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ORIGINAL ARTICLE

Effects of Korean Black Raspberry Wines on Hepatic Cholesterol Metabolism and Retinal Vascular Formation *In Vitro*

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Abstract Toxicity, antioxidant activity, and biological functionality of Korean black raspberry wine (KBRW) extracts on human ARPE-19 and HepG2 cells were determined using MTT and lipid/malonaldehyde (MA) assays and reverse transcription polymerase chain reaction (RT-PCR) as well as anti-obesity effect of KBRW extracts on various cancer cells. All samples inhibited MA formation by 38.0–88.0%. Bokbunja and Sanmaesu exerted greater inhibitory effect than other wines. Both cell lines were each treated with KBRW for 24 h, and viability was measured by MTT assay. No toxicity was found, even at 500 μg/mL. Changes in gene expression for CYP7A1, low-density-lipoprotein (LDL) receptor, 3-hydrozy-3-methylglutaryl-CoA (HMG-CoA) reductase, and vescular endothelial growth factor (VEGF) were assessed by semi-quantitative RT-PCR. CYP7A1 and LDL receptor expressions in ARPE-19 cells were elevated, whereas HMG CoA reductase expression was decreased by Bokbunja and Sanmaesu. Expression of VEGF was decreased by Bokbunja but elevated by Sanmaesu. In HepG2 cells, gene expression was similar after Bokbunja and Sanmaesu treatments. CYP7A1 and HMG CoA expressions were elevated. LDL receptor expression was increased by Bokbunja but decreased by Sanmaesu. Bokbunja and Sanmaesu inhibited VEGF expression but elevated those of CYP7A1 and HMG CoA reductase. Bokbunja and Sanmaesu displayed anti-cholesterol effects that could be attributed to increased gene expression in CYP7A1 and HMG CoA reductase.

Keywords Bokbunja · cholesterol 7α-hydroxylase · 3-hydroxy-3-methyl-glutaryl-CoA · low-density lipoprotein receptor · malonaldehyde · Sanmaesu · vascular endothelial growth factor

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Introduction

Interests in human health, nutrition, and disease prevention have increased consumer demand for functional foods, including fruits and their products such as wine. Epidemiological evidence has shown that fruit constituents are beneficial to human health and contribute to the prevention of degenerative processes caused by oxidative stress (Kaur and Kapoor, 2001). Fruits are one of the most important sources of antioxidants such as carotenoids, phenols, flavonoids, vitamins, and dietary glutathiones (Vinson et al., 2001). Dietary intake of plant phenolic compounds is inversely related to coronary heart disease (Tomas-Barberan et al., 2001), and these compounds act as anti-ulcer, antispasmodic, antisecretory, and antidiarrheal agents in the gastrointestinal tract (Hertog et al., 1997). These antioxidants are capable of acting as free radical scavengers, such as peroxide decomposers, singlet and triplet oxygen quenchers, and enzyme inhibitors and synergists (Carlo et al., 1999). Berry fruits, including raspberries, have high phenolic and anthocyanin contents (Larson, 1998) and have been show to inhibit low-density lipoprotein (LDL) and liposome oxidation (Kähkönen et al., 2001). Thus, various antioxidants found in berry fruits provide significant health benefits.

Rubus is one of a 100 genera in the family Rosaceae, subfamily Rosoideae, tribe Potentilleae; 250 species of *Rubus* exist worldwide. *Rubus coreanus* Miq. (Rosaceae, Rubi Fructus), known as

Bokbunja in Korea, is a type of black raspberry, and is a perennial shrub that is distributed in the southern part of the Korean peninsula. Unripe fruits of *R. coreanus* Miq. have been used in traditional herbal medicine for the treatment of diabetes mellitus and sexual disinclination (Heinonen et al., 1998). In addition, its free radical-scavenging activity (Moon, 1991; Yoon, 2001), inhibitory effect on formation of malonaldehyde (MA), anticancer activity (Lee et al., 2003; Kim et al., 2005), antimicrobial activity, and anti-hepatitis B virus activity (Cha et al., 2001; Lee et al., 2003) have been reported. The fruits are rich in sugars, organic acids, and several vitamins, as well as various antioxidants (Yoon et al., 2001), terpenoids (Ohtani et al., 1990; Yoon et al., 2003), tannins (Kim and Kang, 1993), and phenolic acids (Lee and Lee, 1995). The low rate of cardiovascular disease in France compared to other developed countries with a comparable intake of dietary saturated fat has been called the French paradox. Korean black raspberry wine (KBRW) is produced by fermenting Korean raspberries, and is compared frequently with red wines. Recently, KBRW has received much attention due to its rich aroma and flavor, as well as its bioactivity. Ingredients of KBR are fermented while producing KBRW, and their metabolites are able to exert invaluable bioactivity similar to that of KBRs. In contrast to KBRs, the bioactivity of KBRW has been reported to be low (Lee, 1995), as shown by the significantly lower serum concentration of cholesterol in KBRW intake groups than in control groups (basal diet and water). More recently, total antioxidant capacity, total phenolic content, mineral elements, and histamine concentrations in wines from different fruit sources have been reported (Kwon et al., 2006). Furthermore, the cytotoxic effects of Korean traditional liquors on cancer cells (Vasantha Rupasinghe and Clegg, 2007) and the stomach (Kim et al., 2004) have also been reported. Therefore, elucidating the bioactivity of KBRW is of upmost importance in preparation of the quality control certification for KBRW production.

It is known that age-related macular degeneration (AMD) is a leading cause of adult blindness in western society, and its prevalence has increased dramatically during the past decade in Korea. The main causes of AMD are the degeneration of the macular retina and choroid by atrophy or detachment and scarring by choroidal neovascularization. Retinal pigment epithelium (RPE) forms a blood–retina barrier, and disruption of this barrier by vascular angiogenesis or cholesterol accumulation has been suggested as a potential mechanism of AMD (Lee et al., 2007). In addition, vascular endothelial growth factor (VEGF) plays a key role in the pathogenesis of choroidal neovascularization. The conversion of cholesterol to bile acids is the major pathway through which cholesterol is removed from the body. The initial and rate-limiting steps in this catabolic pathway are catalyzed by the liver-specific enzyme cholesterol 7α-hydroxylase (CYP7A1). Human cells can either synthesize their own cholesterol or they can take it up via the LDL receptor by receptor-mediated endocytosis. The rate-limiting step of the cholesterol biosynthetic pathway is controlled by 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase).

To study the characteristics of KBRW, the antioxidant activity, toxicity, and functions of Bokbunja and Sanmaesu at non-toxic concentrations were investigated, and CYP7A1, HMG CoA reductase, LDL receptor, and VEGF gene expression was detected. Wine and beer acted as controls. It has been shown that wine decreased the risk of cardiovascular disease. Pal et al. (2003) have shown that red wine lowers apolipoprotein B100 by 50% and increases HMG CoA reductase mRNA and LDL receptor in HepG2 cells.

Materials and Methods

Chemicals. *N*-methylhydrazine (NMH), α-tocopherol (vitamin E; 95%), 2-methylpyrazine (MP), sodium dodecyl sulfate (SDS), MTT, DMSO, diethl pyrocarbonate (DEPC), and ferrous chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Dichloromethane, ethylacetate, hydrogen peroxide, cod liver oil, trizma hydrochloride, and trizma base were purchased from Aldrich Chemical Co. (Milwaukee, WI). The 2,6-di-*tert*-butyl-4-methylpheonol (BHT) was purchased from Junsei Chemical Co (Tokyo, Japan). Dulbecco's Modified Eagle's Medium (DMEM): HAM's F-12, 1:1 mixture; DMEM; fetal bovine serum (FBS); penicillin–streptomycin solution (PEST); and trypsin–EDTA solution were purchased from WelGENE Inc. (Seoul, Korea). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA), and PowerScript Reverse Transcriptase was purchased from Clontech (Mountain View, CA). Oligo(dT)$_{15}$ and all PCR primers were purchased from Bioneer (Seoul, Korea).

Preparation of KBRW extract. KBRWs were donated from Heungjin Co. (Gochang, Korea). Ten milliliters of KBRW was evaporated using a reduced pressured rotary evaporator (Büchi R110; Essen, Germany) at 60°C. A condensed extract was cooled at room temperature for 30 min. The extract was freeze dried at −100°C for 48 h (Model 4451F; Labconco, CA) and stored at −20°C until analyzed.

Lipid/MA assay. The antioxidative activity of extracts was determined by analyzing MA formed from cod liver oil upon oxidation after derivatizing to 1-MP with NMH. An aqueous solution (5 mL) that contained 10 μL cod liver oil, 0.25 mmol trizma buffer (pH 7.4), 5 μmol ferrous chloride, 10 μmol hydrogen peroxide, 0.75 mmol potassium chloride, and 0.2% surfactant SDS was incubated with various amounts of the KBRW extracts for 17 h at 37°C in a 20-mL test tube. The oxidation of samples was stopped by adding 50 μL of 4% BHT solution. NMH (30 μL) was added to the oxidized cod liver oil solutions, and the solutions were stirred for 1 h at room temperature. Sep-Pak C_{18} cartridges (Millipore, Milford, MA) were used for clean up and concentration of the analytes. The Sep-Pak C_{18} cartridge was activated with 10 mL each ethylacetate and methanol followed by washing with 10 mL water. Subsequently, the aqueous sample was added, and the sorbent was washed with 5 mL water. The elution was performed

with 10 mL ethylacetate. The eluant was adjusted to exactly 10 mL by adding ethylacetate and 20 μL of 2-MP solution as a gas chromatography (GC) internal standard. The solution was analyzed for 1-MP by GC with a nitrogen–phosphorus detector (NPD).

Instrumental analysis. An HP model 6890 gas chromatograph equipped with a DB-WAX bonded phase fused-silica capillary column [30 mm × 0.32 mm i.d. (d_f=0.25 μm), J&W Scientific, Folsom, CA] and an NPD were used for analysis of 1-MP. The oven temperature was held at 60°C, increased to final temperature of 160°C at 4°C/min, and maintained for 2 min. Injector and detector temperatures were 250°C. The carrier gas was helium at a flow rate of 1.5 mL/min. An HP model 5890 GC interfaced to a 5972A mass selective detector (GC/MS) was used for mass spectral identification of the GC components at an MS ionization voltage of 70 eV. Chromatographic separation was performed with a DB-WAX bonded-phase fused-silica capillary column [60 m × 0.25 mm i.d. (d_f=0.25 μm), J & W Scientific]. The carrier gas was helium at a flow rate of 1.5 mL/min and a linear velocity of 30 cm/s. The injector and detector temperatures were 250°C. The oven temperature was programmed from 50°C (2°C/min isothermal) to 200°C at 3°C/min and held for 10 min.

Cell culture. Adult human ARPE-19 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA) and cultured in DMEM/F12 that contained 10% heat-inactivated FBS and 1% PEST in 75-cm^3 flasks at 37°C in 5% CO_2. HepG2 cells (Korean Cell Line Bank, Seoul, Korea) were cultured in DMEM, which contained 10% FBS and 1% PEST. Medium was replaced three times per week, and cells were subcultured at confluence using trypsin-EDTA solution (0.05%). Cell counts were determined using Neubauer-improved cell counting plates (Lauda-Konigshofen, Germany). Cells were plated in six- or 24-well Falcon plates and used for experiments at 90% confluence.

Viability test. For measuring the cytotoxic effect of KBRW, cell proliferation was measured by MTT assay. This assay is based on the ability of viable cells to convert soluble MTT into insoluble dark blue formazan reaction product, and the bulk conversion of MTT is detected photometrically (Lee and Choi, 2006). MTT was dissolved in PBS at 5 mg mL^{-1} and sterilized by filtering with a 0.22-nm filter. ARPE-19 and HepG2 cells were suspended in full growth medium and seeded at 1.5×10^5 cells cm^{-2} in 24-well plates and cultured for 48 h. Medium was changed to pre-warmed serum-free DMEM:F-12 for ARPE-19 cells and DMEM for HepG2 cells for additional 12 h. Cells were then treated with KBRW at various concentrations of 0, 0.5, 1, 10, 50, 100, 200, and 500 μg/mL for 24 h and then maintained for additional 4 h in no-serum medium that contained 10% (v/v) MTT reagent. Thereafter, cells were solubilized in 1 mL DMSO for 1 h. The viability of cultured cells was quantified by measuring the reduction of MTT by mitochondrial dehydrogenases to produce a dark blue formazan product (Chung et al., 2008). The absorbance was read with a 570-nm filter by using a BioKinetics Microplate reader. This assay was repeated three times, and each concentration was subjected to three replications.

Isolation of total RNA and RT-PCR. Cells were seeded at 1.5×10^5 cells cm^{-2} in six-well plates and cultured for 48 h. Medium was changed to serum-free DMEM:F-12 for ARPE-19 cells and DMEM for HepG2 cells for additional 12 h, and cells were treated with KBRW samples for 24 h. Total RNA was extracted and isolated from cells using TRIzol reagent according to the manufacturer's instructions (Invitrogen) and suspended in DEPC-treated water. For cDNA synthesis, 1 μg total RNA was reverse transcribed using PowerScript Reverse Transcriptase according to the manufacturer's protocol (Clontech), using a combination of oligo$(dT)_{15}$ primer and random hexamers, resulting in 20 μL cDNA. PCR was performed using 1 U Top-Taq™ DNA polymerase, 0.2 mM dNTP, 2 mM $MgSO_4$, 1× High Fidelity PCR buffer and 1 μL of each primer in a total volume of 20 μL with 1 μL cDNA template. *In vitro* PCR primers were designed using published nucleotide sequences (Table 1). PCR using the primers CYP7A1 was performed with an initial cycle of 4 min at 94°C, 30 s at 94°C, 30 s at 57°C, and 30 s at 72°C, with a final extension for 5 min at 72°C. PCR using the HMG CoA reductase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) templates was similar, with the exception of the number of cycles (26 for HMG CoA reductase and 32 for GAPDH). PCR using LDL receptor primer was performed with an initial cycle of 5 min at 95°C, followed by 29 cycles of 30 s at 95°C, 30 s at 62°C, and 30

Table 1 Primer sequence and size of their PCR products

Target mRMA	Primer	Sequence	Fregment size (bp)
CYP7A1	Forward	5' GCATCATAGCTCTTTACCCAC 3'	
	Reverse	5' GGTGTTCTGCAGCAGTCCTGTAAT 3'	416 (Christian et al., 2003)
HMG CoA reducetase	Forward	5' TACCATGTCAGGGGTACGTC 3'	
	Reverse	5' CAAGCCTAGAGACATAATCATC 3'	247 (Christian et al., 2003)
LDL receptor	Forward	5' CAATGTCTCACCAAGCTCTG 3'	
	Reverse	5' TCTGTCTCGAGGGGTAGCTG 3'	258 (Christian et al., 2003)
VEGF	Forward	5' GAAGTGGTGAAGTTCATGGATGTC 3'	
	Reverse	5' CGATCGTTCTGTATCAGTCTTTCC 3'	504 (Ohta et al., 1996)
GAPDH	Forward	5' AGGTCGGAGTCAACGGATTTGG 3'	
	Reverse	5' ACAGTCTTCTGGGTGGCAGTGATG 3'	550 (Christian et al., 2003)

The human RPE cell line ARPE-19 and human liver cell line HepG2 cells were analyzed for expression of RNA.

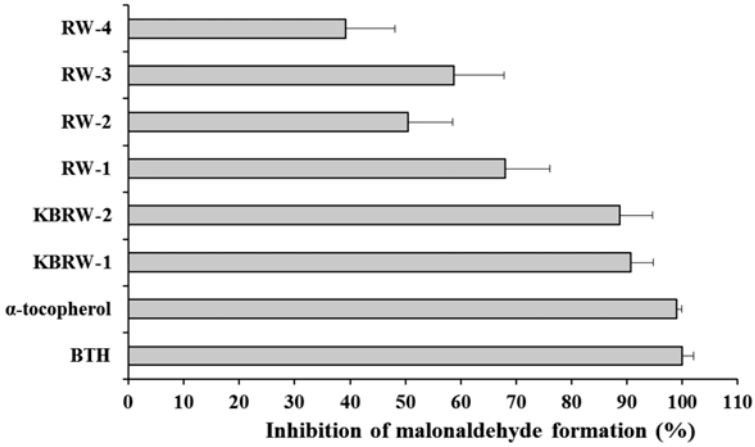

Fig. 1 Inhibitory effect of KBRW and red wine on MA formation from cod liver oil. KBRW-1, Sanmaesu and KBRW-2, Bokbunja. RW-1 to RW-4 are commercially available red wine extracts. Antioxidant activity was measured by inhibition of MA formation from cod liver oil using BHT and α-tocopherol as references. Eight hundred μg/mL of substrate was used for measurement following the procedure described in the method section. The inhibitory effect of BHT was considered as 100%.

s at 72°C, with a final extension for 5 min at 72°C. PCR using VEGF templates was similar, with the exception of 35 cycles and an annealing temperature of 58°C. The GAPDH transcript was used as an internal control. The reaction product was analyzed on a 1% agarose gel, stained with ethidium bromide (EtBr) along with DNA markers, and photographed (Sabine et al., 2006).

Statistical analysis. Values presented in the figures are expressed as means ± SD of at least three independent experiments. The mean and SD were calculated using SAS Statistical Analysis for Windows v9.1 (SAS Institute Inc., Cary, NC). Significance of differences was determined by ANOVA and Duncan's multiple range test at the level of $p < 0.05$.

Results and Discussion

Antioxidant activity of KBRW extracts. The antioxidant activity of KBRW extracts was measured by lipid/MA assay. The antioxidant activity of the two assays was expressed as percentage inhibition. The antioxidant activities of the extracts compared with those of red wines evaluated by the lipid/MA assay are shown in Fig. 1. Assays of antioxidant activity were carried out in triplicate. Four red-wine extracts, RW-1, RW-2, RW-3, and RW-4, were used as a comparison group for the determination of the antioxidant activity of KBRW extracts. The lipid/MA assay is specific for measuring lipid peroxidation products, particularly MA (Wada and Ou, 2002). All samples at 0.5 mL inhibited MA formation by 38.0–88.0% (Fig. 1). KBRW-1 (Sanmaesu) and KBRW-2 (Bokbunja) showed greater inhibitory activities than the other extracts. This indicated that KBRW inhibited the formation of MA. Black raspberries have the highest anthocyanin content among berries (Wada and Ou, 2002). The identities of four anthocyanins in black raspberries were confirmed using HPLC-coupled photodiode array detection and reported the presence of an unidentified anthocyanin (Mazza and Miniati, 1993). In general, the flavylium cation form of the anthocyanins is stable under acidic conditions, but the structure changes under neutral and alkaline conditions and breaks down (Wada and Ou, 2002). When anthocyanins scavenge active oxygen or lipid hydroperoxide radicals, the structure is also broken down, thereby allowing the radicals showing antioxidative activity to be scavenged by the reaction products. Although additional data are needed to better understand this antioxidant activity, the present study confirms that KBRs and KBRWs represent a significant source of phenolic (especially anthocyanic) antioxidants.

Survival of ARPE-19 and HepG2 cells treated with KBRW compounds. Toxicity of KBRW was examined by MTT assay. The cytotoxic effects of Bokbunja, Sanmaesu, wine, and beer on the viability of ARPE-19 and HepG2 cells are shown in Table 2. The viabilities of ARPE-19 and HepG2 cells treated with Bokbunja

Table 2 Viability of ARPE-19 and HepG2 cell treated with ethanol-soluble compounds

		Bokbunja	Sanmaesu	Wine	Beer
ARPE-19	Control	100±0.38	100±0.78	100±1.07	100±3.31
	10	98.2±0.89.	101.67±1.14	99.09±0.31	90.27±4.16
	100	90.92±3.28	98.81±1.60	97.61±0.97	91.28±3.69
	500	84.07±5.73	97.81±0.62	97.94±2.73	87.63±3.93
HepG2	Control	100±0.43	100±0.95	100±0.41	100±7.45
	10	98.39±0.51	101.00±0.06	101.18±2.46	88.9±4.99
	100	97.39±1.88	101.17±0.27	97.94±3.05	90.61±4.4
	500	94.43±0.72	102.25±0.20	98.94±2.04	88.64±4.84

Both ARPE-19 and HepG2 cells were treated with KBRW for 24 h in various concentrations ranging from 0, 0.5, 1, 10, 50, 100, 200 to 500 μg/mL for 24 h. Data are means ± SEM, expressed as a percentage of control, $p < 0.05$ compared to control.

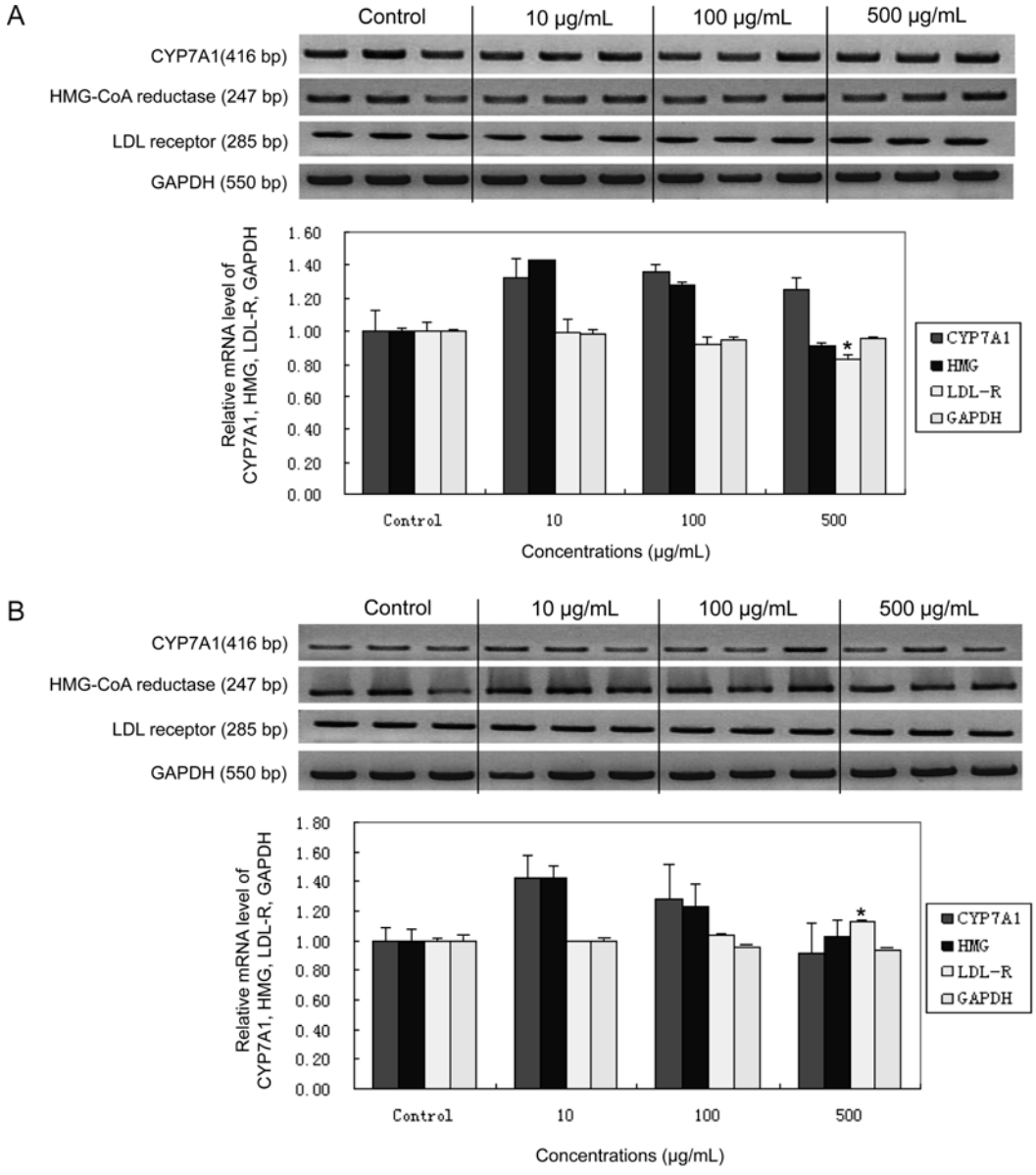

Fig. 2 Relative mRNA expression level for CYP7A1, HMG CoA reductase, LDL receptor, and GAPDH in ARPE-19 cells treated with (A) Bokbunja, (B) Sanmaesu, (C) wine, and (D) beer at 0, 10, 100, and 500 μg/mL for 24 h. The bar graph indicates the means ± SEM of three independent analyses. *, p <0.05 compared with non-treated control group.

was lower than those treated with Sanmaesu, beer, and wine at the same concentration; however, Bokbunja and Sanmaesu compounds were not toxic, even at 500 μg/mL, comparable to regular intake, although they did show some cytotoxicity at extremely high concentrations (data not shown). The gene expression changes were measured at concentrations at which no cellular toxicity occurred.

Effect of KBRW on expression of CYP7A1, HMG CoA reductase, LDL receptor and VEGF in ARPE-19 cells. ARPE-19 cells were treated with four types of ethanol-soluble compounds, Bokbunja, Sanmaesu, wine, and beer, at 0, 10, 100, and 500 μg/mL for 24 h. After isolation of total RNA and synthesis of cDNA, the expressions of CYP7A1, HMG CoA reductase, LDL receptor and VEGF were detected by RT-PCR (Fig. 2). CYP7A1 expression level was elevated significantly by >13% when treated with Bokbunja at 10, 100, and 500 μg/mL. Sanmaesu treatment did not affect CYP7A1, regardless of concentration. In contrast, HMG CoA reductase expression level was decreased when treated with Bokbunja and Sanmaesu at 100 μg/mL, and the expression level was higher than that with wine and lower than that with beer. In addition, LDL receptor expression level was increased by 5% when treated with Bokbunja and by 19% when treated with Sanmaesu at 10 μg/mL. When treated with all three concentrations

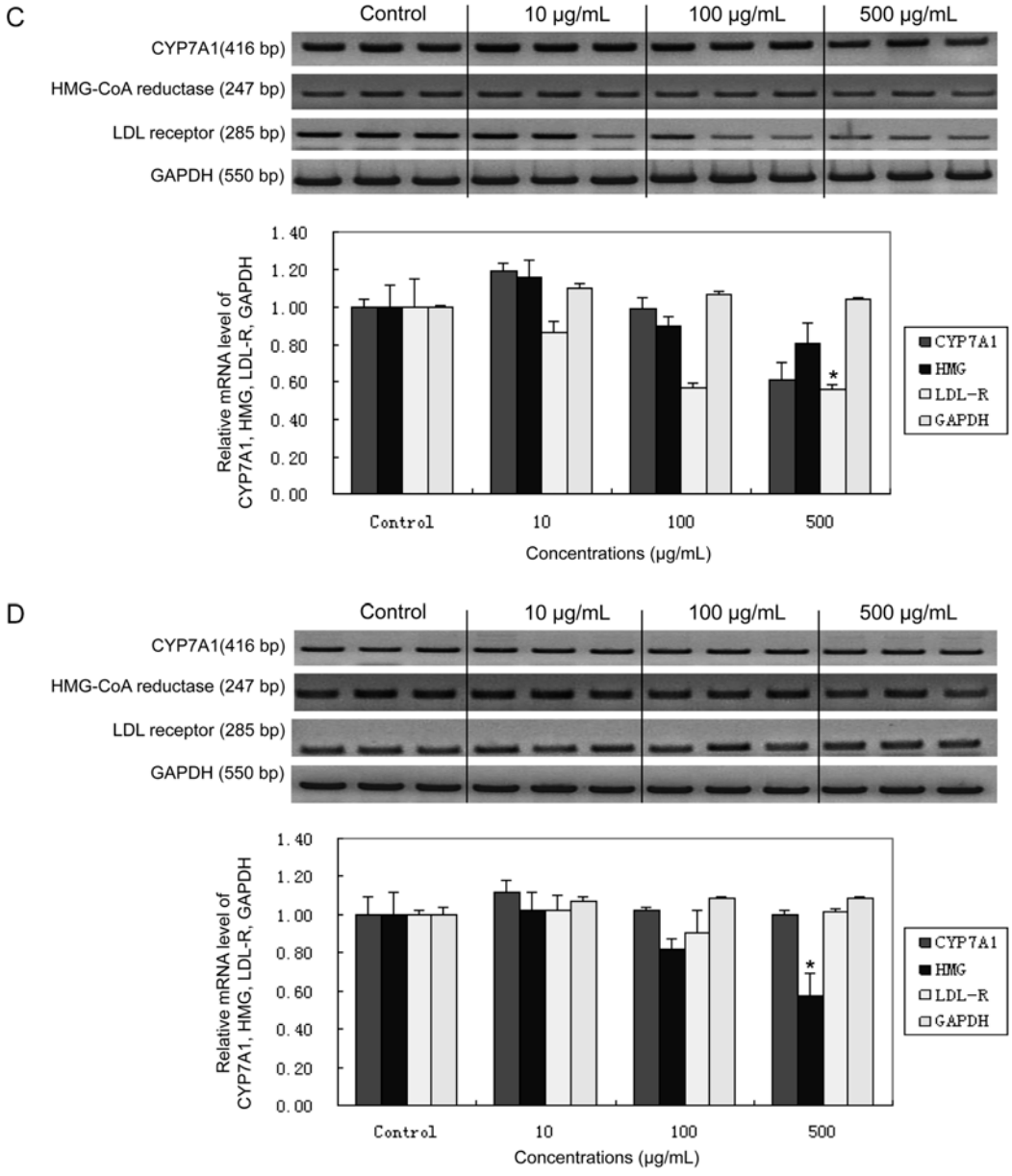

Fig. 2 Continued.

of Bokbunja, VEGF expression was reduced significantly (by 25%) and was almost the same as that treated with wine. However, VEGF expression was elevated by treatment with Sanmaesu at 100 and 500 μg/mL. The results showed that after treating the ARPE-19 cells with Bokbunja-soluble compound, CYP7A1 and LDL receptor expressions were elevated, HMG CoA reductase expression was decreased by both Bokbunja and Sanmaesu, and the expression of VEGF was decreased with Bokbunja but elevated with Sanmaesu treatment.

Effects of KBRW on expressions of CYP7A1, HMG CoA reductase, LDL receptor, and VEGF treatments in HepG2 cells. The treatment conditions for HepG2 cells were the same as those for ARPE-19 cells. CYP7A1 expression was decreased by 11% with Bokbunja treatment at 100 μg/mL, whereas increased significantly (>30%) by Sanmaesu at 10, 100, and 500 μg/mL. HMG CoA reductase level was elevated by both Bokbunja and Sanmaesu. Unexpectedly, LDL receptor expression level was increased by Sanmaesu, whereas was decreased by Bokbunja (Fig. 3). Compared with ARPE-19 cells, gene expression in HepG2 cells after treatment with Bokbunja and Sanmaesu was almost the same. CYP7A1 and HMG CoA expressions were elevated. LDL receptor expression was also elevated by Bokbunja but was decreased by Sanmaesu. Furthermore, the level of cholesterol can be decreased by reducing plasma cholesterol,

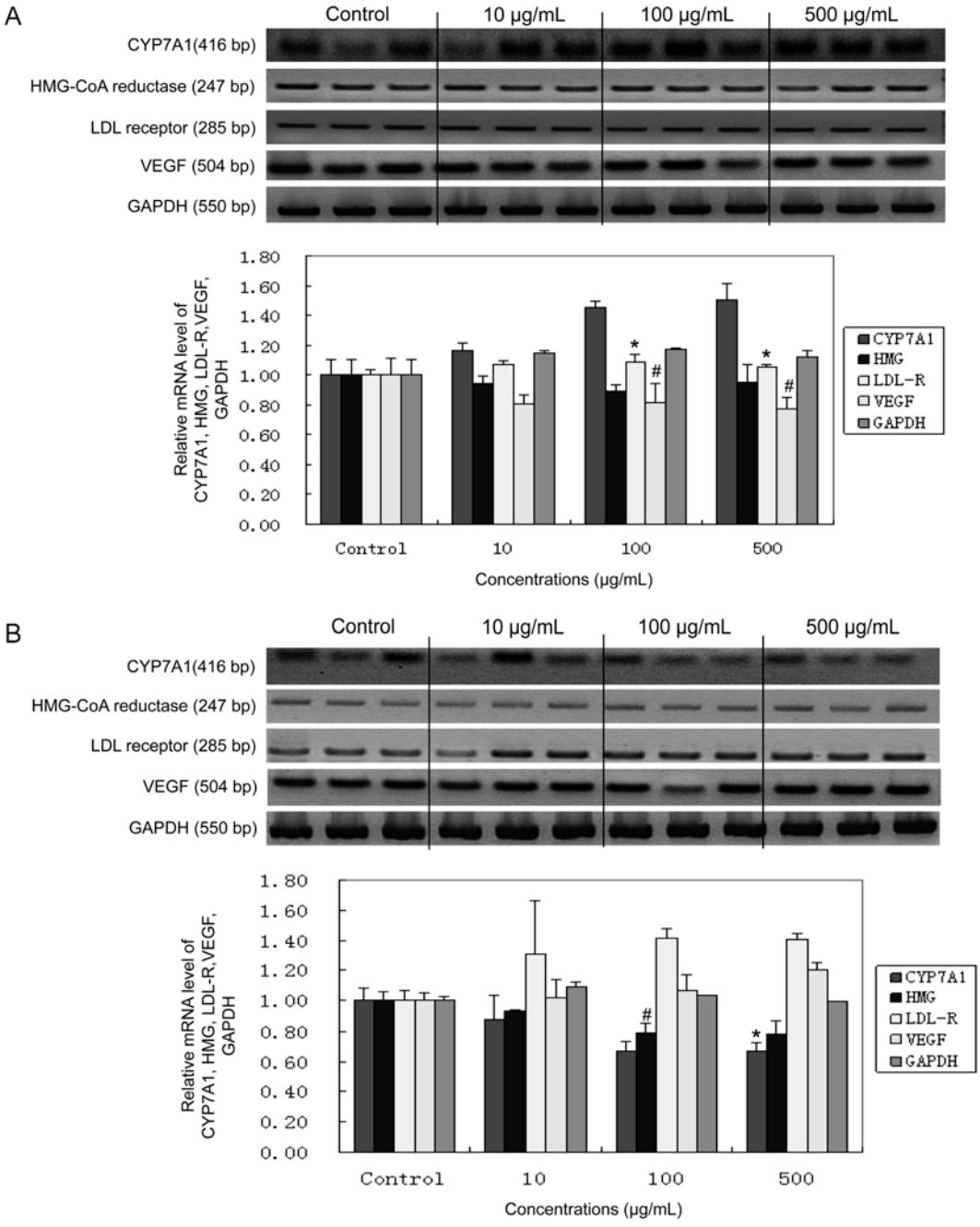

Fig. 3 Relative mRNA expression level of CYP7A1, HMG CoA reductase, LDL receptor, and GAPDH in HepG2 cells treated with (A) Bokbunja, (B) Sanmaesu, (C) wine, and (D) beer at 0, 10, 100, and 500 μg/mL for 24 h. The bar graph indicates the mean ± SEM of three independent analyses. *, $p < 0.05$ compared with non-treated control group. #, $p < 0.01$ compared with non-treated control group.

decreasing hepatic synthesis, or enhancing cholesterol degradation and excretion. The formation of bile acid from cholesterol in the liver is the major pathway of cholesterol reduction. In addition, CYP7A1 is a rate-limiting enzyme in the classical pathway of bile acid formation. Our results showed that CYP7A1 expression was elevated by treatment with Bokbunja and Sanmaesu in ARPE-19 cells, but was decreased in HepG2 cells. Bokbunja and Sanmaesu may have a role in reducing cholesterol but have no positive effect on human macular degeneration. Plasma cholesterol levels could be reduced by the induction of LDL receptor. In addition, inhibition of HMG CoA reductase or its expression could effectively control the hepatic cholesterol biosynthesis.

VEGF is an important signaling protein involved in both vasculogenesis and angiogenesis. *In vitro*, VEGF has been shown

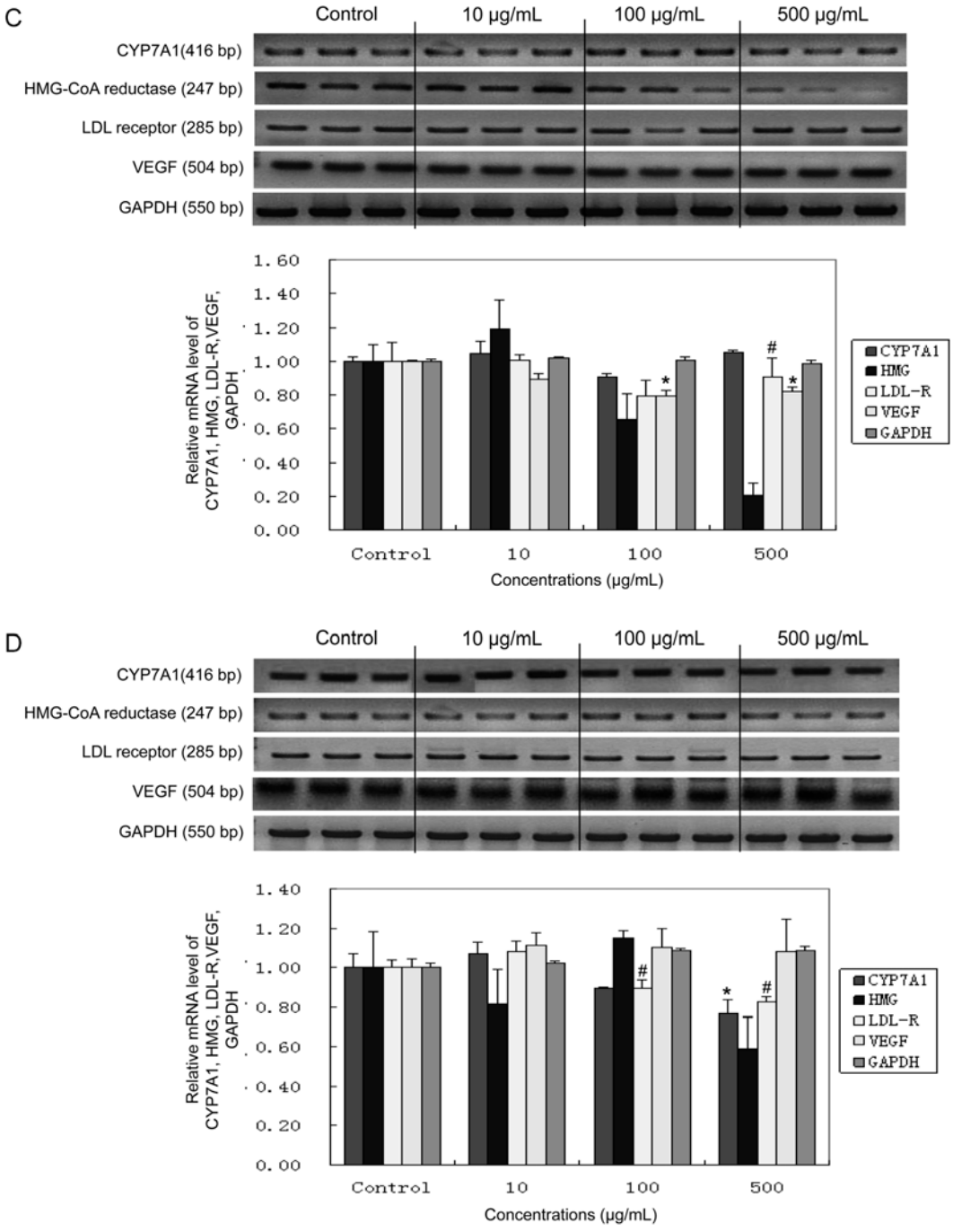

Fig. 3 Continued.

to stimulate endothelial cell mitogenesis and cell migration. In addition, VEGF is a vascular permeability factor and may increase microvascular permeability (Ferrara and Gerber, 2002; Caldwell et al., 2004). Compared with controls, the expression of VEGF was decreased when treated with Bokbunja but was elevated significantly by Sanmaesu treatment. HMG CoA reductase expression was elevated when 3T3-L1 fibroblasts cells were treated with Bokbunja (Choi and Myung, 2005). On the other hand, LDL receptor expression was decreased in HepG2 cells treated with Sanmaesu (Choi and Myung, 2005), and this was confirmed by our data.

Conclusion. The effects of Bokbunja and Sanmaesu were investigated on human ARPE-19 and HepG2 cells, using wine as a positive control and beer as a negative control. The toxicity of

KBRW was examined by MTT assay at concentrations that were not cytotoxic. CYP7A1, HMG CoA reductase, LDL receptor, and VEGF expression levels were detected by RT-PCR. Four types of amplified cDNAs, CYP7A1, HMG CoA reductase, LDL receptor, and VEGF, were detected in ARPE-19 and HepG2 cell lines by RT-PCR. Our results show that ethanol-soluble compounds from Bokbunja and Sanmaesu induce beneficial physiological changes in RPE cells and may prevent AMD by the following mechanisms: hypocholesterolemic effects caused by lowering of cellular cholesterol biosynthesis and lipoprotein uptake, and reduced vascular angiogenesis in RPE cells.

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References

Caldwell RB, Bartoli M, and Behzadian MA (2004) Vascular endothelial growth factor and diabetic retinopathy: Pathophysiological mechanisms and treatment perspectives. *Diabetes Metab Res Rev* **19**, 442–55.

Carlo GD, Mascolo N, Izzo AA, and Capasso F (1999) Flavonoids: old and new aspects of a class of natural therapeutic drugs. *Life Sci* **65**, 337–53.

Cha HS, Park MS, and Park KM (2001) Physiological activities of *Rubus coreanus* Miq. *Korean J Food Sci Technol* **33**, 409–15.

Choi H and Myung KH (2005) Comparative study of red wine and Korean black raspberry wine in adipocyte differentiation and cardiovascular disease-related gene expression. *Food Sci Biotechnol* **14**, 514–7.

Christian S, Fischer S, and Hass OA (2003) Quantitation of the sterol regulatory element-binding protein mRNA in mononuclear blood cells by competitive RT-PCR. *Clinica Chimica Acta* **336**, 27–37.

Chung MJ, Park KW, Kim KH, Kim CT, Baek JP, Bang KH, Choi YM, and Lee SJ (2008) Asian plantain (*Plantago asiatica*) essential oils suppress 3-hydroxy-3-methyl-glutaryl-co-enzyme A reductase expression *in vitro* and *in vivo* and show hypocholesterolaemic properties in mice. *Br J Nutr* **99**, 67–75.

Ferrara N and Gerber HP (2002) The role of vascular endothelial growth factor in angiogenesis. *Acta Haematol* **106**, 148–56.

Heinonen IM, Meyer AS, and Frankel EN (1998) Antioxidant activity of berry phenolics on human low-density-lipoprotein and liposome oxidation. *J Agric Food Chem* **46**, 4107–12.

Hertog MG, Feskens EJ, and Kromhout D (1997) Antioxidant flavonols and coronary heart disease risk. *Lancet* **349**, 699.

Kähkönen MP, Hopis AI, and Heinonen M (2001) Berry phenolics and their antioxidant activity. *J Agric Food Chem* **49**, 4076.

Kaur C and Kapoor HC (2001) Antioxidants in fruits and vegetables-the millennium's health. *Int J Food Sci Technol* **36**, 720–5.

Kim JS, Ko SH, Lee WY, and Kim GW (2004) Cytotoxic effects of Korean Rice-Wine (*Yakju*) on cancer cells. *Korean J Food Sci Technol* **36**, 812–7.

Kim SH, Chung HG, Jang YS, Park YK, Park HS, and Kim SC (2005) Characteristics and screening of antioxidative activity for the Fruit by *Rubus coreanus* Miq. *Clone. J Korean Forest Soc* **94**, 11–5.

Kim SJ, Baek JY, Park CK, and Kim GW (2004) Gastroprotective effect of Korean Rice-Wine (*Yakju*). *Korean J Food Sci Technol* **36**, 818–22.

Kim YH and Kang SS (1993) Trietpenoids from *Rubi Fructus* (Bokbunja). *Arch Pharm Res* **76**, 109–13.

Kwon KH, Cha WS, Kim DC, and Shin HJ (2006) A research and application of active ingredients in Bokbunja (*Rubus coreanus* Miquel). *Korean J Biotechnol Bioeng* **21**, 405–9.

Larson RA (1998) The antioxidants of higher plants. *Phytochem* **4**, 969–978.

Lee KH and Choi EM (2006) *Rubus coreanus* Miq. Extract promotes osteoblast differentiation and inhibits bone-resorbing mediators in MC3-3-E1cells. *Am J Chinese Med* **34**, 643–54.

Lee MK, Lee HS, Choi GP, Oh DH, Kim JD, Yoo CY, and Lee HY (2003) Screening of biological activity of the extract of *Rubus coreanus* Miq. *Korean J Medical Crop Science* **11**, 5–12.

Lee MW (1995) Phenolic compounds from the leaves of *Rubus coreanum*. *Yakhak Hoeji* **39**, 200–4.

Lee SJ, Kim JH, Kim JH, Chung MJ, Wen QC, Chung H, Kim KY, and YU YS (2007) Human apolipoprotein E2 transgenic mice show lipid accumulation in Retin l pigment epithelium and altered expression of VEGF and bFGF in the eyes. *J Microbiol Biotechnol* **17**, 1024–30.

Lee YA and Lee MW (1995) Tannins from *Rubus coreanum*. *Korean J Pharmacogn* **26,** 27–30.

Mazza G and Miniati E (1993) In *Anthocyanins in Fruits, Vegetables, and Grains*. pp. 211–12, CRC Press, Boca Raton, FL.

Moon GS (1991) In *Constituents and Uses of Medicinal Herbs*. pp. 310–11. Ilweolseogak, Seoul, Korea.

Ohta Y, Endo Y, Tanaka M, Shimizu J, Oda M, Hayashi Y, Watanabe Y, and Sasaki T (1996) Significance of vascular endothelial growth factor messenger RNA expression in primary lung cancer. *Clin Cancer Res* **2**, 1411–6.

Ohtani K, Miyajima C, Takahasi T, Kasai R, Tannaka O, Hahn DR, and Naruhasi N (1990) A dimeric triterpene-glycoside from *Rubus coreanus*. *Phytochem* **29**, 3275–80.

Pal S, Ho N, Santos C, Dubois P, Mamo J, Croft K, and Allister E (2003) Red wine pholiphenlicis increase LDL receptor expression and activity and suppress the secretion of ApoB 100 from human HepG2 cells. *J Nutr* **133**, 700–6.

Sabine A, Zbang M, Bacber D, Yee J, Brunken WJ, and Hunter DD (2006) Retinal pigment epithelial cells synthesize laminins, including laminen 5, and adhere to them through α3- and α6-containing integrins. *IOVS* **47**, 5337–44.

Tomas-Barberan FA, Gil MI, Ceremin P, Waerhous AL, Hess-Pierce B, and Kader AA (2001) HPLC-DADESIMS analysis of phenolic compounds in nectarines, peaches, and plums. *J Agric Food Chem* **49**, 4748–60.

Vasantha Rupasinghe HP and Clegg S (2007) Total antioxidant capacity, total phenolic content, mineral elements, and histamine concentrations in wines of different fruit sources. *J Food Composition Anal* **20**, 133–7.

Vinson JA, Xuehui S, Ligia Z, and Bose P (2001) Phenol antioxidant quantity and quality in foods: Fruits. *J Agric Food Chem* **49**, 5315–21.

Wada L and Ou B (2002) Antioxidant activity and phenolic content of Oregon caneberries. *J Agric Food Chem* **50**, 3495–500.

Yoon I, Cho JY, Kuk JH, Wee JH, Jang MY, and Ahn TH (2001) Identification and activity of antioxidative compounds from *Rubus coreanum* Fruit. *Korean J Food Sci Technol* **34**, 898–904.

Yoon I, Wee JH, Moon JH, Ahn TH, and Park KH (2003) Isolation and identification of quercetin with antioxidative activity from the fruits of *Rubus coreanus* Miq. *Korean J Food Sci Technol* **35**, 499–502.