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Napiergrass (*Pennisetum purpureum* S.) protects oxidative damage of biomolecules and modulates antioxidant enzyme activity

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Abstract

The effects of water extract of napiergrass (*Pennisetum purpureum* S.) (WEN) on oxidative damage of biomolecules and modulation of antioxidant enzyme activity were investigated. The results showed that WEN displayed marked free radical scavenging, reducing power, as well as ferrous ions chelating effects. WEN has a dose-dependent response for protective action on oxidation of phospholipid, deoxyribose and low-density lipoprotein (LDL) in the range of 0–0.5 mg/ml, indicating that WEN had in vitro protective action on oxidative damage of biomolecules. Oxidative stress induced by H_2O_2 significantly decreased the viability of BNL cells. However, addition of WEN in the medium protected cells from H_2O_2-induced cytotoxicity. Furthermore, treatment of cells with WEN in the range of 0–0.2 mg/ml displayed protective effect from H_2O_2 induced oxidation in a concentration dependent manner. With respect to the effect of WEN on antioxidant enzymes, the results showed the WEN at 0.2 mg/ml enhanced activities of glutathione peroxidase (GPX), glutathione reductase (GR), glutathione transferase (GST) and catalase (CAT) in BNL cells by 2.93-, 35.8-, 4.23-, and 2.78-fold, respectively, compared to the control; WEN increased the GSH content by 3.2-fold, implying that WEN may up-regulate the levels of GSH and antioxidant enzymes in BNL cells. WEN scavenged NO generated by a NO donor, sodium nitroprusside (SNP) and suppressed NO production in lipopolysaccharide (LPS)-activated macrophage RAW 264.7 cells. The determination of ascorbic acid and total anthocyanins as well as HPLC analysis revealed that ascorbic acid, rutin, epicatechin, anthocyanins, *p*-coumaric acid, quercetin and catechin were present in WEN, which function as in vitro antioxidants by virtue of their ability to scavenge ROS and RNS. Overall, the results obtained showed that WEN is rich in antioxidant components and they can serve as an excellent potential for use as a natural phytochemicals source.

Keywords: Napiergrass; Low-density lipoprotein (LDL); Oxidative stress; Glutathione; Antioxidant enzymes; Nitric oxide

1. Introduction

Epidemiological studies have provided evidence that oxidative stress caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS) induced oxidation of low-density lipoprotein (LDL) mainly leads to development of atherosclerosis (Pryor, 2000). Many reports showed that natural phytochemicals present in fruits, vegetables and herbs may be in possibly contributing to reducing overall cardiovascular disease risks. Moreover, dietary intake of nutraceuticals correlated with lower oxidative stress, inflammation, coronary heart disease and cancer (Ness & Powles, 1997). Thus, supplementation of antioxidants and phytochemicals has attracted considerable attention due to their biofunctional activity. Halliwell (1990) defined biological antioxidants as molecules that can prevent or reduce the extent of oxidative destruction of biomolecules, which include enzymatic antioxidants (e.g. catalase, superoxide dismutase, glutathione peroxidase) and non-enzymatic antioxidants. These biological antioxidants produced in the body, namely endogenous, and other

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supplied with the diet, namely, exogenous (Masella, Di Benedetto, Vari, Filesi, & Giovannini, 2005).

Halliwell (1999) noted that when the level of ROS and RNS exceed the biological antioxidant capacity of the cells, the intracellular redox homeostasis is altered and oxidative stress ensues. On the contrary, diets rich in biological antioxidants appear to contribute to reduction of oxidative stress. Thus, it is worthy goal to investigate the prevention of oxidative stress by antioxidants and dietary ingredients. Accumulating evidence in recent years indicates that the plant-derived dietary displayed protective action on the oxidative damage and modulating oxidative stress. Therefore, consumption of the natural food and drinks derived from plants due to their biofunctional benefits is becoming popular.

Napiergrass (*Pennisetum purpureum* S.) used as livestock feed is one of two major forage species grown in Taiwan. Recently, napiergrass is used to prepare drinks for health promotion, whereas it has not been scientifically investigated for its health promoting potential. The literature related to napiergrass is replenished with investigations on their forage yield and silage quality (Hong, Hsu, & Lu, 2000). Analysis of its antioxidative components has been reported (Wu et al., 2002). Apart from these, the literature regarding the effects on oxidative stress and antioxidant enzymes by napiergrass is limited. Although napiergrass extracts showed antioxidant activity, whether it has any effects on protecting biomolecules as well as enzymatic and non-enzymatic antioxidant function remains unclear. The aims of the present study were to explore the protective effect of napiergrass on oxidative damage of biomolecules, and to investigate the modulation of antioxidant enzyme activity and non-enzymatic antioxidants.

2. Materials and methods

2.1. Materials

Horseradish peroxidase, 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium ferricyanide, deoxyribose, thiobarbituric acid, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), Ferrozine, NADPH, 1-chloro 2,4-dinitrobenzene (CDNB), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), trolox (a water-soluble analogue of vitamin E), lipopolysaccharide (LPS, *Escherichia coli* 0127: B8) and sodium nitroprusside were purchased from Sigma (St. Louis, MO, USA). 4,5-diaminofluorescein (DAF-2) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Napiergrass (*Pennisetum purpureum* S.), donated by Tsou-Ma-Lai Farm, Tainan County, Taiwan, was cut into small pieces and stored at cooling room (4 °C) until used.

2.2. Sample preparation

Napiergrass (100 g) was extracted with boiling water (1000 ml) for 5 min, and the filtrate was freeze-dried. The final dehydrated power was then dissolved in phosphate buffer saline. This sample was named as water extracts of napiergrass (WEN).

2.3. Determination of the trolox equivalent antioxidant capacity

This method is based on the capacity of WEN to scavenge the $ABTS^{\cdot+}$ radical cation compared with trolox in a concentration-dependent response. The $ABTS^{\cdot+}$ radical-scavenging activity was measured as previously described (Miller, Rice-Evans, Davis, Gopinathan, & Milner, 1993). The $ABTS^{\cdot+}$ radical was generated by reacting 1 mM ABTS, 0.5 mM hydrogen peroxide and 10 units/ml horseradish peroxidase in dark at 30 °C for 2 h. After 1 ml $ABTS^{\cdot+}$ was added to WEN or trolox standards, the absorbance at 734 nm was recorded after 10 min. The radical-scavenging capacity was plotted as a function of concentration and the trolox equivalent antioxidant capacity (TEAC) was calculated against a trolox calibration curve.

2.4. Determination of chelating activity

The chelating activity of WEN on Fe^{2+} was measured as previously described (Carter, 1971). Various concentrations of WEN with 0.1 mM $FeCl_2$ were incubated at 25 °C for 10 min. The mixture was treated with 0.1 mM ferrozine and absorbance determined at 562 nm. Ethylenediaminetetraacetic acid (EDTA) was used as positive control.

2.5. Determination of reducing activity

The reducing power of WEN was determined as previously described (Oyaizu, 1986). WEN in phosphate buffer (2.5 ml, 0.2 M, pH 6.6) were added to potassium ferricyanide (2.5 ml, 10 mg/ml), and the mixture was incubated at 50 °C for 20 min. TCA (2.5 ml, 100 mg/ml) was added to the mixture, which was then centrifuged at 650*g* for 10 min. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 1.0 mg/ml), and then the absorbance was read at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power.

2.6. Protective action on liposome oxidation

Lecithin (580 mg) was sonicated in an ultrasonic cleaner (Branson 8210, Branson Ultrasonic Corporation, Danbury, CT, USA) in 58 ml, 10 mM phosphate buffer (pH 7.4) for 2 h in ice-cold water bath. The various concentrations of WEN (1 ml) was mixed with liposome (1 ml), 0.4 mM $FeCl_3$ (1 ml), 0.4 mM ascorbic acid (1 ml). The final mixtures were incubated at 37 °C for 1 h and tested by the thiobarbituric acid (TBA) method (Tamura & Shibamoto, 1991). The absorbance of the sample was read at 532 nm against a blank, which contained all reagents except lecithin.

2.7. Protective action on deoxyribose oxidation

Degradation of deoxyribose was evaluated by Fenton reaction that produced thiobarbituric acid reactive substance (TBARS) (Halliwell, Gutteridge, & Aruoma, 1987). The reaction mixture contained 5 mM deoxyribose, 0.05 mM $FeCl_3$, 0.1 mM ascorbic acid, 0.1 mM H_2O_2 in 20 mM KH_2PO_4 buffer (pH 7.4) and various concentrations of WEN. The final reaction mixtures were incubated at 37 °C for 30 min. Then, 1 ml of 1% thiobarbituric acid and 1 ml of 3% trichloroacetic acid were added and heated at 90 °C for 20 min. The TBARS was measured, as previously described, by reading absorbance at 532 nm.

2.8. Protective action on low-density lipoprotein (LDL) oxidation

Human LDL ($d = 1.02$–1.06 g/ml) was prepared from fasting plasma, routinely pooled from five healthy normolipemia individuals. Lipoproteins were isolated by sequential preparative ultra-centrifugation and dialyzed overnight as previously described (Viana et al., 1996). Freshly prepared native LDL (0.1 mg/ml) was treated with $CuSO_4$ (0.01 mM) in the presence of WEN or not for 24 h at 37 °C and oxidation was stopped by addition of BHT. LDL oxidation was analyzed by TBARS assay as previously described, by measuring absorbance at 532 nm. The results were expressed as percentage inhibition of LDL oxidation.

2.9. Protective effect on BNL cells viability

BNL cells (ATCC number: TIB-73) were purchased from Bioresources Collection and Research Center (Shin-chu, Taiwan) and cultured in DMEM medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM pyruvate and maintained in humidified 5% CO_2/95% air at 37 °C. After cells were cultured with 1 mM H_2O_2 in the presence of WEN or not for 24 h, cell viability was determined by colorimetric measurement of the reduction product of MTT. Briefly, the original medium was removed, then, MTT (final 0.5 mg/ml) were added to each well. After 1 h incubation, the reaction was terminated and the plates were incubated for 30 min to solubilize the formazan dye by addition of dimethyl sulfoxide. The optical density of each well was measured at 570 nm (Wang, Chen, Liang, & Duh, 2005). On the other hand, after BNL cells (1×10^7 cells) were cultured with $FeCl_3$ (3.12 μM), ascorbic acid (125 μM) and WEN at 37 °C for 1 h, the oxidation of cells were determined by the thiobarbituric acid (TBA) method (Tamura & Shibamoto, 1991).

2.10. Effect on glutathione and antioxidant enzyme activities in BNL cells

After BNL cells were cultured in the presence of WEN for 24 h, cells (1×10^7 cells) were collected and centrifuged at 1000*g* for 10 min. The pellets were then resuspended in 10 mM phosphate buffer, pH 7.4, containing 1.5% (w/v) KCl and 1 mM EDTA, and lysed by three cycles of freezing, thawing and sonication for 10 s at 60 W. The cellular homogenates were centrifuged at 10,000*g* for 30 min and the supernatants for the subsequent determination of antioxidant enzyme activities were stored at −80 °C. Intracellular activities of glutathione peroxidase (GPX) (Lawerence & Burk, 1976) and glutathione reductase (GR) (Bellomo et al., 1987) were measured by following the decrease in the absorbance due to oxidation of NADPH. Briefly, in a reaction mixture containing 1 mM GSH, 1 unit/ml GR, 1 mM NaN_3, 1 mM EDTA, 0.2 mM NADPH and 0.1 ml of cellular extract added 0.1 ml of 2.5 mM H_2O_2 for GPX activity determination. In another reaction containing mixture 1 mM $MgCl_2$, 1 mM GSSG, 0.2 mM NADPH added 0.1 ml of cellular extract for GR activity determination. The decreased absorbance at 340 nm was measured for 3 min. Cellular glutathione-S-transferase (GST) activity was assayed by measuring the increment of absorbance at 340 nm due to the formation of 2,4-dinitrophenyl-S-glutathione from 1-chloro 2,4-dinitrobenzene (CDNB) and GSH (Habig, Pabst, & Jakoby, 1974). Briefly, a reaction mixture containing 1 mM GSH, 1 mM CDNB added 0.1 ml of cellular extract. The activity of catalase (CAT) was measured as described previously (Armstrong & Browne, 1994). Cellular extracts were reacted with 20 mM H_2O_2 in 50 mM potassium phosphate buffer, pH 7.0. The change in absorbance at 240 nm at 30 and 120 s was monitored. The cellular GSH levels were measured as trichloroacetic acid (TCA) soluble sulfhydryl group by measurement at 412 nm using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in 0.2 M Tris–HCl buffer, pH 8.9 (Anderson, 1985).

2.11. Assay of scavenging activity against nitric oxide

Nitric oxide (NO) scavenging activity was determined by using the DAF-2 fluorescence probe as previously described (Shin, Kim, Chung, & Jeong, 2005). DAF-2 reacts with NO to produce a highly fluorescent triazolofluorescein product, which emits fluorescence when excited at 485 nm. Sodium nitroprusside (SNP), at physiological pH, spontaneously produce NO (Marcocci, Maguire, Droy-Lefaix, & Packer, 1994). SNP (0.005 M) and various concentrations of WEN in PBS (pH 7.4) were incubated at 25 °C for 30 min in the presence of DAF-2 (5 μM). After incubation, NO produced from SNP was determined using a Bio-Tek FLx800 microplate fluorescence reader with excitation and emission wavelengths of 485 and 535 nm, respectively.

2.12. Inhibitory action on NO production in RAW 264.7 cells

RAW 264.7 cells (ATCC number: TIB-71) were purchased from Bioresources Collection and Research Center (Shin-chu, Taiwan) and cultured in RPMI-1640 medium

containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM pyruvate and maintained in humidified 5% CO_2/95% air at 37 °C. Nitrite levels in the cultured media, which reflect intracellular nitric oxide synthase activity, were determined by Griess reaction. Briefly, cells were cultured with WEN with or without LPS for 24 h. Then, the growth medium was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in water); absorbance of the mixture at 550 nm was determined by using an Anthos 2010 microplate reader (Wang et al., 2005).

2.13. Total polyphenolics assay

Total polyphenolics were determined as gallic acid equivalents (Taga, Miller, & Pratt, 1984). The different concentrations of WEN were added to a 10 ml volumetric flask, to which 2 ml sodium carbonate (20% (w/v)) was added. After 5 min, 0.1 ml Folin–Ciocalteu reagent (50% (v/v)) was added and the volume were made up to 10 ml with H_2O. After 1 h incubation at 30 °C, the absorbance were measured at 750 nm and compared to a gallic acid calibration curve.

2.14. Total flavonoid content assay

One millilitre of WEN was incubated with 0.1 ml (2-aminoethyl) diphenyl borate (0.2% in ethanol). After 20 min of incubation, the absorbance was measured at 405 nm (Hairi, Salle, & Andary, 1991). The absorbance of rutin solutions was detected under the same conditions. The amount of flavonoids in WEN (in rutin equivalents) was calculated.

2.15. Total anthocyanins assay

The content of total anthocyanins of WEN was determined as previously described (Cheng & Breen, 1991). Absorbance of 0.2 mg/ml WEN (pH 1.0 and 4.5) was measured at 510 and 700 nm using a spectrophotometer. The total anthocyanins absorbance was calculated using the following formula: $A = [(A_{510} - A_{700})_{\text{pH 1.0}} - (A_{510} - A_{700})_{\text{pH 4.5}}]$. Delphidin was used as the standard for a calibration curve.

2.16. Ascorbic acid content assay

The ascorbic acid in WEN was determined by HPLC performed with Hitachi liquid chromatography (Hitachi, Ltd., Tokyo) consisting of a model L-7100 pump, and a model L-7420 UV–vis detector set at 234 nm. A hypersil BDS RP-18 reverse-phase column (5 μm, 250 × 4.6 mm, i.d., Hypersil) was used for analysis. The volume injected was 20 μl. The elution solvents were A and B containing KH_2PO_4 (10 mM, pH 2.5) and acetonitrile. The gradient elution program was set at 1.0 ml/min, starting with 100% A in 3 min, 97% A and 3% B in 4 min, and 100% in 5–12 min. The content of ascorbic acid in WEN was calculated from the standard curve of ascorbic acid.

2.17. High-performance liquid chromatography (HPLC) analysis

HPLC analyses of phenolic and nonphenolic compounds were based on the previous method (Hakkinen, Karenlampi, Heinonen, Mykkanen, & Torronen, 1998). WEN (50 mg/ml) was filtered through a 0.45 μm filter and injected onto the HPLC column. HPLC was performed with a Hitachi Liquid Chromatograph (Hitachi, Ltd., Tokyo, Japan), consisting of a model L-7100 pump, and a model L-7455 photodiode array detector. The injection volume was 20 μl and the flow rate was 1.0 ml/min. A Hypersil BDS RP-18 reversed-phase column (5 μm, 250 mm × 4.6 mm, i.d.), protected with a guard column RP-18 (5 μm, 10 mm × 4 mm), was used for analysis. The solvent were: (A) 50 mM ammonium dihydrogen phosphate, pH 2.6; (B) 0.2 mM *ortho*-phosphoric acid, pH 1.5; and (c) 20% solvent A in 80 % acetonitrile. The solvent gradient elution program was as follows:

Time (min)	Solvent (%)		
	A	B	C
0	100	0	0
5	96	0	4
10	92	0	8
10.1	0	92	8
20	0	80	20
30	0	70	30
60	0	50	50
75	0	20	80
80	100	0	0

2.18. Statistical analysis

Statistical analysis involved use of the Statistical Analysis System software package. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests at a level of $P < 0.05$.

3. Results and discussion

The polyphenolics contribute to antioxidant potential and therefore the identification or analysis of WEN phenolics by HPLC was undertaken. The HPLC analyses of WEN were identified as rutin, epicatechin, *p*-coumaric acid, quercetin, and catechin. These observations were in agreement with the report of Wu et al. (2002) who showed the same polyphenolics mentioned above from napiergrass. The content of polyphenolics in WEN was in the order rutin (0.816 mg/g) > epicatechin (0.611 mg/g) > *p*-couma-

ric acid (0.528 mg/g) > quercetin (0.144 mg/g) > catechin (0.123 mg/g). Of the five compounds, rutin, epicatechin, quercetin and catechin are flavonoids and *p*-coumaric acid is a phenolic acid. Rutin and *p*-coumaric acid were selected as the representatives of flavonoid and phenolic acid, respectively, as the reference compounds for comparison for subsequent studies.

In the present study, several assays were used to determine antioxidant action of napiergrass extracts. Table 1 summarizes the antioxidant properties of WEN. Of these models, TEAC method is generally recognized as useful method to determine antioxidant capacity, which is based on the activity of the antioxidant to scavenge the blue–green colored ABTS radical cation relative to the ABTS radical-scavenging activity of the water-soluble vitamin E analogue, trolox (Miller et al., 1993). It was found that all the samples were able to scavenge ABTS radical cation. In particular, rutin and *p*-coumaric acid displayed marked scavenging capacity at low concentration. There was concentration dependent antioxidant activity of WEN in the range of 0–0.5 mg/ml. However, the reference compounds, rutin and *p*-coumaric acid, in the range of 0–0.5 mg/ml possessed high antiradical activity under these experimental condition, consequently, show no significant differences ($P > 0.05$) in scavenging ABTS radical action. These observations imply that WEN, rutin and *p*-coumaric acid had significant antioxidant activity. Also, decolourization of ABTS reflects the capacity of WEN, rutin and *p*-coumaric acid to donate electron or hydrogen atoms to inactivate this radical cation. In other words, WEN, rutin and *p*-coumaric acid showed marked scavenging activity on free radical, which make these good antioxidants as a result of free radical inhibitors.

Table 1
Antioxidant properties of water extract of napiergrass (WEN)

Sample	Concentration (mg/ml)	Total antioxidant activity (TEAC[A] ppm)	Chelation (%)	Reducing ability (Abs. at 700 nm)	Inhibition (%)	
					Deoxyribose	LDL
WEN	0	0.0 ± 0.0^{e}	0.0 ± 0.0^{d}	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}	0.0 ± 0.0^{d}
	0.05	14.8 ± 1.90^{d}	5.8 ± 0.00^{c}	0.1 ± 0.02^{d}	45.4 ± 0.00^{d}	31.7 ± 4.54^{c}
	0.1	34.5 ± 2.62^{c}	7.19 ± 0.01^{c}	0.2 ± 0.01^{c}	53.0 ± 0.01^{c}	57.8 ± 5.19^{b}
	0.2	54.4 ± 0.76^{b}	27.2 ± 0.01^{b}	0.39 ± 0.01^{b}	62.7 ± 0.01^{b}	93.6 ± 1.3^{a}
	0.5	63.9 ± 0.11^{a}	44.9 ± 0.01^{a}	0.85 ± 0.00^{a}	71.5 ± 0.00^{a}	97.4 ± 0.65^{a}
Rut	0	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{b}
	0.05	63.77 ± 0.11^{a}	1.46 ± 0.01^{a}	1.4 ± 0.04^{c}	20.0 ± 0.01^{c}	97.4 ± 2.64^{a}
	0.1	63.73 ± 0.06^{a}	3.58 ± 0.00^{a}	2.43 ± 0.01^{b}	20.7 ± 0.00^{c}	97.8 ± 2.11^{a}
	0.2	63.69 ± 0.22^{a}	3.82 ± 0.00^{a}	2.60 ± 0.01^{a}	30.8 ± 0.01^{b}	97.4 ± 0.53^{a}
	0.5	63.69 ± 0.00^{a}	2.52 ± 0.03^{a}	2.46 ± 0.03^{b}	39.9 ± 0.00^{a}	97.0 ± 1.06^{a}
Cou	0	0.0 ± 0.0^{d}	00.0 ± 0.0^{b}	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}	0.0 ± 0.0^{c}
	0.05	61.87 ± 0.06^{c}	1.79 ± 0.00^{a}	0.1 ± 0.00^{d}	38.0 ± 0.01^{d}	12.7 ± 2.11^{b}
	0.1	62.26 ± 0.06^{b}	1.79 ± 0.01^{a}	0.17 ± 0.01^{c}	46.8 ± 0.01^{c}	11.2 ± 2.11^{b}
	0.2	62.30 ± 0.11^{b}	1.30 ± 0.01^{a}	0.29 ± 0.00^{b}	56.7 ± 0.01^{b}	16.0 ± 4.75^{b}
	0.5	63.07 ± 0.00^{a}	1.79 ± 0.00^{a}	0.50 ± 0.01^{a}	81.4 ± 0.00^{a}	50.0 ± 1.06^{a}
EDTA	0		0.0 ± 0.0^{b}			
	0.05		99.35 ± 0.00^{a}			
	0.1		99.47 ± 0.00^{a}			
	0.2		99.55 ± 0.00^{a}			
	0.5		99.47 ± 0.00^{a}			
AA	0			0.0 ± 0.0^{d}		
	0.05			2.75 ± 0.00^{a}		
	0.1			2.83 ± 0.04^{a}		
BHA	0				0.0 ± 0.0^{e}	
	0.05				33.3 ± 0.00^{d}	
	0.1				40.3 ± 0.01^{c}	
	0.2				47.0 ± 0.00^{b}	
	0.5				54.9 ± 0.01^{a}	
Toc	0					0.0 ± 0.0^{c}
	0.05					84.9 ± 1.26^{b}
	0.1					84.5 ± 0.77^{b}
	0.2					85.0 ± 0.68^{b}
	0.5					92.5 ± 0.39^{a}

[A] Trolox equivalent antioxidant capacity (TEAC) is the ppm concentration of a trolox solution having the antioxidant capacity equivalent to mg/ml solution of the sample under investigation. Results are mean ± SD for $n = 3$. Values with different superscripts in a column are significantly different ($p < 0.05$). LDL, low-density lipoprotein; Rut, rutin; Cou, *p*-coumaric acid; EDTA, ethylenediaminetetraacetic acid; AA, ascorbic acid; BHA, butylated hydroxyanisole; Toc, Tocopherol.

In order to test the possibility of metal ions chelating activity of WEN, the experiment to study chelation of ferrous ions. WEN in the range of 0–0.5 mg/ml inhibited the chromogen formation in a concentration-dependent fashion, indicating that WEN chelated ferrous ions (Table 1). However, the chelating of ferrous ions by rutin and *p*-coumaric acid is negligible in comparison with EDTA, which is a chelating agent. In addition, when compared to EDTA, WEN had moderate chelating action on ferrous ions. WEN may change the ratio of Fe^{3+}/Fe^{2+} or reduce the rate of conversions of ferrous to ferric or chelate the iron itself (Braughler, Duncan, & Chase, 1986). Thus, there is a possibility that WEN chelated the ferrous form and thereby removes the free ion out of the reaction system (Narasimhan, Govindarajan, Vijayakumar, & Mehrotra, 2006).

The reducing ability of WEN, rutin, and *p*-coumaric acid is also shown in Table 1. The reducing ability of WEN and *p*-coumaric acid in the range of 0–0.5 mg/ml and 0–0.2 mg/ml of rutin are concentration-dependent manners, respectively, which are inferior to ascorbic acid, a strong reducing agent. Tanaka, Kuei, Nashima, and Taguchi (1988) reported that antioxidant activity is concomitant with the development of reducing ability. Thus, WEN showed remarkable reducing ability. This may be attributed to reducing compounds in WEN, which consequently react with free radicals to stabilize and terminate radical chain reaction.

The protective effect of WEN on deoxyribose measured in terms of TBARS, compared to the standard antioxidant, butylated hydroxyanisole (BHA) was shown in Table 1. The protective effect of samples increased with increasing concentration of WEN in the range of 0–0.5 mg/ml. It can be found that TBARS were induced in this model system. However, simultaneous addition of samples at 0.2 mg/ml prevented the damage of deoxyribose by 62.7%, 56.7%, 47% and 30.8% for WEN, *p*-coumaric acid, BHA and rutin, respectively. The protective effect of WEN at 0.2 mg/ml on oxidative damage of deoxyribose was higher than BHA, *p*-coumaric acid and rutin. Apparently, WEN showed significant inhibitory effect on oxidative damage of deoxyribose.

Lipid peroxidation is an important process occurring in the cells. In order to elucidate the protective effect of WEN, rutin, and *p*-coumaric acid on lipid peroxidation in cells, liposomes were prepared from phospholipid and used as a liposome model system. WEN, rutin, *p*-coumaric acid and tocopherol (Toc) at 0.5 mg/ml showed 65.6%, 91.3%, 81.9% and 92.2% inhibition in TBARS formation, respectively. The protective effect of WEN was less than Toc, rutin and *p*-coumaric acid which showed significant inhibitory on TBARS formation. These observations indicated potent antioxidant action of WEN, rutin, and *p*-coumaric acid.

In liposome model system, WEN had the lowest antioxidant activity. However, WEN showed the most protective action in deoxyribose model system. These results imply that antioxidant activity of extracts varies between various model systems. In addition, TBARS are final stable aldehyde product of peroxidation, generated by breakdown of peroxides, which are the crucial indicators of cellular damage (Tripathi, Mohan, & Kamat, 2007). Obviously, the oxidative damage of biomolecules such as phospholipid, deoxyribose, results in TBARS formation, subsequently, they form adducts with various compounds in cells and showed many adverse effects on the organism (Esterbauer, 1996; Halliwell, 1999; Tripathi et al., 2007). According to the data, WEN, rutin and *p*-coumaric acid exhibited marked protective action on oxidative damage of lipid and deoxyribose, indicating that WEN, rutin and *p*-coumaric acid may prevent biomolecules from oxidative damage in cells.

Many evidence showed that oxidized LDL appear to play a significant role in atherosclerosis. Thus, prevention of LDL oxidation by antioxidants may contribute to prevention of atherosclerosis. The data obtained from Table 1 shows the protective effect of WEN, rutin, *p*-coumaric acid and Toc on the LDL oxidation, induced by Cu^{2+}. In the presence of WEN, rutin, and *p*-coumaric acid, the peroxidation of LDL was significantly inhibited. The protective action of WEN and *p*-coumaric acid on LDL oxidation was in a concentration-dependent manner. The inhibition of LDL oxidation was 97.4%, 97.0%, 50.0% and 92.5% for WEN, rutin, *p*-coumaric acid and Toc at 0.5 mg/ml, respectively, indicating that these phenolic compounds prevented oxidation of LDL. Lipid peroxidation resulting in ox-LDL production is a common occurrence in patients with systemic autoimmune diseases and in chronic inflammatory disorders (McMurray, Parthasarathy, & Steinberg, 1993). Moreover, ox-LDL can stimulate endothelial cells and monocytes to make tissue factor, which may contribute to thrombus formation in retyped plaques and enhance spontaneous fibrin deposition (Berliner & Heinecke, 1996). This phenomenon result in gradual thickening of arteries, causing decreased elasticity, narrowing and reduced blood supply leading to atherosclerosis (Stoll & Bendszus, 2006). Based on the data obtained from Table 1, WEN has potential for preventing atherosclerosis because of suppression of LDL oxidation. Collectively, these remarkable properties indicate that WEN has significant antioxidant activity.

The levels of polyphenoilc compounds and flavonoids in WEN were 36.325 and 14.695 mg/g, respectively; the levels of polyphenolics in WEN are relatively higher compared to the results of our previous studies, which showed levels of polyphenolics at 21.6 and 17.2 mg/g for roasted coffee (Yen, Wang, Chang, & Duh, 2005) and flower of *Chrysanthemum morifolium* Ramat (Duh & Yen, 1997), respectively. Witzum (1994) reported that phenolic compounds that can bind LDL are likely to exert their peroxyl radical-scavenging activity in the arterial intima, where oxidation of LDL mainly occurs in microdomains. Lamuela-Raventos, Covas, Fito, Marrugat, and de La Torre-Boronat (1999) noted that phenolic compounds can bind tryptophans of apolipoprotein B in LDL to inhibit lipid

oxidation. Apparently, the high levels of polyphenolics in WEN may contribute to the inhibitory effect on LDL oxidation. In addition, phenolic compounds may scavenge some radical species and chelate pro-oxidant metal ions as well as regenerate other antioxidants. In the present study, rutin and *p*-coumaric acid, the main phenolic compounds, present in WEN showed remarkable inhibition of LDL oxidation. This observation may be in part associated to protect LDL against oxidation. According to the data mentioned above, WEN is a free radical inhibitor and a chelator of metals ions as well as protector of lipid peroxidation. These appear to be associated to explain that WEN can prevent the oxidation of LDL. Combining this fact with the results obtained, we suggested that WEN might exert antioxidant activity to inhibit LDL oxidation by different mechanisms, including free radicals scavenging, metal chelating, and lipid peroxidation inhibiting (Table 1).

To further understand the protective action of WEN, oxidative damage in cellular system was investigated. To explore whether WEN protected BNL cells against oxidative stress, the cytotoxic action of H_2O_2 on BNL cells in the presence and absence of WEN was determined by MTT assay. Hydrogen peroxide at 1 mM decreased the viability of BNL by 47.34% (Fig. 1). However, WEN in the range of 0.01–0.2 mg/ml alone did not have any harmful effect on the viability of BNL cells. Meanwhile, simultaneous addition of WEN in the culture medium protected cells against H_2O_2-induced cytotoxicity. As can be seen in Fig. 1, WEN at 0.01 mg/ml significantly increased the BNL cell viability up to >100% from 52.66% of H_2O_2-treated cells. Apparently, WEN not only has no cytotoxicity toward the viability of BNL cells, but also protects BNL cells from oxidative damage induced by H_2O_2. Fig. 2 shows the protective effect of WEN on cellular oxidation on BNL cells induced by H_2O_2. Incubation cells with various concentrations (0–0.2 mg/ml) of WEN, rutin, and *p*-coumaric acid displayed protective effect against H_2O_2-induced oxidation in a concentration-dependent manner. The protective effect on cell oxidation induced by H_2O_2 was 71.91%, 59.58% and 40.42% inhibition for WEN, rutin and *p*-coumaric acid, respectively, at 0.2 mg/ml. H_2O_2 is produced from the reaction of superoxide anion and dismutase of cells as well as some enzymes, including NADPH oxidase, amino acid oxidase, and uric acid oxidase (Park et al., 2003). H_2O_2 can cause membrane damage to release arachidonic acid, which is responsible for the prolonged damage of cells. In addition, H_2O_2 can penetrate the cell membrane and then react with metal ions in cells through the Fenton reaction to form extremely highly toxic hydroxyl radicals, which cause molecular damage and cell injury leading to accelerated aging and diseases (Halliwell & Gutteridge, 1989). In the present study, WEN did not show any interaction with H_2O_2 (data not shown), indicating that WEN did not react directly with H_2O_2. On the other hand, the data in Table 1 showed that WEN displayed marked chelating activity. This observation clearly indicated that

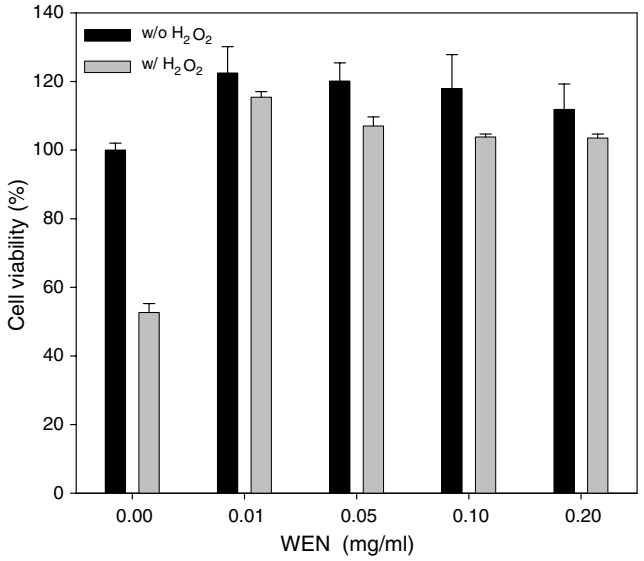

Fig. 1. Effect of water extract of napiergrass (WEN) on BNL cell viability induced by H_2O_2.

chelating effect of WEN on chelating Fe^{2+} may result in the inhibition of Fenton reaction, derived from interaction of Fe^{2+} and H_2O_2, and may contribute to inhibit hydroxyl radicals; consequently, protect cells from oxidative damage. Also, these results suggested that WEN is an effective protector against H_2O_2 cytotoxicity and protect BNL cells from oxidative stress related cellular injuries.

The effect of WEN, rutin, and *p*-coumaric acid on GSH levels in BNL cells are shown in Table 2. The samples in the range of 0–0.2 mg/ml induced an increase in GSH levels in

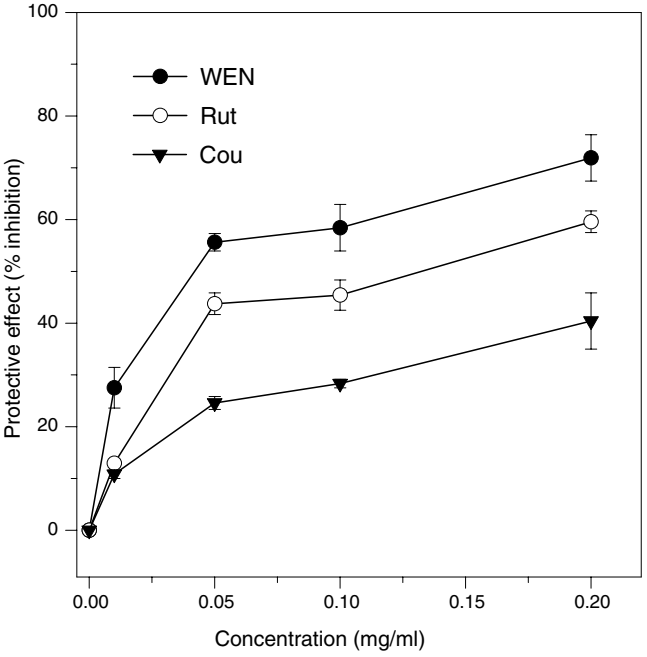

Fig. 2. Effect of water extract of napiergrass (WEN) on oxidation of BNL cell induced by H_2O_2. Rut, rutin; Cou, *p*-coumaric acid.

a concentration-dependent manner when compared to control. WEN, rutin and *p*-coumaric acid at 0.2 mg/ml, the GSH levels were 3.2-, 47.7- and 11.3-fold compared to the control, respectively. These observations imply that WEN, rutin, and *p*-coumaric acid positively regulated the GSH concentrations in BNL cells. GSH is known to function both as a reductant and as a nucleophile due to its side chain sulfhydryl (–SH) residue in cysteine of GSH (Anderson, 1985). The intracellular concentration of GSH in most mammalian cells is relatively high (in the millimolar range) and is maintained at these levels by continuous synthesis (Orrenius, 1994). However, the intracellular concentration of GSH decreases because of GSH oxidation and depletion during oxidative stress. In addition, Martensson and Meister (1992) noted that ascorbate may decrease GSH consumption by minimizing the GSH dependent reduction of dehydroascobate to ascobate, and by providing an alternative cellular reducing agent. As can be seen in Table 1, WEN, rutin, and *p*-coumaric acid had a significant reducing ability. In the present work, up-regulation of GSH concentration in BNL cells may in part be attributed to the reducing ability of WEN, rutin, and *p*-coumaric acid.

To defend against oxygen toxicity, many endogenous non-protein antioxidant molecules are important in scavenging free radicals. However, the scavenging reactions lead to lower the levels of antioxidant molecules in cells. Therefore, along with antioxidant molecules, endogenous antioxidant enzymes are considered essential for alleviating oxidative stress (Larson, 1995). As can be seen in Table 2, the activity of GPX, GR, GST, and CAT increased with increasing the concentration of WEN, rutin, and *p*-coumaric acid, respectively. When WEN was added at 0.2 mg/ml, the activity of GPX, GR, GST, and CAT were 2.93-, 35.8-, 4.23-, and 2.78-fold higher compared to the control, respectively. These observations indicate that the samples tested positively induced GPX, GR, GST, and CAT activities. GPX, present in the cell cytosol and mitochondria, is regarded as a crucial enzyme which catalyses the reduction of H_2O_2, ethyl hydroperoxides, *tert*-butyl-hydroperoxide, cumene hydroperoxide, linoleic acid hydroperoxide and nucleotide or steroid derived hydroperoxide (Ahmad, 1995). GR catalyzes the NADPH-dependent reduction of oxidized GSH (Bellomo et al., 1987). Thus, GSH levels are in part associated with GR activity. In other words, the more activity of GR there is in the cells, the greater is the GSH levels in cells. GST, which plays an important role in detoxification and excretion of xenobiotics, is a soluble protein. GST functions in catalyzing the conjugation of the thiol functional groups of GSH to electrophilic xenobiotics and results in increasing solubility. Consequently, GST increases solubility of hydrophobic substances and thereby reduces the liver damage (Ahmad, 1995). Therefore, compounds that elevate GST activity positively regulate metabolism of toxic substances to nontoxic ones. This study indicates that WEN may have potential for increasing antihepatotoxic action. The primary role of CAT is to decompose H_2O_2. Collectively, these oxidative enzymes provide a first line of defense against oxidative stress. According to the data in Table 2, WEN, rutin, and *p*-coumaric acid may play an important role in limiting ROS-mediated damage to biological macromolecules because of providing a positive upregulation of antioxidant enzymes. This finding also may provide clues on mechanism of WEN against oxidative damage in cells. Puiggros et al. (2005) noted that phytochemicals regulated glutathione related enzymes by transcriptional control to increase mRNA and consequently enzyme activity. In addition, Masella et al. (2005) reported that dietary polyphenols could stimulate the transcription of antioxidant responsive elements (AREs), which are found in the promoters of many of genes that are inducible by oxidative and chemical stress. In this regard, the investigation regarding whether

Table 2
The effects of water extract of napiergrass (WEN) on glutathione and antioxidant enzymes

Sample	Concentration (mg/ml)	mg/mg protein GSH (10^{-3})	Activity (*n* mol/min/mg protein)			
			GPX	GR	GST (10^{-3})	CAT
WEN	0	50 ± 8^{c}	1.363 ± 0.042^{d}	0.077 ± 0.004^{d}	1.71 ± 0.03^{c}	160.13 ± 24.46^{e}
	0.01	59 ± 14^{c}	2.443 ± 0.374^{c}	0.095 ± 0.007^{d}	1.74 ± 0.09^{c}	147.38 ± 19.02^{d}
	0.05	106 ± 16^{b}	2.849 ± 0.089^{bc}	0.612 ± 0.008^{c}	4.55 ± 1.20^{b}	276.12 ± 19.24^{c}
	0.1	145 ± 7^{a}	3.115 ± 0.025^{b}	0.890 ± 0.02^{b}	5.45 ± 0.85^{ab}	364.77 ± 30.07^{b}
	0.2	161 ± 4^{a}	4.000 ± 0.030^{a}	2.755 ± 0.105^{a}	7.23 ± 0.430^{a}	445.17 ± 10.95^{a}
Rut	0	15 ± 5^{c}	1.394 ± 0.07^{d}	1.085 ± 0.055^{d}	0.79 ± 0.07^{c}	163.50 ± 27.04^{e}
	0.01	45 ± 5^{c}	2.440 ± 0.101^{c}	2.600 ± 0.450^{c}	1.41 ± 0.43^{c}	362.54 ± 17.21^{d}
	0.05	105 ± 15^{c}	2.849 ± 0.089^{c}	3.770 ± 0.230^{b}	2.87 ± 0.50^{b}	466.65 ± 10.30^{c}
	0.1	535 ± 45^{b}	3.115 ± 0.025^{ab}	4.635 ± 0.285^{b}	5.91 ± 0.39^{a}	558.84 ± 32.48^{b}
	0.2	715 ± 55^{a}	3.535 ± 0.292^{a}	5.930 ± 0.340^{a}	7.23 ± 0.43^{a}	678.21 ± 17.16^{a}
Cou	0	15 ± 5^{d}	1.394 ± 0.07^{c}	1.085 ± 0.055^{c}	0.79 ± 0.07^{d}	163.50 ± 27.04^{b}
	0.01	75 ± 5^{c}	1.491 ± 0.041^{c}	1.595 ± 0.40^{bc}	2.45 ± 0.25^{c}	158.59 ± 19.45^{b}
	0.05	105 ± 15^{bc}	2.900 ± 0.090^{b}	1.745 ± 0.405^{bc}	4.22 ± 0.26^{b}	187.00 ± 33.33^{b}
	0.1	135 ± 15^{ab}	2.962 ± 0.088^{b}	3.175 ± 0.615^{ab}	4.94 ± 0.34^{b}	256.87 ± 31.45^{ab}
	0.2	170 ± 10^{a}	3.565 ± 0.225^{a}	3.900 ± 0.300^{a}	6.29 ± 0.48^{a}	345.25 ± 35.00^{a}

Values with different superscripts in a column are significantly different ($p < 0.05$). Rut, rutin; Cou, *p*-coumaric acid.

positive modulation of WEN on antioxidant enzymes is caused by transcription of genes is in progress.

It has been proven that adequate quantity of nitric oxide (NO) plays a protective role against oxidative stress. However, the large levels of NO contribute to tissue injury (Licinio, Prolo, McCann, & Wong, 1999). In the present work, the scavenging action of WEN on NO was determined. SNP is known to decompose in PBS solution and then generate NO. Nitric oxide, under aerobic conditions, reacts with oxygen to form nitrate and nitrite, which can be determined using Griess reagent (Rejdak et al., 2003). Fig. 3 shows the scavenging activity of WEN, rutin and *p*-coumaric acid on NO generated from SNP. WEN, rutin and *p*-coumaric acid in the range of 0–0.5 mg/ml showed a concentration-dependent scavenging NO. As compared to rutin and *p*-coumaric acid, the scavenging activity of WEN was relatively less. However, these observations implied that WEN could react with NO and act as scavenger of NO. To explore the effect of WEN on LPS-induced NO production in RAW 264.7 cells, the concentration of accumulation of nitrite, the oxidative product of NO, was determined by Griess method. As can be seen in Fig. 4, WEN showed concentration-dependent inhibition of NO accumulation in LPS-activated RAW 264.7 cells. The NO production significantly increased by almost 4.84-fold compared to the control by addition of 200 ng/ml LPS to the culture system. This enhancement of NO production was reduced to 80.54% by addition of 0.5 mg/ml WEN, which were superior to rutin (25%) and *p*-coumaric acid (40%), respectively, at the same concentration. In addition, the

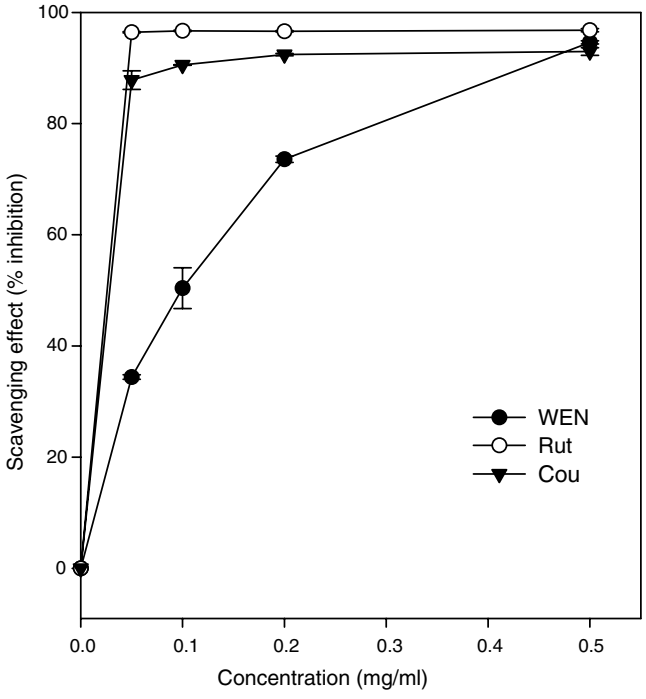

Fig. 3. Scavenging effect of water extract of napiergrass (WEN) on nitric oxide generated with sodium nitroprusside (SNP). Rut, rutin; Cou, *p*-coumaric acid.

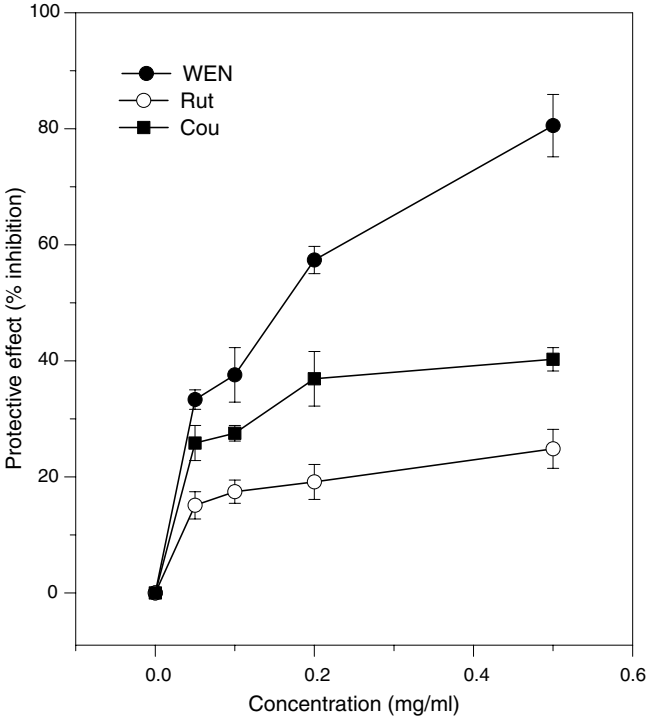

Fig. 4. Effect of water extract of napiergrass (WEN) on production of nitric oxide in RAW 264.7 macrophages induced by lipopolysaccharide. Rut, rutin; Cou, *p*-coumaric acid.

inhibitory effect of WEN on NO production in LPS-activated RAW 264.7 cells was in a concentration–dependent manner. Therefore, LPS-activated nitrite production was significantly reduced by incubation with WEN. Kobuchi, Virgili, and Packer (1999) noted that flavonoids, flavones, flavonols, and catechins inhibited the NO production of inducible nitrite oxide synthase. In the present study, WEN exhibited an inhibitory effect on NO production in LPS-activated RAW 264.7 cells that may be attributed to rutin and *p*-coumaric acid and other active compounds such as catechin present in WEN.

To analyze the main bioactive compounds in WEN in addition to the five phenolic compounds identified above, ascorbic acid and total anthocyanins were also determined. The contents of ascorbic acid and total anthocyanins are 1.30 mg/g and 0.555 mg/g, respectively. These compounds mentioned above have been identified as bioactive agents. For example, ascorbic acid is well known as an antioxidant and has been found to decrease oxidation of LDL particles at the early phase of oxidation, whereas it increases at the late phase of oxidation (Ashidate et al., 2003). Nakagawa and Yokozawa (2002) noted that green tea can scavenge NO and O_2- and their scavenging action are attributed to their tea catechins with the galloyl group displayed significantly scavenging action. Faria, Calhau, de Freitas, and Mateus (2006) reported that procyandins could exert a protective action in the organism against protein and lipid oxidation, reducing the risk of chronic disease and certain type of cancer. Rutin which is well known to have

antioxidant activity has displayed considerable activity in suppression the hyperproliferation of colonic epithelial cells, and thereby reducing focal areas of dysplasia and ultrimately colon tumor incidence (Ho, 1991). Quercetin is an effective scavenger of free radical due to its catechol and phenol function and its low redox potential (Salvi, Carrupt, Tillement, & Testa, 2001). Rutin and *p*-coumaric acid had shown significant antioxidant effect in the present study. In addition, based on HPLC analysis there are other peaks present in WEN, suggesting that other uncharacterized phenolic compounds may be present in WEN. Taken together; WEN showed marked biological action as well as scavenging effect on NO could be correlated with their contents of phenolics compounds, anthocyaninis, ascorbic acid and some bioactive compounds. These compounds in WEN may be due to synergism of the components with one another and therefore responsible for the functional properties of WEN. Therefore, taking WEN dietary supplements that contain bioactive compounds may benefit consumers.

In conclusion, the results reported in this research indicate that WEN showed protective effect against oxidative damage of biomolecules, including phospholipid, deoxyribose and LDL. In addition, WEN showed significant upregulation of GSH levels as well as the activities of antioxidant enzymes. Moreover, WEN protected cells from oxidation induced by H_2O_2. In addition, by directly scavenging NO radicals WEN contributed to suppress NO production in RAW 264.7 macrophages. Most likely WEN exerted their protective action on oxidation damage because of phenolic related bioactive compounds in WEN. Further studies of protective effects of WEN on oxidative stress in vivo are in progress.

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