Food Sci. Biotechnol. Vol. 18, No. 3, pp. 689 ~ 693 (2009)

Food Science and Biotechnology

ⓒ The Korean Society of Food Science and Technology

Proliferative and Differentiative Effects of Trachelogenin Isolated from Germinated Safflower (*Carthamus tinctorius*) Seeds on Calvarial Bone Cells

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Abstract Germination is well-known to enhance the digestibility, functionality, and palatability of plant seeds. To examine the functionality of germinated-safflower seed (GSS), proliferative and differentiative effects of GSS extract on the mouse calvarial bone cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinbromide (MTT) assay and alkaline phosphatase activity, respectively. Water extract of GSS increased dose-dependently proliferative and differentiative effects on calvarial bone cell, and its effects were stronger than those of ungerminated-safflower seeds (UGSS) extract. One major component was isolated from GSS extract by a series of purification procedure of solvent fractionation, Diaion HP-20, and Sephadex LH-20 column chromatographies. Its chemical structure was identified as trachelogenin (TC) by nuclear magnetic resonance (NMR) and mass spectrometry (MS) spectral analysis. Trachelogenin showed significant proliferative (125.7%) and differentiative (132.1%) effects on calvarial bone cells at 10^{-8} M, and its effects were significantly higher than those of 17β-estradiol (E_2). TC was found to be a major active compound responsible for high proliferative and differentative effects of the water extract of GSS. Therefore, these results suggest that TC in GSS may be useful as potential therapeutic agent for the prevention and treatment of bone loss.

Keywords: safflower (*Carthamus tinctorius* L.) seed, germination, proliferation and differentiation, mouse calvarial bone cell, trachelogenin

Introduction

Estrogen deficiency in postmenopausal women is a major risk factor of osteoporosis (1). Hormone replacement therapy (HRT) is widely used for the treatment of postmenopausal osteoporosis and bone fracture (2,3). However, use of HRT in postmenopausal women has been limited, because of possible increased risks of breast and endometrial cancers with long-term use (4). Therefore, an alternative therapy of the conventional HRT is required. Phytoestrogens are currently well-known as a potentially alternative to HRT in the prevention of postmenopausal osteoporosis.

Safflower (*Cathamus tinctorius* L.) seeds have long been clinically used as Oriental herbal medicine for the treatment of osteoporosis and bone resorption (5). Recent studies showed that safflower seeds stimulated proliferation and differentiation of osteoblastic cells (6-8) and increased bone density in 3 osteoporotic patients (9). In addition, previous studies revealed that the defatted safflower seeds containing phenolic compounds markedly attenuated bone loss in ovariectomized rats (10,11) and phenolic compounds in safflower seed, including lignans and flavones, were acted to be as phytoestrogens with mammalian estrogen-like activity (10,12). Thus, phenolic compounds in safflower seed are recently receiving much attention as dietary supplements for prevention and treatment of postmenopausal osteoporosis.

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Received October 13, 2008; Revised November 24, 2008; Accepted December 7, 2008

Safflower seed powder is not suitable for dietary supplement source because of indigestible and impalatable hard husks (13). In addition, lignan and serotonin glycosides in safflower seed were known to have bitter taste and cathartic effect, and thereby partly restricting current use of safflower meal (14,15). Thus, development of proper processing technology is required to increase use of safflower meal as a dietary supplement. Germination (16,17) and heat pretreatments (18-20) have been reported to improve the digestibility, functionality, and palatability of plant seeds. In particular, germination has been known to induce important phytochemicals of plant seeds (21-23). Our previous study showed that germination was one of effective way to enhance the palatability and functionality of safflower seed (24).

In the present study, the proliferative and differentative effects of water extract of germinated-safflower seed (GSS) and ungerminated-safflower seed (UGSS) on the mouse calvarial bone cells were evaluated through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinbromide (MTT) assay and alkaline phosphatase (ALP) activity. Futhermore, major active constituent responsible for bone proliferation and differentiation was isolated and identified from GSS extract, and its effects were also determined.

Materials and Methods

Reagents α-Minimum essential medium (α-MEM), fetal bovine serum (FBS), and penicillin-streptomycin solution were obtained from Gibco BRL (Gland Island, NY, USA). Bovine serum albumin (BSA), trypsin-ethylenediamine tetraacetic acid (EDTA), 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolinbromide (MTT), dimethylsulfoxide (DMSO), 17β-estradiol, genistein, *p*-nitrophenol (PNP), and *p*-nitrophenylphosphate (PNPP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Packing materials for column chromatography were as follows; Diaion HP-20 resin (Mitsubishi Chem, Co., Tokyo, Japan) and Sephadex LH-20 (Pharmacia Biotech., Uppsala, Sweden). All other chemicals were of analytical grade.

Plant materials Safflower seeds were directly harvested on late July 2007 at the farm, Sancheong, Gyeongnam, Korea. The harvested seeds were completely sun-dried for 1 week to 6-8% of moisture content. Safflower seeds were soaked in water at 25°C for overnight, drained, and then layered on a plastic seed germinator on which a layer of cheesecloth had been laid. The germinator was kept in the dark, and the seeds were germinated at 25°C for 3 days. Water sprayed 6 times/day and then cultivated safflower seeds with 0.5-1.5 cm length of sprouts. GSSs were harvested and immediately freeze-dried. The dried GSSs were ground into fine powder with a coffee maker (recovery: 110.4±5.8) and stored at 4°C until use.

Preparation of the water extract of GSS and UGSS and high performance liquid chromatography (HPLC) analysis Germinated-safflower seed (GSS) and ungerminated-safflower seed (UGSS) powder (100 g) was extracted twice with distilled water under reflux at 90-100°C for 2 hr, filtered and partially evaporated under reduced pressure. The water extract was finally freeze-dried and obtained 2 water extracts (yields, GSS: 10.5%, UGGS: 11.3%). Each extract (1.0 g) was further suspended in water (100 mL) and then partitioned twice with ethylacetate (EtOAc) (200 mL). The EtOAc extracts were analysed by HPLC to differentiate phenolic compositions between GSS and UGSS water extracts according to a previous method (25).

Cell culture and MTT assay For the neonatal osteoblast cell culture, the osteoblast-like cells were isolated from sequential digestion from calvaria of 1-day-old newborn mice skull. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in with α-MEM containing 10% FBS and 1% penicillin-streptomycin. Cell growth was assessed by MTT assay, which stain viable cells with MTT (26). The cells were seeded at a density of 5×10^3/well in 96 well plates. Sub-confluent cells were growth arrested to reach a quiescent state for 24 hr in 1% FBS. Then, cells were treated with 5-100 μg/mL water extracts of GSS and UGSS, trachelogenin (TC) isolated from GSS, and positive controls, such as genistein, a soy isoflavone, and 17β-estradiol in α-MEM containing 5% FBS. After 24 hr incubation, 50 μL of MTT solution (2 mg/mL) was added to each well and then incubated for an additional 4 hr. The colored formazan crystal produced from MTT was dissolved in 150 μL of DMSO and then the optical density was measured at 562 nm by an enzyme-linked immuno-sorbent assay (ELISA) reader (Model 550; Bio-Rad, Hercules, CA, USA).

Alkaline phosphatase (ALP) activity Calvarial cells were seeded at 1×10^5 cell/well in 6-well plates with α-MEM containing 10% FBS. After 24 hr culture, subconfluent cells were starved with same culture medium only 1% FBS for 24 hr and treated with the water extracts of GSS and UGSS, and TC isolated from GSS extract in α-MEM containing 5% FBS for 48 hr. At the end of the indicated culture period, calvarial cells were washed with PBS, scraped into a 1.0 mL of 10 mM Tris-HCl buffer (pH 7.6) containing 0.1% Triton-X 100 on ice and centrifuged. An aliquot of the supernatant was used for the determination of ALP activity by measuring the release of PNP from PNPP (27). ALP activity is expressed as nmol PNP formed/min/mg protein.

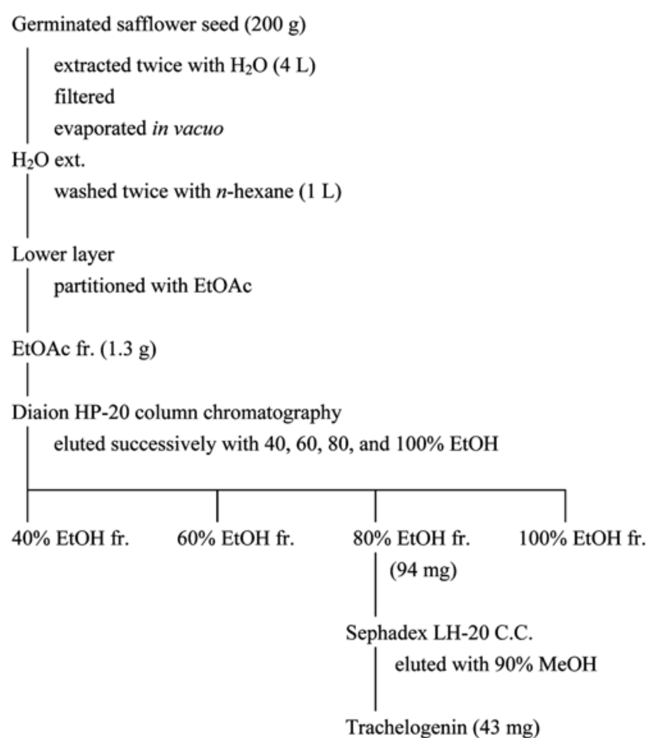

Fig. 1. Schematic procedure for extraction, isolation, and purification of trachelogenin from germinated-safflower seeds.

Isolation and identification of trachelogenin GSS water extract (20.1 g) obtained previously was successively partitioned with *n*-hexane and EtOAc. The EtOAc fraction (1.3 g) was solubilized in 40% aq. MeOH (100 mL) and subjected onto a Diaion HP-20 column (4×50 cm). The column was eluted successively with 40% (2 L), 60% (1 L), 80% (1 L), and 100% MeOH (1 L), and each fraction was then evaporated to yield 40% MeOH fr. (0.42 g), 60% MeOH fr. (0.64 g), 80% MeOH fr. (94 mg), and 100% MeOH fr. (62 mg), respectively. The 80% MeOH fr. was chromatographed on a Sephadex LH-20 column with 90% aq. MeOH and yielded a pure compound (43 mg). Its chemical structure was elucidated as TC by NMR and MS spectral analysis, and comparison of published spectral data (28,29). Schematic procedure for isolation and purification of TC from GSS extract is shown in Fig. 1, and its chemical structure is shown in Fig. 2. The detailed MS and NMR spectral data were as followed. $[M+H]^+$ at *m/z* 389 in the positive fast atom bondardment (FAB)-MS spectrum. ^{1}H-

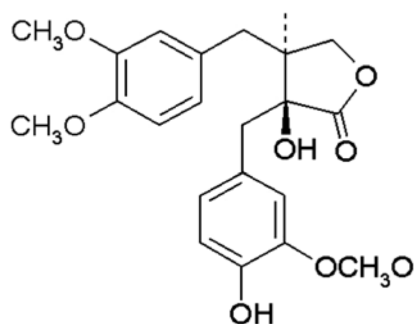

Fig. 2. Chemical structure of trachelogenin isolated from germinated-safflower seeds.

NMR spectral data [δ 6.55 (1H, dd, J=1.6, 8.0 Hz, H-6), 6.60 (1H, dd, J=1.6, 8.0 Hz, H-6'), 6.64 (1H, d, J=8.0 Hz, H-5), 6.65 (1H, d, J=1.8 Hz, H-2), 6.71 (1H, d, J=1.8 Hz, H-2'), 6.79 (1H, d, J=8.0 Hz, H-5'), and butylolactone signals [2.38 (1H, m, H-8), 2.59 (1H, dd, J=3.6, 12.0 Hz, H-7), 2.79 & 2.96 (1H, d, J=13.2 Hz, H-7'), 3.90 & 3.92 (1H, brs, H-9)], 3 methoxyl signals (δ 3.66, 3.66, 3.68). ^{13}C-NMR spectral data [δ 178.69 (CO, C-9'), 149.31 (C-3), 147.87 (C-3'), 147.85 (C-4), 146.01 (C-4'), 132.35 (C-1'), 127.07 (C-1), 123.31 (C-6'), 121.06 (C-6), 115.87 (C-5'), 115.09 (C-2'), 113.07 (C-2), 112.50 (C-5), 76.03 (C-8'), 70.61 (C-9), 49.25 (C-8'), 43.44 (C-7'), 31.42 (C-7), 3 methoxyl carbons (55.97, 56.11, 56.18).

Statistical analysis All data were represented as mean± standard error (SE). Statistical analysis was performed using one-way ANOVA (p<0.05). The analysis was performed using SAS statistical software.

Results and Discussion

Proliferative and differentiative effects of water extracts of GSS and UGSS on calvarial cell Proliferative effects of the water extracts of GSS and UGSS on the mouse calvarial bone cells were determined by MTT assay. As shown in Fig. 3, GSS and UGSS extracts dose-dependently stimulated the proliferation of calvarial cells at 5-100 μg/mL, and especially significantly stimulated cell growth by 121.4 and 112.2% at 50 μg/mL, respectively, as compared to control. The proliferative effect of GSS extract was higher than that of UGSS extract. Meanwhile, to compare the differentiative effect of the water extracts of GSS and UGSS on calvarial cell, the activity of ALP, an estrogen-inducible marker enzyme, of 2 extracts were determined. As shown in Fig. 4, GSS extract dose-dependently increased the ALP activity of calvarial cells at 5-100 μg/mL, and especially significantly increased the activity by 108.2 and 144.8% at 50 and 100 μg/mL, respectively, as compared to control. In contrast, UGSS extract did not show an appreciable activity at 5-100 μg/mL. Thus, the proliferative and differentiative effect of GSS extract on calvarial cell were higher than those of UGSS. These results indicate that GSS extract stimulates the proliferation and differentiation of calvarial cell. Germination is known to increase phenolic phytochemicals in plant seeds and convert phenolic glycosides into their corresponding aglycones (16,23). In addition, our previous study found that phenolic

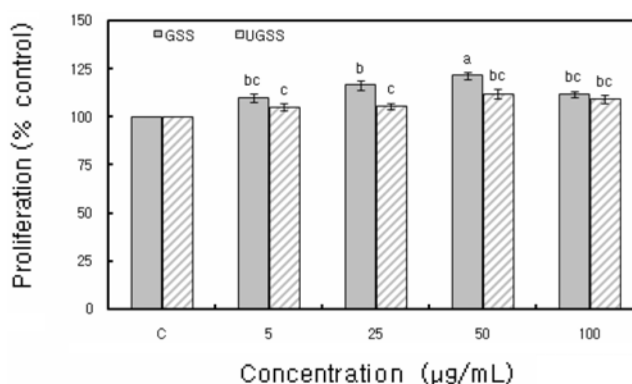

Fig. 3. Proliferative effect of the water extracts of germinated (GSS)- and ungerminated (UGSS)-safflower seeds on calvarial bone cells. Values are mean±SE (n=3); Different superscript letters show significant differences at p<0.05 by Duncan's test.

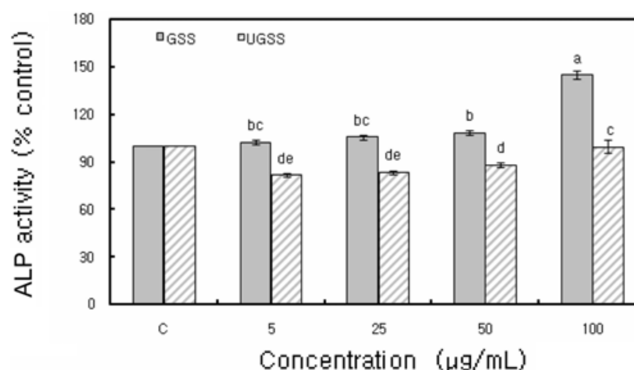

Fig. 4. Alkaline phosphatase (ALP) activity of the water extracts of germinated- (GSS) and ungerminated- (UGSS) safflower seeds. Values are mean±SE (n=3); Different superscript letters show significant differences at p<0.05 by Duncan's test.

compounds in safflower seed stimulated the proliferation and differentiation of osteoblastic cells (10,11). These facts suggest that there are chemical differences in GSS and UGSS, especially phenolic compositions as a biomarker of bone formation. Thus, we further analysed phenolic profiles in GSS and UGSS by HPLC.

HPLC analysis of water extracts of GSS and UGSS To differentiate phenolic compositions of 2 water extracts of GSS and UGSS, 2 EtOAc fractions obtained previously were analysed by HPLC. As shown in Fig. 5, 1 peak with retention time at about 37 min was found in GSS extract, not in UGSS extract, suggesting that this compound may be responsible for strong proliferation and differenation effects of GSS extract. Therefore, GSS water extract was further fractionated and chromatographed, and finally isolated and identified TC, as previously described. TC in safflower seed is mainly present as its glycoside, tracheloside (14,30), which is degraded to TC during roasting processing (31). In addition, it was found that phenolic compositions and contents of safflower seeds varied with cultivars and maturation (24,31). These facts suggest that TC in safflower seeds may be affected by maturity, genotype, and processing.

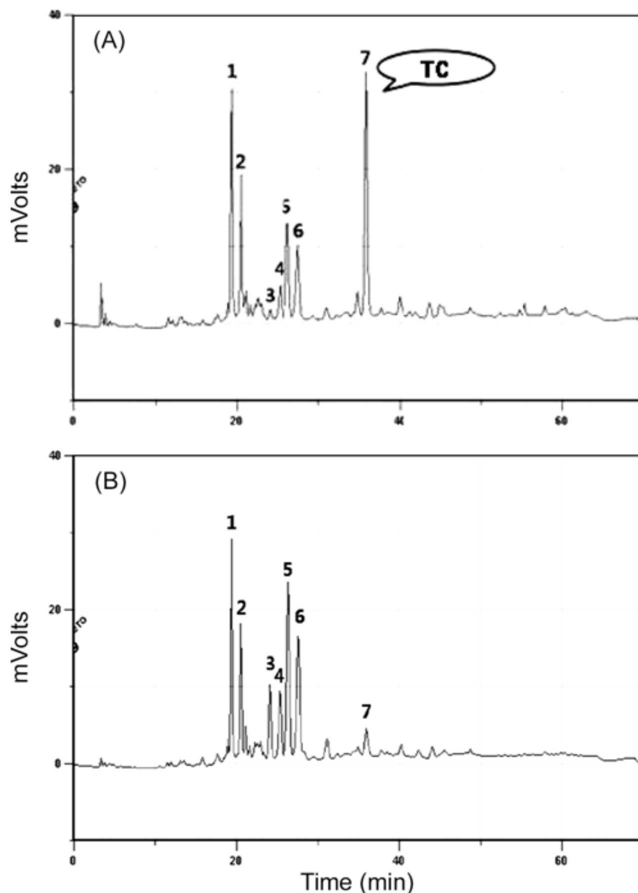

Fig. 5. HPLC chromatograms of ethylacetate fractions from the water extracts of germinated- (GSS, A) and ungerminated- (UGSS, B) safflower seeds.

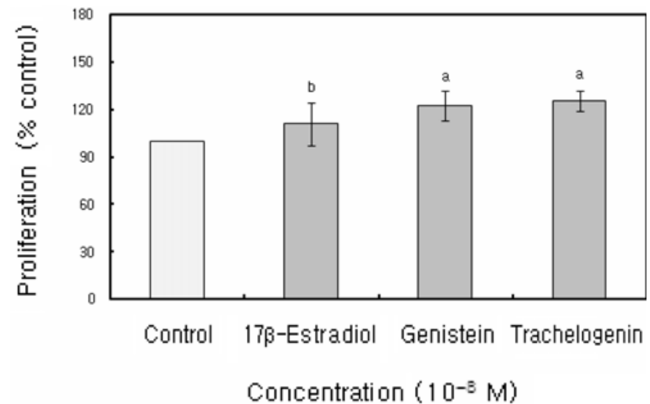

Fig. 6. Proliferative effect of trachelogenin isolated from germinated-safflower seeds on calvarial bone cells. Values are mean±SE (n=6); Different superscript letters show significant differences at p<0.05 by Duncan's test.

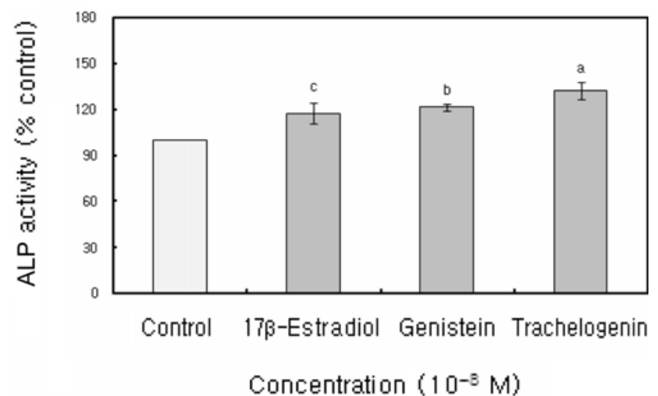

Fig. 7. Alkaline phosphatase (ALP) activity of trachelogenin isolated from germinated-safflower seeds on calvarial bone cells. Values are mean±SE (n=3); Different superscript letters show significant differences at p<0.05 by Duncan's test.

Proliferative and differentiative effects of TC on calvarial cells Proliferative effects of TC isolated from GSS on calvarial cells is shown in Fig. 6. TC significantly stimulated the proliferation of calvarial cells by 125.7% at 10^{-8} M. When 17β-estradiol (E_2) and genistein, a soy isoflavone, were tested to compare their effects, they also increased the proliferation of calvarial cells by 110.3 and 123.3% at 10^{-8} M, respectively. Thus, the proliferative effect of TC on calvarial cells was higher than that of 17β-estradiol (E_2), and comparable to that of genistein (p<0.05). To investigate the differentiative effect of TC on calvarial cell, ALP activity was also determined. As shown in Fig. 7, TC significantly increased ALP activity of calvarial cells by 132.1% at 10^{-8} M, as compared to control. ALP activities of 2 positive references, 17β-estradiol (E_2) and genistein were 111.0 and 121.3% at 10^{-8} M, respectively. ALP activity of TC was the highest of the 3 compounds. Thus, TC in GSS significantly stimulated the proliferation and differentiation of calvarial cells. These results indicate that TC in GSS could be mainly responsible for strong proliferative and differentiative effects of GSS extract. TC had no effect on the cell viability at any of the concentrations tested (data not shown). Trachelogenin glycoside, tracheloside, is generally known to be a phytoestrogen because of structurally similar to the well-known lignan phytoestrogens, matairesinoside, and arctiin (32). It is generally accepted that tracheloside is transformed into TC and further to estrogenic and anti-estrogenic substances by human intestinal bacteria (33,34). Additionally, tracheloside in safflower seeds was recently found to act as an anti-estrogenic component (35). The fact supports earlier report that phytoestrogens, such as isoflavones and lignans, have been reported to have estrogenic and/or anti-estrogenic properties (36). Therefore, these results suggest that TC in GSS may be useful as potential therapeutic agent for the prevention and treatment of bone loss. This study is the first time that germination of safflower seed is one of effective way to improve health-related benefits and TC acted as a active principle for bone proliferative and differentiative activities of GSS. Further *in vivo* and *in vitro* studies will be needed to investigate the bone formation effect of TC.

In summary, water extract of germinated-safflower seed (GSS) stimulated the proliferation and differentiation of calvarial bone cells. Trachelogenin (TC) in GSS could be acted as major constituent responsible for bone cell formation. Thus, GSS powder containing TC may be utilized as dietary supplement for the prevention and treatment of

bone loss, although further studies are needed to clarify beneficial effects of TC on bone formation.

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