

Full Length Research Paper

Anti-oxidant and anti-diabetic activities of *Lespedeza cuneata* water extract

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Received 6 June, 2014; Accepted 11 July, 2014

Oxidative damage induced by free radicals are the attendant causes of many chronic diseases. The purpose of this study was to investigate whether *Lespedeza cuneata* water extract (LCW) has anti-oxidant and anti-diabetic activities *in vitro*. Anti-oxidant activity was measured by superoxide anion (O_2^-), hydroxyl ($\cdot OH$) radical, nitric oxide (NO) and peroxynitrite ($ONOO^-$) scavenging assays. Further, radical-mediated protein oxidation was measured by an allophycocyanin assay. Alpha-glucosidase, dipeptidyl peptidase (DPP)-IV and anti-glycation assays were conducted to measure its anti-diabetic activities. LCW significantly inhibited O_2^- , $\cdot OH$ and $ONOO^-$ formation. LCW strongly protected radical-induced allophycocyanin degradation during 60 min from the initiation of the reaction. LCW significantly inhibited α -glucosidase, DPP-IV and advanced glycation end product formation. Our results suggest that LCW had both anti-oxidant and anti-diabetic activities, and could be used for the prevention and treatment of oxidative stress and diabetes.

Key words: Free radicals, dipeptidyl peptidase (DPP)-IV, α -glucosidase, *Lespedeza cuneata*, diabetes.

INTRODUCTION

Free radicals, unstable organic molecules, dominate antioxidant defense mechanism for their stability, and stimulate excessive oxidative damages through electron pairing with biological macromolecules, including proteins, DNA and lipids in healthy cells (Florence, 1995). Chronic hyperglycemia induces the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are associated with oxidative stress, which is the root cause of glucose auto-oxidation, lipid peroxide formation,

and non-enzymatic protein glycosylation that forms advanced glycation end products (AGEs) (Gillery, 2001). Also, free radical-induced oxidative stress causes β -cell dysfunction, thereby impairing insulin secretion and glucose metabolism for instance in pancreatic β cells, which contain very low level of antioxidant enzymes (Oberley, 1988). Therefore, free radicals and oxidative stress play an important role in the development of diabetes and its complications. Recently, many papers reported the direct

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relationship between anti-oxidant and anti-diabetic activities (Pandey et al., 2011). Therefore, the development of anti-diabetic drugs with anti-oxidant potential will have promising effects on diabetes. Moreover, natural antioxidants that can enhance insulin secretion and control hyperglycemia would be mystical for the prevention and treatment of diabetes.

Lespedeza cuneata, a common native plant, is distributed in Korea, China, Taiwan, India, Australia and many states of the USA. It contains useful ingredients such as β -sitosterol, succinic acid, triacontan-1-ol, quercetin, kaempferol, pinitol, avicularin, juglanin and trifolins (Matsuura et al., 1978). It has been traditionally used for the prevention and treatment of various biological disorders, including diabetes, premature ejaculation, impotence, involuntary emission of semen, coughing and asthma for thousands of years (Huang and Williams, 1999). Recently, radical scavenging activity of flavonoid compounds isolated from *Lespedeza cuneata* has been reported, however, no reports have mentioned its anti-diabetic effects, yet (Kim et al., 2011). Therefore, we hypothesized to investigate *L. cuneata* has anti-oxidant and anti-diabetic activities *in vitro*. To test this hypothesis, we measured antioxidative effects of *L. cuneata* on superoxide anion ($O_2^{\cdot-}$), hydroxyl ($\cdot OH$) radical, nitric oxide (NO) and peroxynitrite ($ONOO^{\cdot}$). Then, we determined its effect on radical-mediated protein oxidation and AGEs formation. Finally, we examined its activities on two key enzymes, α -glucosidase and dipeptidyl peptidase (DPP)-IV, which are linked to diabetes.

MATERIALS AND METHODS

Chemicals

Ascorbic acid, curcumin, penicillamine, thiourea, trolox, aminoguanidine, α -glucosidase from *Saccharomyces cerevisiae*, acarbose, sodium niroprusside (SNP), N-(1-naphthyl)ethylene-diamine dihydrochloride (NED), β -nicotinamide adenine dinucleotide reduced disodium salt (β -NADH), dihydrorhodamine (DHR) 123, nitro-blue-tetrazolium (NBT), phenazine methosulfate (PMS), diethylenetriamine-pentaacetic acid (DTPA), 3-morpholinopyridone (SIN-1), and 4-nitro-phenyl, α -D-glucopyranoside were purchased from Sigma (St. Louis, MO, USA). Similarly, 2,2'-azobis(2-aminopropane) dihydrochloride (AAPH) and H-Gly-Pro-AMC were purchased from Wako Pure Chemical (Osaka, Japan) and Anaspec (San Jose, CA, USA), respectively. Unless otherwise specified, all reagents and solvents were of high analytical grade.

Preparation of *L. cuneata* water extract

The whole plant (herb, stem, and flower) of *L. cuneata* was collected from Geumsan (Chungnam), Korea. It was dried in an oven at 40°C for 24 h, and 315 g of dried form was boiled with water for 1 h. The water extract of *L. cuneata* (LCW) was then concentrated by

evaporation using rotary evaporator under reduced pressure to obtain the viscous residue, which was converted to powder form by freeze drying. The yield of water extract was about 20% of the starting material.

$O_2^{\cdot-}$ scavenging assay

Following the method described by Ewing and Janero (1995), the reaction solution containing 98 μ M β -NADH, 125 μ M EDTA, 62 μ M NBT, and samples in 50 mM phosphate buffer (pH 7.4) was initiated by the addition of 33 μ M PMS and then incubated at room temperature for 5 min. The absorbance was measured at 540 nm using Tecan SPECTRAFlour (Tecan UK, Goring-on-Thames, UK). Ascorbic acid was used as a standard.

NO scavenging assay

SNP (10 mM) was mixed with different concentrations of the sample for two and a half hours at 25°C. Then, 100 μ l of the incubated solution was mixed with equal volume of Griess reagent (0.1% NED and 1% sulfanilamide in 5% phosphoric acid). Absorbance was measured at 540 nm using Tecan SPECTRAFlour (Tecan UK, Goring-on-Thames, UK). Curcumin was used as a standard.

ONOO $^{\cdot}$ scavenging assay

The oxidation of non-fluorescent DHR 123 to fluorescent DHR 123 is a sensitive indicator of ONOO $^{\cdot}$. According to the method described by Kooy et al. (1994), the reaction solution containing 90 mM NaCl, 5 mM KCl, 5 mM DTPA, and 5 mM DHR 123 in 50 mM sodium phosphate buffer (pH 7.4) was initiated by treatment of samples and 10 μ M SIN-1. After 5 min, fluorescent intensity of rhodamine 123 was measured with Tecan SPECTRAFlour (Tecan UK, Goring-on-Thames, UK) at an excitation wavelength of 485 nm and emission wavelength of 530 nm. Penicillamine was used as a standard.

$\cdot OH$ radical scavenging assay

The method described by Miura et al. (1993) was slightly modified to evaluate $\cdot OH$ radical scavenging activity. Fenton reaction was conducted by mixing 10% 5,5 dimethyl-1-pyrroline N-oxide (DMPO, v/v), 0.2 mM Fe_2SO_4 , 1 mM DTPA, and 1 mM hydrogen peroxide in 1.5 ml tube. The influence of samples on the formation and stabilization of $\cdot OH$ radical was measured using ESR spectrophotometer (JEOL, Tokyo, Japan). The resonance condition was as follows: microwave power 1.02 mW, modulation frequency 9.4397 GHz, field modulation 339.458 mT, sweep width 5.000 mT, and time constraint 0.03 s. Thiourea was used as a standard.

Allophycocyanin assay

According to the method described by Vergely et al. (1998), reaction mixture containing AAPH and sample solution prepared in 75 mM phosphate buffer (pH 7.0) was incubated at 37°C during 0, 5, 15, 30, 45, 60, 75 and 90 min. Loss of fluorescence was measured from fluorescence spectrophotometer (Tecan SPECTRA Flour, UK) at 598 nm excitation and 651 nm emission. Trolox was used as a standard.

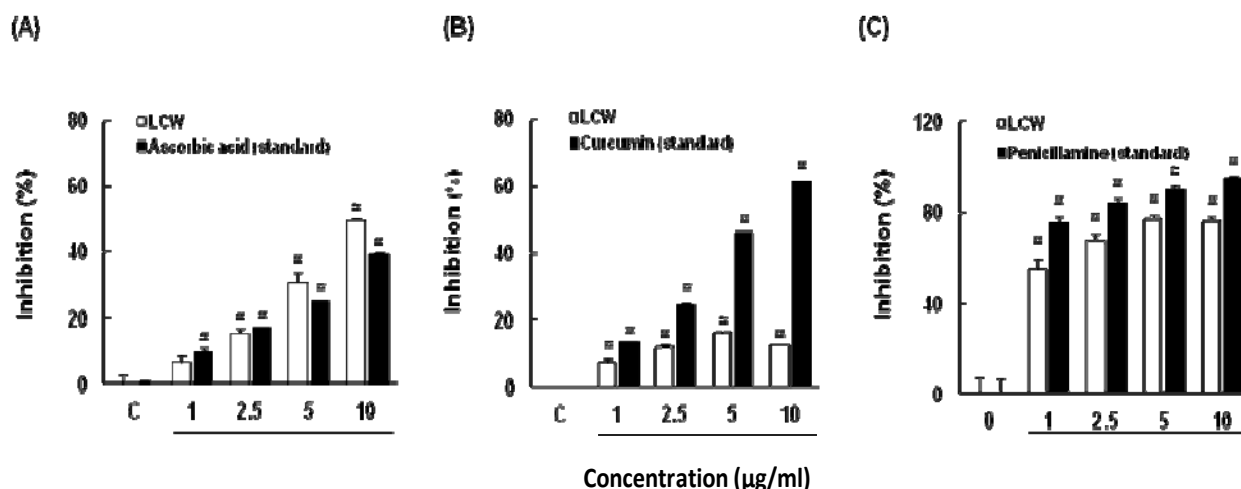


Figure 1. Effect of LCW on $O_2^{\cdot-}$ (A), NO (B), and $ONOO^{\cdot-}$ (C) radicals. Each assay was measured with respective standard drugs. The value is the inhibitory percentage of free radicals. Each value represents mean \pm SE (N = 6). #p < 0.05 vs control.

Antiglycation assay

Bovine serum albumin (10 mg/ml), prepared in phosphate buffer (pH 7.4), was mixed with the solution containing 25 mM glucose and 25 mM fructose. This reaction mixture was incubated with different concentrations of samples at 37°C for 2 weeks. Then, the fluorescence of reaction was measured from Victor³ Multilabel Counter (Perkin Elmer, USA) at 370 nm excitation and 440 nm emission.

α -Glucosidase assay

In brief, reaction mixture containing 50 μ l of phosphate buffer (50 mM, pH 6.8), 10 μ l of yeast α -glucosidase enzyme (U/ml), and 20 μ l of samples were incubated in a 96-well plates for 5 min at 37°C. The reaction mixture was further incubated during another 10 min with 50 μ l substrate solution (5 mM, p-nitrophenyl α -D-glucopyranoside), and finally the released nitrophenol was read at 405 nm using spectrophotometer (Immuno Mini NJ-2300, Japan). Acarbose was used as a standard.

DPP-IV assay

Briefly, an aliquot of 10 μ l serum was incubated with 25 μ l of 50 mM HEPES buffer (pH 7.4) containing Gly-Pro-AMC substrate (final substrate concentration 1 mM). Then, appropriate concentration of the samples was added to the reaction mixture. After 1 h incubation, the reaction was stopped by the addition of 3 M acetic acid. Then, released AMC was measured using Victor³ Multilabel Counter (Perkin Elmer, USA) at an excitation wavelength of 370 nm and an emission wavelength of 440 nm.

Statistical analysis

Statistical package for social sciences (SPSS) was used to perform

data analysis. All data are presented as mean \pm standard error (SE) (n = 6), and were analyzed by one way analysis of variance (ANOVA) followed by Tukey's post hoc tests. Significance was assigned at $p \leq 0.05$.

RESULTS

Effect of LCW on $O_2^{\cdot-}$, NO, $ONOO^{\cdot-}$, and \cdot OH radicals

LCW (10 μ g/ml) inhibited $O_2^{\cdot-}$, NO and $ONOO^{\cdot-}$ formation by 49.7, 12.5 and 76%, respectively; whereas, the reference drugs, ascorbic acid, curcumin and penicillamine, inhibited them by 39.2, 61.4, and 95.2%, respectively (Figure 1). Similarly, 2 mg/ml LCW decreased signal peak to 0.87, which is comparable to the signal peak of thiourea (0.40), a reference drug (Figure 2).

Effect of LCW on radical-mediated protein oxidation

As shown in Figure 3, there is a rapid loss of fluorescence by AAPH treatment, decreasing to 3% at 60 min. However, the addition of 10 μ g/ml LCW showed a right shift of the extinction curve. The fluorescence values of LCW and trolox, a reference drug, remained at 96.8 and 104%, at 60 min.

Effect of LCW on glycated proteins

LCW (500 μ g/ml) inhibited AGEs formation by 38.1%,

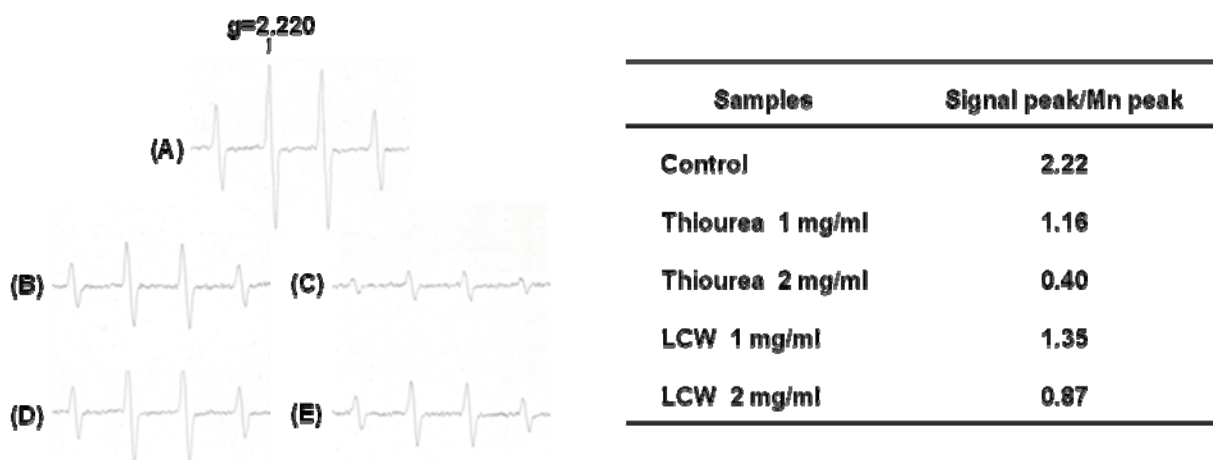


Figure 2. Effect of LCW on ·OH radical. ESR spectra of control (A), thiourea 1 mg/ml (B), thiourea 2 mg/ml (C), LCW 1 mg/ml (D), and LCW 2 mg/ml (E). Thiourea is a positive control.

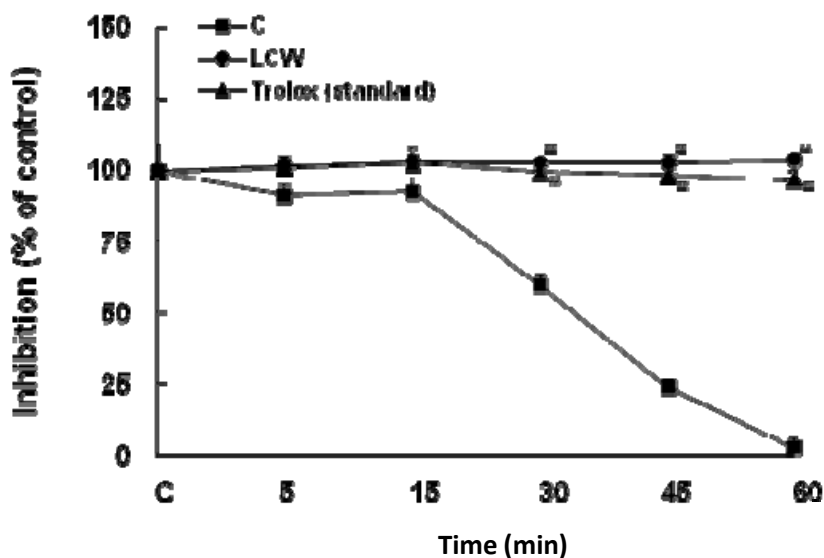


Figure 3. Effect of LCW on radical-mediated protein oxidation. AAPH is a radical inducer. The value is the protective effects of LCW (10 µg/ml) and trolox (10 µg/ml) on AAPH-induced allophycocyanin damage. Trolox is a positive control. Each value represents mean±SE (N=5). #p<0.05 vs control.

whereas aminoguanidine, a reference drug, inhibited AGEs formation by 92.1% (Figure 4).

Effect of LCW on α-glucosidase and DPP-IV activities

100 µg/ml LCW inhibited α-glucosidase by 74%, whereas 10 µg/ml acarbose, a reference drug, inhibited α-glucosidase by 84%. Similarly, 1000 µg/ml LCW inhibited

DPP-IV by 32.9% (Figure 5).

DISCUSSION

During the progression of diabetes, chronic hyperglycemia causes an imbalance between the generation of free radicals and antioxidant capacity that increases intracellular oxidative stress. Numerous studies have reported

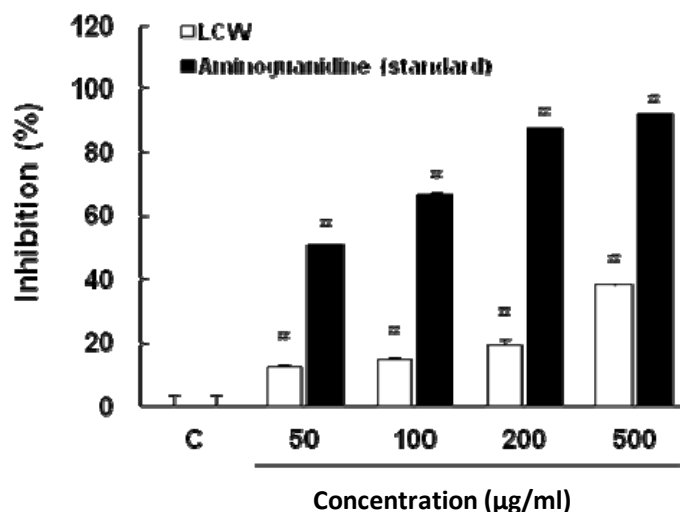


Figure 4. Effect of LCW on glycated proteins. Aminoguanidine is a positive control. The data represent the inhibitory percentage of AGEs formation. All data are expressed as mean ± SE (N = 6). #p < 0.05 vs control.

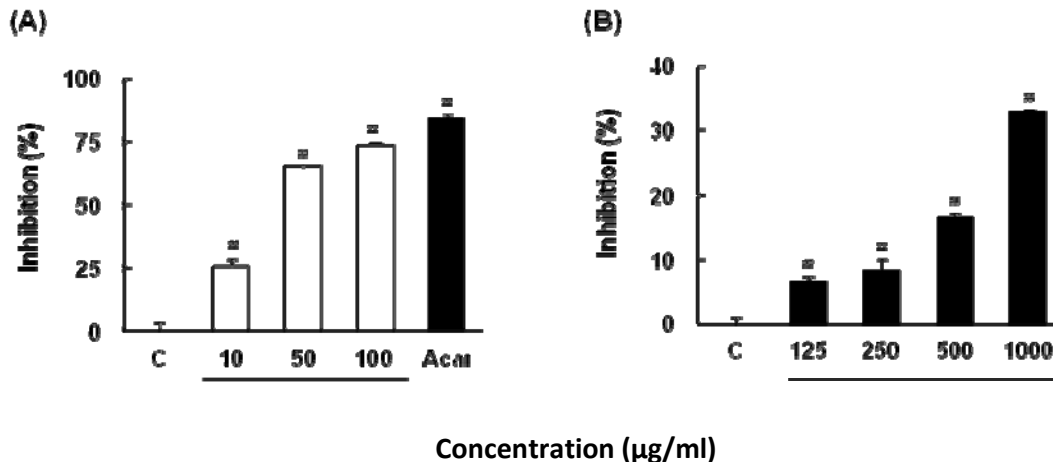


Figure 5. Effect of LCW on α-glucosidase (A) and DPP-IV (B) activities. The data represent the percentage of inhibition as compared to control. Acarbose (Acar) was used as a standard drug for α-glucosidase inhibition. All data are expressed as mean±SE (N=6). #p<0.05 vs control.

the beneficial roles of antioxidants in the management of diabetes (Salehi et al., 2013). In this study, we found that LCW effectively inhibited O_2^- , $\cdot OH$, NO and $ONOO^-$ radicals, and strongly suppressed protein oxidation and glycosylation. Moreover, the activities of two key enzymes, α-glucosidase and DPP-IV, directly linked to diabetes, were also decreased by the addition of LCW.

High glucose in a chronic diabetic condition enhances the production of mitochondrial ROS that increases the formation of O_2^- which reacts with the sustained level of NO , drives the formation of $ONOO^-$, and induces the oxidation of proteins and lipids, affecting cell metabolism, insulin signaling and vascular endothelial dysfunction in diabetes (Son, 2012). Similarly, $\cdot OH$ radical, highly reactive

free radical, reacts with all types of bio-molecules, including carbohydrates, nucleic acids, lipids and amino acids, causing different complications of diabetes (Lipinski, 2011). Our result showed that LCW strongly inhibited the production of O_2^- , $ONOO^-$ and $\cdot OH$ radical, suggesting its antioxidant activities. Teugwa et al. (2013) found that polyphenol-rich plant extracts decreased both hyperglycemia and hyperglycemia induced oxidative stress in an animal model. Recently, Kim, et al. (2011) reported the correlation between the hepato-protective effects and free radical scavenging activities of the flavonoid glycosides isolated from *L. cuneata* in tert-butyl hydroperoxide-induced HePG2 cells. Therefore, we speculated that the antioxidant or radical scavenging effects of *L. cuneata* could regulate hyperglycemia and hyperglycemia-induced oxidative stress.

High glucose-mediated oxidative damage decreases the antioxidant enzyme activities and increases lipid peroxidation. Therefore, the marker of lipid peroxidation, thiobarbituric acid reactive substances (TBARS) provides useful information for the prognosis of diabetes. Recent studies have suggested that AGEs activation after its interaction with intracellular receptors for AGEs (RAGE) alters mitogen-activated protein kinases (MAPKs) phosphorylation and release of pro-inflammatory molecules and free radicals (Pandey et al., 2011). Furthermore, AGEs play an important role in the pathogenesis of diabetic complications, including retinopathy, neuropathy, nephropathy and cardiomyopathy. Therefore, the prevention of AGEs formation may be a novel therapeutic target for improving blood glucose or insulin resistance in diabetes. Our results showed that LCW effectively inhibited peroxyl radical formation by shifting the curve towards the right on the AAPH-induced decrease in allophycocyanin fluorescence and significantly reduced the formation of AGEs. Thus, these results indicate that anti-oxidant and anti-diabetic effect of LCW is able to regulate the lipid peroxidation and protein glycation in hyperglycemic condition.

α -Glucosidase digests carbohydrates into glucose, making its prompt absorption through the intestine. Therefore, α -glucosidase inhibitors prevent postprandial elevation of blood glucose. DPP-IV inhibitors play an important role in glucose metabolism by intensifying insulin secretion, since DPP-IV degrades incretin hormones that enhance insulin secretion from pancreatic β -cells (McIntosh et al., 2005). Therefore, α -glucosidase and DPP-IV inhibitors had been developed as a class of drug for the treatment of diabetes over the past few years.

Several studies have suggested that natural remedies that inhibit α -glucosidase and DPP-IV could regulate glucose metabolism and insulin secretion in diabetic conditions (Sharma and Rhyu, 2014).

In this study, we found that LCW strongly inhibited α -glucosidase and DPP-IV enzyme activities. Thus, anti-diabetic effect of LCW is linked to the inhibition of α -glucosidase and DPP-IV enzyme activities. However, to address the key issue of whether *L. cuneata* has anti-oxidant and anti-diabetic activities in an animal model, further studies will be required. Nevertheless, our results validate the folk use of *L. cuneata* for the treatment of diabetes. Taken together, our findings indicate that *L. cuneata* could be used as a promising plant resource for the prevention of, and therapy for, oxidative disorders and diabetes.

ACKNOWLEDGEMENT

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (NRF-2009-0077541).

Conflict of interest

The authors have declared that there is no conflict of interests.

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