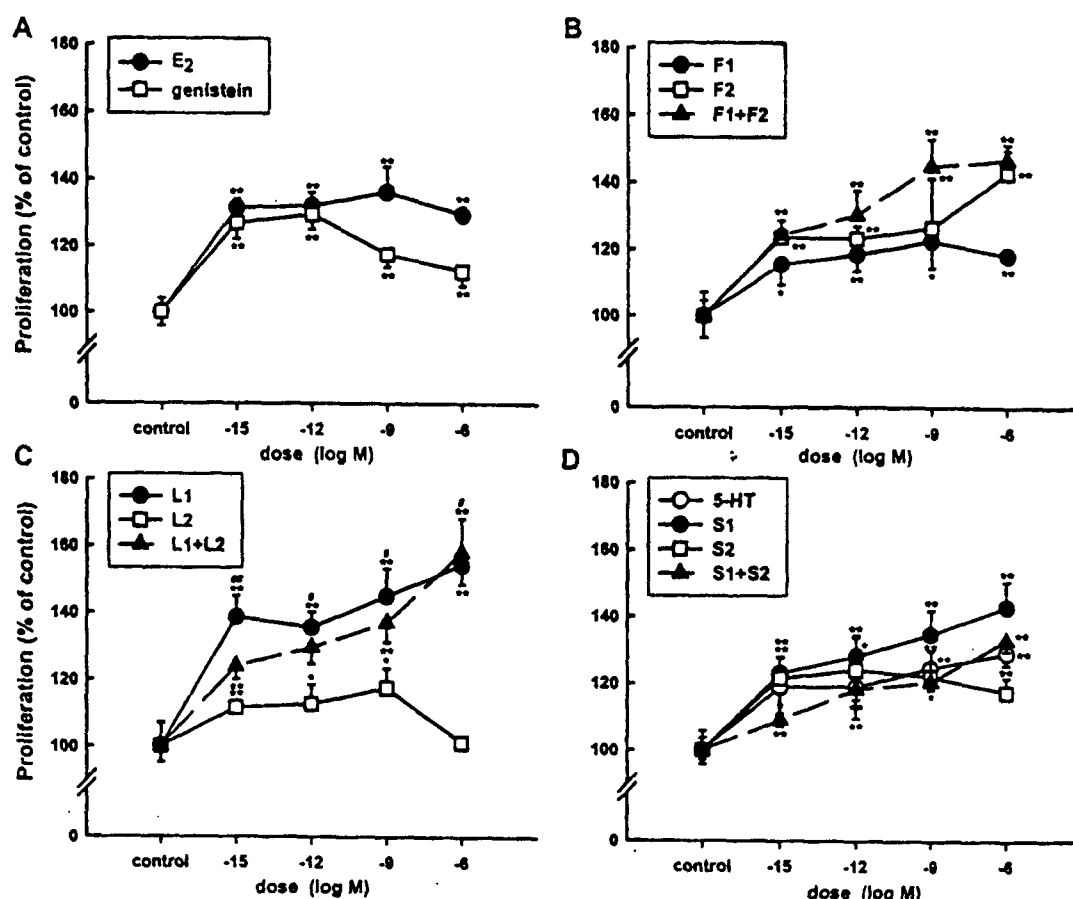


Fig. 6. Dose-dependent effects of lignans, flavones and serotonin derivatives isolated from the defatted safflower seeds, and genistein and 17 β -estradiol (E₂) on proliferation of ROS 17/2.8 osteoblast-like cells. Cell proliferation was determined by MTT assay as described in Materials and Methods. Results are expressed as percentage of the control and values represent mean \pm SE from 6 separate experiments. L1, matairesinol; L2, 8'-hydroxyarctigenin; F1, Tilianine; F2, acacetin; S1, *N*-feruloylserotonin; S2, *N*-(*p*-coumaroyl)serotonin. * $p < 0.05$, ** $p < 0.01$ vs. control; # $p < 0.05$, ## $p < 0.01$ vs. L2.

ACKNOWLEDGMENTS

This work was partly supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare (HMP-00-B-22000-0152), the RRC program of MOST and KOSEF and the Woori Safflower Seed Farmer's Association in Korea.

REFERENCES

1. Yoshikawa, T., Naito, Y. and Kondo, M. The role of free radicals in the pathogenesis of human diseases. *J. Act. Oxyg. Free Rad.* **1**, 83 (1990)
2. Halliwell, B. and Gutteridge, J. M. C. Free radicals, ageing, and disease. In “*Free radicals in biology and medicine*” Clarendon Press, London, P. 416 (1996)
3. Frei, B. Nonenzymatic antioxidant defense systems. In “*Natural antioxidants in human health and disease*” Briviba, K. and Sies, H. (eds.), Academic Press, London, p. 107 (1994)
4. Fukuzawa, K. and Takaishi, Y. Antioxidants. *J. Act. Oxyg. Free Rad.* **1**, 55 (1990)
5. Shutt, D.A. and Cox, R.I. Steroid and phytoestrogen binding to sheep uterine receptors in vitro. *J. Endocrinol.* **52**, 299 (1972)
6. Adlercreutz, H., Fotsis, T., Heikkinen, R., Dwyer, J.T., Woods, M., Goldin, B.R. and Gorbach, S.L. (1982). Excretion of the lignans enterolactone and enterodiol and of equol in omnivorous and vegetarian postmenopausal women and in women with breast cancer. *Lancet* **II**, 1295 (1982)
7. Potter, S. M., Baum, J.A., Teng, H., Stillman, R. J., Shay, N. F., and Erdman, J. W. Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. *Am. J. Clin. Nutr.* **68** (suppl), 1375S (1998).
8. Setchell, K.D.R., and Adlercreutz, H. Mammalian lignans and phytoestrogens: Recent studies on their formation, metabolism and biological role in health and disease. *In: The role of gut microflora in toxicity and cancer.* Academic Press, New York, pp 315-345, 1988.
9. Persson, I., Weiderpass, E., Bergkvist, L., Bergstrom, R., and Schairer, C. Risks of breast and endometrial cancer after estrogen and estrogen-progestin replacement. *Cancer Causes Control* **10**, 253 (1999)
10. Stumpf, P.G. and Trolice, M.P. Compliance problems with hormone replacement therapy. *Obstet. Gynecol. Clin. North. Am.* **21**, 219 (1994)
11. Jeon, S.M., Kim, J.H., Lee, H.J., Lee, I.K., Moon, K.D. and Choi, M.S. Effects of Korean safflower (*Carthamus tinctorius* L.) seed powder supplementation diet on bone metabolism indices in rats during the recovery of rib fracture. *Kor. J. Nutr.* **31**, 1049 (1998)
12. Chung, S.Y., Choi, H.J., Chung, M.W., Ahn, M.R., Yoo, T.M., Rhee, H.M., and Yang, J.S. Effects of safflower seed on the fracture healing in rat tibia. *Yakhak Hoehi*, **43**, 526 (1999)
13. Seo, H.J., Kim, J.H., Kwak, D.Y., Jeon, S.M., Ku, S.K, Lee, J.H., Moon, K.D., and Choi, M.S. The effects of safflower seed powder and its fraction on bone tissue in rib-fractured rats during the recovery. *Kor. J. Nutr.*, **33**, 411 (2000)
14. Kim, J.H., Jeon, S.M., Kim, Park, Y.A., Choi, M.S. and Moon, K.D. Effects of safflower seed (*Carthamus tinctorius* L.) powder on lipid metabolism in high fat and high cholesterol-fed rats. *J. Kor. Soc. Food Sci. Nutr.*, **28**, 625 (1999)
15. Cho, S. H., Choi, S. W., Choi, Y. S. and Lee, W. J. Effects of defatted safflower and perilla seed powders on lipid metabolism in ovariectomized female rats fed high cholesterol diets. *J. Kor. Soc. Food Sci. Nutr.*, **30**, 112 (2001)

16. Moon, K.D., Back, S.S., Kim, J.H., Jeon, S.M., Lee, M.K. and Choi, M.S. Safflower seed extract lowers plasma and hepatic lipids in rats fed high-cholesterol diet. *J.Nutr. Res.* **21**, 895 (2001)
17. Sakamura, A., Terayama, Y., Kawakatsu, S., Ichihara, A. and Saito, H. Conjugated serotonins and phenolic constituents in safflower seed (*Carthamus tinctorius* L.). *Agric. Biol. Chem.* **44**, 2951 (1980)
18. Lee, J.Y., Kang, G.H. and Choi, S.W.: Changes in phenolic compounds in safflower seed during growth stage and roasting process. *J. Agric. Food Chem.*, (2003) submitted
19. Baek, N.I., Kim, Y.H., Ahn, E.M., Bang, M.H., Nam, J.Y., and Kwon, B.M., Isolation of biologically active compounds from the flower petals of *Carthamus tinctorius* L. *Agr. Chem. Biotechnol.*, **41**, 197 (1998)
20. Zhang, H. L., Nagatsu, A. and Sakakibara, J. Novel antioxidants from safflower (*Carthamus tinctorus* L.) oil cake. *Chem. Pharm. Bull.*, **44**, 874 (1996)
21. Zhang, H. L., Nagatsu, A., Watanabe, T., Sakakibara, J. and Okuyama, H. Antioxidative compounds isolated from safflower (*Carthamus tinctorus* L.) oil cake. *Chem. Pharm. Bull.*, **45**, 1910 (1997)
22. Kang, G. W., Chang, E. J., and Choi, S. W. Antioxidative activity of phenolic compounds in roasted safflower (*Carthamus tinctorus* L.) seeds. *J. Food Sci. Nutr.* **4**, 221 (1999)
23. Kawashima, S., Hayashi, M., Takii, T., Kimura, H., Zhang, H. L., Nagatsu, A., Sakakibara, J., Murata, K., Oomoto, Y., and Onozaki, K. Serotonin derivative, N-(*p*-coumaroyl)serotonin, inhibits the production of TNF- α , IL-1 α , IL-1 β , and IL-6 by endotoxin-stimulated human blood monocytes. *J. Interfer. Cytokin. Res.* **18**, 423 (1998)
24. Takii, T., Hayashi, M., Hiroyuki, H., Chiba, T., Kawashima, S., Zhang, H. L., Nagatsu, A., Sakakibara, J., and Onozaki, K. Serotonin derivative, N-(*p*-coumaroyl) serotonin, isolated from safflower (*Carthamus tinctorius* L.) oil cake augments the proliferation of normal human and mouse fibroblasts in synergy with basic fibroblast growth factor (β FGF) or epidermal growth factor(EGF). *J. Biochem.*, **125**, 910 (1999)
25. Roh, J. S., Sun, W. S., Oh, S. U., Lee, J. I., Oh, W. T. and Kim, J. H.: *In vitro* antioxidant activity of safflower (*Carthamus tinctorus* L.) seeds. *Food Sci. & Biotechnol.*, **8**, 88 (1999)
26. Bae, S. J., Shim, S. M., Park, Y. J., Lee, J. Y., Chang, E. J., and Choi, S. W., Cytotoxicity of phenolic compounds isolated from seeds of safflower (*Carthamus tinctorius* L.) on cancer cell lines. *Food Sci. Biotechnol.*, **11**, 140 (2002)
27. Kim, J.H., Park, Y.H., Choi, S.W., Yang, E.K. and Lee, W.J. Lignan from safflower seeds induces apoptosis in human promyelocytic leukemia cells, *Nutraceuticals & Food* (2003) submitted
28. Kim, H.J., Bae, Y.C., Choi, S.W., Cho, S.H., Park, R.W., Choi, Y.S., and Lee, W.J. Bone-protecting effect of safflower seeds in ovariectomized rats. *Calcified Tissue International* **71**, 88 (2002)
29. Song, H.R., Ra, D.K., Kim, G.S., Chang, K.C., Hwang, J.M., Yeon, S.C., Park, H.B. and Choi, S.W. The effect of safflower seed or matairesinol on spontaneous bone formation in surgically induced bone defect in young rabbit. *J. Kor. Soc. Fractures* **15**, 87 (2002)
30. Bae, Y. C., Choi, M.K., Kim, H.J., Park, R.W., Choi, S. W., Cho, S. H., and Lee, W. J. Phenolic compounds in safflower seeds are responsible for bone-protecting effects in ovariectomized rats. *Bone* (2003) submitted.

31. Palter, R. and Lundin, R. E.: A bitter principle of safflower, matairesinol monoglucoside. *Phytochemistry* **9**, 2407 (1970)
32. Palter, R., Lundin, R. E. and Haddon, W. F.: A cathartic lignan glycoside isolated from *Carthamus tinctorius*. *Phytochemistry* **11**, 2871 (1972)
33. Sakumura, S., Terayama, Y., Kawakatsu, S., Ichihara, A. and Saito, H.: Conjugated serotonins related to cathartic activity in safflower seed, *Agric. Biol. Chem.*, **42**, 1805 (1978)
34. Choi, S.W. and Lee, J.Y. Method for preparation of high quality tea extract of safflower leaf containing antioxidative flavonoids. Kor. patent No. 30363 (2002)
35. Lee, J.Y., Chang, E.J, Park, J.H. and Choi, S.W. Antioxidative flavonoids from leaves of safflower (*Carthamus tinctorius* L.). *Arch. Pharm. Res.* **25**, 313 (2002)
36. Brand-Williams, W., Cuvelier, M. E. and Berset, C.: Use of a free radical method to evaluate antioxidant activity. *Lebensm. Wiss. Technol.*, **28**, 25 (1995)
37. Kim H.J., Chang, E.J. park, H.D., Chung, S.K., and Choi, S.W. Antioxidative activity of resveratrol and its derivatives from seeds of paeonia lactiflora. *Biosci. Biochem. Biotechnol.* **66**, 1990 (2002)
38. Park, Y.H., park, H.K., Lee, H.J., Park, S.M., Choi, S.W., Lee, W.J. Phytoestrogen-induced phosphorylation of MAP kinase in osteoblasts is mediated by membrane estrogen receptor. *Kor. J. Physiology Pharmacology* **6**, 165 (2002)