Food Sci. Biotechnol. Vol. 16, No. 3, pp. 439 ~ 443 (2007)

Food Science and Biotechnology

Effects of the Methanol Extract of the Leaves of *Brassica juncea* and Its Major Component, Isorhamnetin 3-O-β-D-Glucoside, on Hepatic Drug Metabolizing Enzymes in Bromobenzene-treated Rats

Jong Moon Hur, Jong Won Choi[1], and Jong Cheol Park*

Department of Oriental Medicine Resources and Research Institute of Korean Oriental Medicine, Sunchon National University, Suncheon, Jeonnam 540-742, Korea

[1]Department of Pharmacy, Kyungsung University, Busan 608-706, Korea

Abstract The effects of the methanol extract of the leaves of *Brassica juncea* and isorhamnetin 3-O-β-D-glucopyranoside, major compound isolated from the ethyl acetate fraction of this plant on hepatic lipid peroxidation and drug-metabolizing enzymes, were evaluated in rats treated with bromobenzene. The extract and isorhamnetin 3-O-β-D-glucopyranoside of oral administration did not show any significant effects on activities of aminopyrine *N*-demethylase and aniline hydroxylase, enzymes forming toxic epoxide by bromobenzene as well as on glutathione content. However, both methanol extract and isorhamnetin 3-O-β-D-glucopyranoside significantly recovered the decreased activities of glutathione *S*-transferase and epoxide hydrolase, and also reduced the lipid peroxide level in rats treated with bromobenzene. From the results, the protections of this plant against bromobenzene-induced hepatotoxicity are thought to be via enhancing the activities of epoxide hydrolase and glutathione S-transferase, enzymes removing toxic epoxide, and reducing the lipid peroxide level.

Keywords: *Brassica juncea*, isorhamnetin 3-O-β-D-glucopyranoside, bromobenzene, glutathione S-transferase, epoxide hydrolase

Introduction

Three types of mustards are *Sinapsis alba* (white mustard), *Brasscia juncea* (brown or oriental mustard), and *Brasscia nigra* (black mustard), which belong to Cruciferaceae family (1). Apart from their seeds has been popularly used as a spicy, mustard leaves are widely used as green vegetables such as salad and *kimchi*.

The seeds of *B. juncea* (Korean name; *gat*) have been used as a spice and in traditional folk medicine as a treatment agent of several diseases such as arthritis, lumbago, dyspepsia, neuralgia, and rheumatism (2). And the leaves of this plant are used as a major or supplemental ingrident of *kimchi* which is a traditional fermented vegetable food in Korea. The *kimchi* made of the leaves of *B. juncea* is usually called by *gatkimchi* (3) and in watery radish *kimchi* (*dongchimi*), comprised over 30% of the constituents (4).

From the leaves of *B. juncea*, flavonoids (5) such as kaempferol and isorhamnetin glycosides including isorhamnetin 3-*O*-β-D-glucopyranoside (IG), β-carotene (6), and essential oils (7) were previously isolated, and several biological activities such as antifungal (7) and antioxidant (8, 9) effects, effects on cholesterol metabolism (10) in rats, and protective effects on renal ischemia-reperfusion in rats (11) and on diabetic oxidative stress (12). No study has been conducted yet on the effect of this plant on hepatic drug metabolizing emzymes.

Xenobiotic chemicals are mainly metabolized by hepatic drug metabolizing enzymes to either toxic intermediates or nontoxic metabolites. The main metabolizing enzymes are microsomal cytochrome P450 (CYP) and glutathione *S*-transferase (GST), which are responsible for the phase I oxidation-reduction reactions and phase II conjugation reactions. The oxidation of xenobiotics sometimes converts into toxic metabolites.

Bromobenzene (BB) is metabolized into toxic epoxide intermediate, BB 3,4-oxide by hepatic CYP mono-oxygenase. And then this toxic epoxide readily conjugate with glutathione (GSH) to result in the depletion of the hepatic GSH content and covalently bind to cellular macromolecules, which cause lipid peroxidation in membrane phospholipids associated with hepatic cell death. The toxic intermediate can be detoxified either by hydration to 3,4-dihydrodiol by epoxide hydrolase (EH) or by conjugation with reduced GSH catalyzed by GST.

In the present study, effects of the methanol extract (ME) of the leaves of *B. juncea* and IG, one of major component of this plant on lipid peroxidation and the drug metabolizing enzymes were investigated in the liver of rats treated with BB.

Materials and Methods

Plant material The leaves of *B. juncea* were purchased from a farmer, Dolsan region, Yeosu, Jeonnam, South Korea in April, 2005. A voucher specimen has been deposited at the Herbarium of Sunchon National University.

Extraction and isolation of IG The air-dried leaves of *B. juncea* (3.7 kg) was refluxed with methanol 4 times for 3 hr and evaporated to gain extract (996 g). This extract was successively partitioned with dichloromethane, ethyl

*Corresponding author: Tel: 82-61-750-3662; Fax: 82-61-750-3608
E-mail: flavonoid@empal.com
Received January 17, 2007; accepted February 26, 2007

acetate, *n*-butanol, and aqueous fraction using organic solvents of different polarities. The ethyl acetate fraction (18 g) was carried out silica gel (70-230 mesh) column (6.3×70 cm) chromatography by elution with CH_2Cl_2 and CH_2Cl_2-CH_3OH-H_2O mixtures (5:1:1 → 65:35:10, lower layer) as elution solvents to gain eight fractions (BJ1-8). And the BJ8 fraction was chromatographed on a Sephadex LH-20 column with acetone to yield 2 subfractions (BJ8A and B). BJ8A fraction was recrystalized with methanol to gain IG (947 mg, Fig. 1) as one of major component of this plant and its structure was elucidated by the comparison NMR spectral data with those of reference (13). And its nuclear magnetic resonance (NMR) data was summarized as follow.

^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 7.93 (1H, d, J=2.04 Hz, H-2'), 7.50 (1H, dd, J=2.04 & 8.4 Hz, H-6'), 6.92 (1H, d, J=8.4 Hz, H-5'), 6.46 (1H, d, J=2.04 Hz, H-8), 6.22 (1H, d, J=2.04 Hz, H-6), 5.56 (1H, d, J=7.4, anomeric H of glucose), 3.84 (3H, s, -OCH_3); ^{13}C-NMR (DMSO-d_6, 100.5 MHz) δ: 177.4 (C-4), 164.1 (C-7), 161.2 (C-5), 156.4 (C-2), 156.3 (C-9), 149.3 (C-3'), 146.9 (C-4'), 132.9 (C-3), 122.0 (C-6'), 121.0 (C-1'), 115.2 (C-2'), 113.4 (C-5'), 104.0 (C-10), 100.7 (C-1"), 98.7 (C-6), 93.7 (C-8), 77.3 (C-3"), 76.3 (C-5"), 74.3 (C-2"), 69.8 (C-4"), 60.6 (C-6"), 55.6 (-OCH_3).

Animals Male Sprague-Dawley rats, 6 to 7 weeks old weighing about 180-200 g, were used for the study. Animals were fed with commercial standard rat diet and water *ad libitum*, and maintained at 20±2°C and with a 12 hr light/dark cycle. Animals were orally administered daily with ME (250 or 500 mg/kg) or IG (1, 3, or 5 mg/kg) for one week. Ascorbic acid (20 mg/kg, AA) was used as a reference compound. Samples were dissolved in 1% Tween 80. Normal group was given 0.2 mL of 1% Tween 80 solution per 200 g.

Induction of hepatic injury BB (460 mg/kg) was *i.p.* injected four times with 12 hr interval during final 2 days of the oral treatment of ME, IG, or AA. The animals were starved overnight before sacrificed in order to reduce the variation of hepatic metabolism. Animals were killed through exsanguinations under anesthesia condition induced by CO_2 gas after 12 hr since final BB injection.

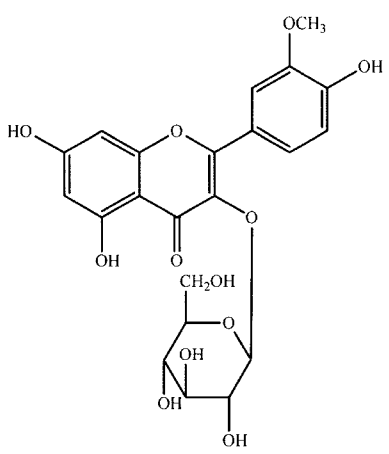

Fig. 1. Structure of isorhamnetin 3-*O*-β-D-glucopyranoside.

Enzyme source The liver was immediately removed and weighed after exhaustive perfusion with ice-cold saline through portal vein until uniformly pale. After trimmed and minced, the liver was homogenized with 4 volumes of ice cold 0.1 M potassium phosphate buffer (pH 7.5). The homogenate was centrifuged at 600×g for 10 min. The resulting supernatant was recentrifuged at 10,000 ×g for 20 min, and the supernatant was further centrifuged at 15,000×g for 60 min to obtain the upper fraction as cytosol. The pellet was resuspended in the same volume of 0.1 M potassium phosphate buffer and centrifuged at 15,000×g for 60 min to obtain the microsomal fractions.

The cytosolic fraction was used as the enzyme sources of GST. And the microsomal fraction was used for the measurement of the activities of aminopyrine *N*-demethylase (AD), aniline hydroxylase (AH), and EH.

Enzyme assays ***AD***: AD activity was assayed by measuring the production of formaldehyde formed by the demethylation of aminopyrine (14). The reaction mixture consisted of 300-400 μg microsomal protein, 0.1 M Na^+/K^+ phosphate buffer (pH 7.5) and 2.0 mM aminopyrine in a total volume of 2.0 mL. The mixture was preincubated for 3 min at 37°C and 0.5 mM NADPH was added to initiate the reaction. After 30 min the reaction was stopped by the addition of 0.5 mL of 15% $ZnSO_4$ and saturated $Ba(OH)_2$, and cooled at room temperature. After centrifugation at 1,000×g for 10 min, 1 mL of the supernatant was mixed with 5 mL of Nash reagent. Then the tubes were capped and heated at 60°C for 30 min. After cooling in tap water, the absorbance was read at 415 nm against water blank. The activity was expressed as nmol of formaldehyde per mg protein per min.

AH: AH activity was assayed by determining *p*-aminophenol formation from aniline (15). The basic incubation system was same as described above, except that 1 mM aniline was used as the substrate. The reaction was initiated by the addition of 0.5 mM NADPH. After shaking for 30 min at 37°C, the reaction was terminated by the addition of 0.5 mL of 20% trichloroacetic acid. The mixture was centrifuged at 1,000×g for 10 min and 1 mL of the supernatant was added to 1 mL of 0.2 N NaOH containing 2% phenol. After mixing, 1 mL of 10% Na_2CO_3 was added. After 20 min at room temperature the absorbance was read at 640 nm against a water blank. The activity was expressed as nmol of *p*-aminophenol per mg protein per min.

GST: GST activity was assayed by conjugated glutathione 2,4-dinitrobenzene formation from 1-chloro-2,4-dinitrobenzene (16). The reaction mixture consisted of 100 μL cytosol fraction, 0.1 M potassium phosphate buffer (pH 6.5), 1 mM 1-chloro-2,4-dinitrobenzene, and 1 mM glutathione in a total volume of 3.5 mL. The mixture was incubated at 25°C for 2 min. The measurement was carried out by spectrometric changes at maximal absorbance wavelength (340 nm) per unit time and calculated with molar extinction coefficient (9.6/mM/cm). The activity was expressed as nmol of 1,2-dinitro-4-nitro-benzene per mg protein per min.

EH: Epoxide hydrolase activity was measured spectro-

photometically by monitoring the rate of decrease in trans-stilbene oxide (TSO) at 229 nm (17). The reaction mixture was consisted of 100-200 μg microsomal protein and 3.0 mM TSO in 0.05 M potassium phosphate buffer (pH 7.0) with a total volume of 3.0 mL. The mixture was incubated for 20 min at 37°C. The activity was defined as nmol of TSO per mg protein per min.

Lipid peroxide level: As a marker for lipid peroxidation the content of malondialdehyde (MDA) the liver was measured (18). Mixture of 0.4 mL of 10% liver homogenate, 1.5 mL of 8.1% sodium dodecyl sulphate, 1.5 mL of 20% acetate buffer (pH 3.5) and 1.5 mL of 0.8% TBA solution was heated at 95°C for 1 hr. After cooling, 5.0 mL of *n*-butanol-pyridine (15:1) was added for extraction, and the absorbance of the *n*-butanol-pyridine layer was measured at 532 nm.

GSH content: Reduced GSH content of liver was measured by a colorimetric method (19). Mixture of 0.5 mL of liver homogenate and 0.5 mL of 4% sulfosalicylic acid was centrifuged at 2,500×g for 10 min. To 0.3 mL of the resulting supernatant 2.7 mL of disulfide reagent was added and absorbance at 412 nm was measured after standing at room temperature for 20 min.

Protein assay: Protein content was determined by Lowry method (20) using bovine serum albumin as a standard. Statistical differences between the experimental groups were determined by using analysis of variance and Duncan's multiple range tests.

Results and Discussion

The seed of *B. juncea* has been popularly used as a spice because of its pungent taste. The glucosinolates such as sinigrin, major constituent of this plant seed were hydrolyzed by enzyme (myrosinase) to yield allyisothiocyantes which produce burning sensation on the tongue (1). Recently, many studies have been conducted the chemical composition and biological activities for the *gatkimchi* as well as raw leaves of *B. juncea* (6, 8-12, 21, 22). *B. juncea* has been in large quantity cultivated in the Dolsan region, Yeosu, South Korea and *gatkimchi* made in this region are very popular.

In the present study, ME of the leaves of *B. juncea* collected in the region of Dolsan and its major compound, IG, were investigated for effects on the enzyme activities associated with hepatic drug metabolism in rats treated with BB.

In the BB metabolism, the nontoxic BB 2,3-epoxide readily forms 2-bromophenol and toxic BB 3,4-epoxide are produced by oxidation induced CYP monooxygenases such as AD and AH. The toxic BB 3,4-oxide covalently bind with GSH and protein thiol groups or is further metabolized into bromophenol. The postulated toxic mechanisms induced by reactive metabolites of BB result from arylation of critical cellular macromolecules, imbalance of Ca^{2+} homeostasis, and lipid peroxidation in membrane phospholipids associated with hepatic cell death (23).

Several pathways can protect the hepatic toxicity induced by BB 3,4-oxide; rearrangement to the 4-bromophenol spontaneously, hydration to the 3,4-dihydrodiol by EH and conjugation with GSH resulting in the formation of mercapturic acid.

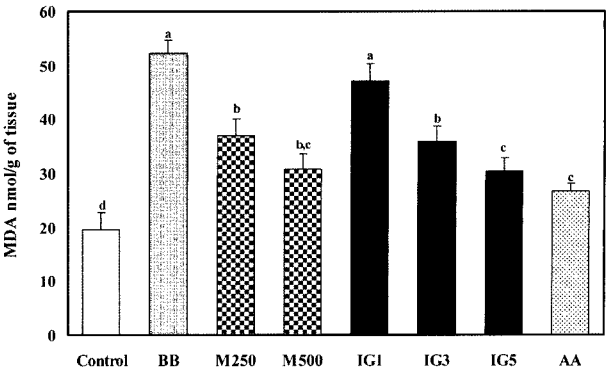

Fig. 2. Hepatic lipid peroxide contents of bromobenzene (BB)-intoxicated rats pretreated with the methanol extract (ME) and isorhamnetin 3-O-β-D-glucopyranoside (IG), major comopound of the leaves of *B. juncea*. Each bar represents the mean± SD ($n = 6$). Values followed by the same letter are not significantly different ($p < 0.05$).

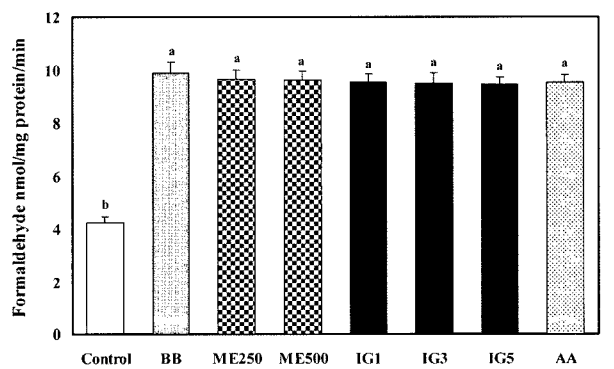

Fig. 3. Aminopyrine *N*-demethylase activities in the liver of BB-intoxicated rats treated with the ME and IG, major comopound of the leaves of *B. juncea*. Each bar represents the mean±SD ($n = 6$). Values followed by the same letter are not significantly different ($p<0.05$).

Hepatic lipid peroxide levels in the BB-intoxicated rats with ME (250 or 500 mg/kg) or IG (1, 3, or 5 mg/kg) isolated from this plant are shown in Fig. 2. Lipid peroxide levels (MDA) were elevated by BB treatment, oral administration of ME (500 mg/kg, 41% of BB) or IG (5 mg/kg, 42% of BB) provided mild protection against lipid peroxidation. However, AA (20 mg/kg, 49% of BB) used as a positive control showed similar effect of ME or IG. Figure 3 and 4 show the effects of ME (500 mg/kg) or IG (5 mg/kg) on hepatic AD and AH activities increased by BB injection. These two hepatic enzymes activities of rat increased with BB treatment were not affected by pretreatment of ME or IG in all concentrations tested. However, the hepatic EH activity (Fig. 5) lowered than that of normal control in the BB-treated rat was greatly recovered by pretreatment with ME or IG into 68 (ME, 500 mg/kg) or 84% (IG, 5 mg/kg) of normal value activity in a dose dependent manner.

The GST family enzymes serve as catalysts for the reaction of electrophilic compounds with glutathione. BB treatment induced a marked decrease in hepatic and an

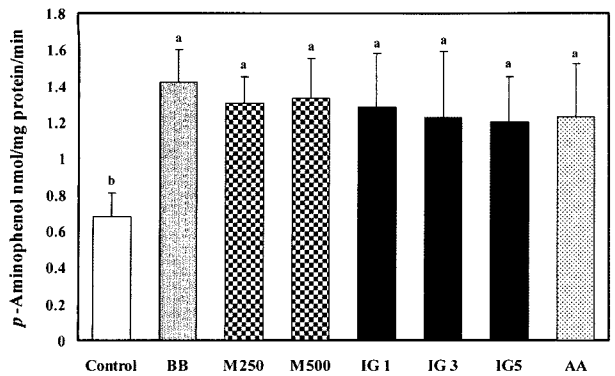

Fig. 4. Aniline hydroxylase activities in the liver of BB-intoxicated rats treated with the ME and IG, major comopound of the leaves of *B. juncea*. Each bar represents the mean±SD (n = 6). Values followed by the same letter are not significantly different (p<0.05).

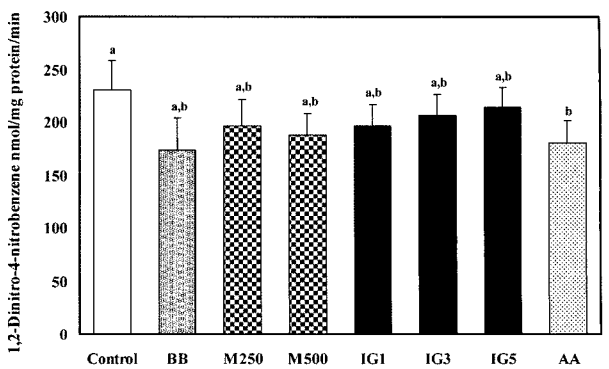

Fig. 6. Glutathione *S*-transferase activities in the liver of BB-intoxicated rats treated with the ME and IG, major comopound of the leaves of *B. juncea*. Each bar represents the mean±SD (n = 6). Values followed by the same letter are not significantly different (p<0.05).

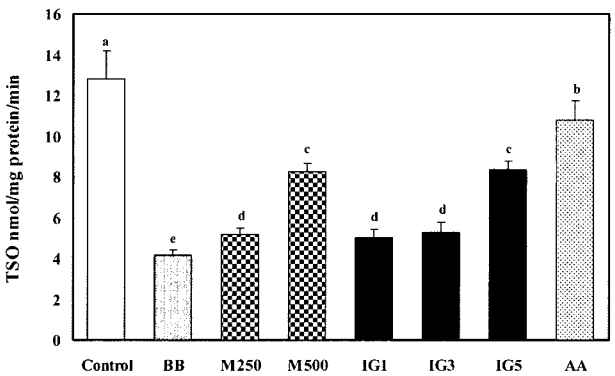

Fig. 5. Epoxide hydrolase activities in the liver of BB-intoxicated rats treated with the ME and IG. Each bar represents the mean±SD (n = 6). Values followed by the same letter are not significantly different (p<0.05).

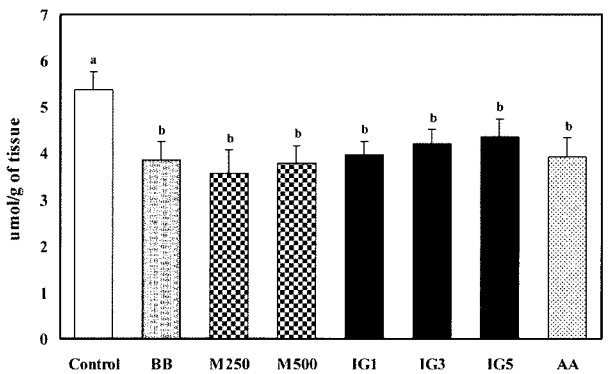

Fig. 7. Hepatic glutathione contents in the liver of BB-intoxicated rats treated with the ME and IG, major comopound of the leaves of *B. juncea*. Each bar represents the mean±SD (n = 6). Values followed by the same letter are not significantly different (p<0.05).

increase in serum GST (25).

As shown in Fig. 6, the hepatic GST activity decreased by BB treatment was greatly recovered by pretreatment with ME or IG while AA used as a positive control gave no effect on GST activity.

GSH plays an important role in numerous cellular functions such as DNA synthesis and regulation of cytosolic Ca^{2+} homeostasis (26, 27). And GSH works together with GST and glutathione reductase, in detoxifying toxic intermediates and maintaining cellular glutathione status. Accordingly, the homeostasis of hepatic mitochondrial glutathione level plays important role in cell survival (28). However, Oral administration of ME or IG did not show any significant effect on the GSH content level (Fig. 7).

In conclusion, ME of the leaves of *B. juncea* and its major component, IG were significantly recovered the decreased activities of EH and GST associated with detoxifying the BB 3,4-oxide, and prevented the lipid peroxidation. IG can be considered as one of the active compounds responsible for the activities of *B. juncea* on hepatic drug metabolizing enzymes in rat treated with BB, and also be a candidate for the new hepatoprotective agent originated from medicinal plants such as cynaroside (lutelolin 7-O-β-D-glucoside) isolated from *Angelica keiskei* (29), showing the effects on the lipid peroxide level and the EH activity. And further studies using the experimental methods of molecular biology will help to elucidate the mechanism of the compound.

Acknowledgments

One of the authors (Park, JC) wishes to thank Planning and Promotion Unit for *Kimchi* Industry, RIS (2006).

References

1. Thomas J, Kuruvilla KM, Hrideek TK. Mustard. Vol. 2, pp. 193-201. In: Handbook of Herbs and Spices. Peter KV (ed). CRC Press, Inc., Boca Raton, FL, USA (2000)
2. Kimura T, But PPH, Guo JX, Sung CK. International collation of traditional and folk medicine. Vol. 3. World Scientific Publishing Co., Singapore, Singapore. pp. 54-55 (1996)
3. Editor Committee. Encyclopedia of *Kimchi*. Yuhanmunhwasa, Seoul, Korea. pp. 133-150 (2004)
4. Park JE, Moon SW, Jang MS. Physicochemical properties of

dongchimi added with *gat* (*Brassica juncea*). Food Sci. Biotechnol. 14: 21-27 (2005)
5. Choi JS, Kim JE, Kim JI, Cheigh, HS, Yokozawa T. A flavonol diglucoside from the leaves of *Brassica juncea*. Nat. Prod. Sci. 6: 199-200 (2000)
6. Song, ES, Jeon YS, Cheigh HS. Isolation of chlorophyll derivatives and β-carotene from mustard leaf and their antioxidant activities on the lipid peroxidation. J. Korean Soc. Food Sci. Nutr. 30: 377-381 (2001)
7. Shin SW, Kang CA. Studies on composition and antifungal activities of essential oils from cultivars of *Brassica juncea* L. Korean J. Pharmacogn. 32: 140-144 (2001)
8. Yokozawa T, Kim HY, Cho EJ, Choi JS, Chung HY. Antioxidant effects of isorhamnetin 3,7-di-o-β-glucopyranoside isolated from mustard leaf (*Brassica juncea*) in rats with streptozotocin-induced diabetes. J. Agr. Food Chem. 50: 5490-5495 (2002)
9. Kim HY, Yokozawa T, Cho EJ, Cheigh HS, Choi JS, Chung HY. *In vitro* and *in vivo* antioxidant effects of mustard leaf (*Brassica juncea*). Phytother. Res. 17: 465-471 (2003)
10. Jo YS, Park JR, Park SK. Effects of mustard leaf (*Brassica juncea*) on cholesterol metabolism in rats. Korean J. Nutr. 26: 13-20 (1993)
11. Yokozawa T, Kim HY, Cho EJ, Choi JS. Protective effects of the BuOH fraction from mustard leaf in a renal ischemia-reperfusion model. J. Nutr. Sci. Vitaminol. 48: 384-389 (2002)
12. Yokozawa T, Kim HY, Cho EJ, Yamabi N, Choi JS. Protective effects of mustard leaf (*Brasscia juncea*) against diabetic oxidative stress. J. Nutr. Sci. Vitaminol. 49: 87-93 (2003)
13. Lim SS, Jung YJ, Hyun SK, Lee YS, Choi JS. Rat lens aldose reductase inhibitory constituents of *Nelumbo nucifera* stamens. Phytother. Res. 20: 825-830 (2006)
14. Nash T. The colorimetric estimation of formaldehyde by means of the Hentisch reaction. J. Biol. Chem. 55: 412-416 (1953)
15. Bidlack WR, Lowery GL. Multiple drug metabolism: *p*-nitroanisole reversal of acetone enhanced aniline hydroxylation. Biochem. Pharmacol. 31: 311-317 (1982)
16. Habig WH, Pabist MJ, Jakoby WB. The first step in mercapturic acid formation. J. Biol. Chem. 249: 7130-7139 (1974)
17. Hammock BD, Hasegawa LS. Spectrophotometric assay for mammalian cytosolic epoxide hydrolase using *trans*-stilbene oxide as the substrate. Biochem. Pharmacol. 31: 1979-1984 (1982)
18. Ohkawa H, Ohishi N, Yaki K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95: 351-358 (1979)
19. Boyne AF, Ellman GL. A methodology for analysis of tissue sulfhydryl components. Anal. Biochem. 46: 639-653 (1972)
20. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275 (1951)
21. Pyo YH, Kim JS, Hahn YS. Volatile compounds of mustard leaf (*Brassica juncea*) *kimchi* and their changes during fermentation. Korean J. Food Sci. 32: 56-61 (2000)
22. Choi YY, Yoo EJ, Lim HS, Kang DS, Nishizawa N, Choi MR. The relationship between physiological activity and cell number in Dolsan leaf mustard *kimchi* (*Brassica juncea*). J. Food Sci. Nutr. 6: 117-121 (2001)
23. Jollow DJ, Mitchell JR, Zampaglione N, Gillette JR. Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3,4-bromobenzene oxide as the hepatotoxic metabolite. Pharmacology 11: 151-169 (1974)
24. Caymañska JA. Hepatotoxicity of brominated benzenes: relationship between chemical structure and hepatotoxic effects in acute intoxication of mice. Arch. Toxicol. 72: 93-103 (1998)
25. Aniya Y, Anders MW. Alteration of hepatic glutathione *S*-transferases and release into serum after treatment with bromobenzene, carbon tetrachloride, or *N*-nitrosodimethylamine. Biochem. Pharmacol. 34: 4239-4244 (1985)
26. Meister A, Anderson ME. Glutathione. Annu. Rev. Biochem. 52: 711-760 (1983)
27. Smith CV, Jones DP, Guenthner TM, Lash LH, Lauterburg BH. Compartmentation of glutathione: implications for the study of toxicity and disease. Toxicol. Appl. Pharmacol. 140: 1-12 (1996)
28. Pascoe GA, Reed DJ. Cell calcium, vitamin E, and the thiol redox system in cytotoxicity. Free Radical Bio. Med. 6: 209-224 (1989)
29. Park JC, Park JG, Kim HJ, Hur JM, Lee JH, Sung NJ, Chung SK, Choi JW. Effects of extract from *Angelica keiskei* and its component, cynaroside on the hepatic bromobenzene-metabolizing enzyme system in rats. Phytother. Res. 16: 24-27 (2002)