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Inhibition of Lipid Peroxidation by Enzymatic Hydrolysates from Wheat Bran

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Summary

Wheat bran, an important by-product of the cereal industry, is rich in potentially health-promoting phenolic compounds. The phenolics are mainly esterified to the cell wall polysaccharides. In our previous paper, wheat bran was destarched and deproteinated by α-amylase, protease and amyloglucosidase successively and further hydrolyzed using *Bacillus subtilis* xylanases, and the enzymatic hydrolysates from wheat bran (EHWB) showed good scavenging activity *in vitro*. The aim of this study is to further characterize the antioxidant potential of EHWB against various systems, both *ex vivo* and *in vivo*, namely, rat liver microsomal lipid peroxidation systems induced by Fe^{2+}/H_2O_2 and Fe^{3+}-adenosine diphosphate (ADP)/dihydronicotinamide adenine dinucleotide phosphate (NADPH), copper- and 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH)-induced human low-density lipoprotein (LDL) oxidation systems, and alloxan-induced *in vivo* lipid peroxidation in mice. EHWB inhibited lipid peroxidation in rat liver microsomes induced by Fe^{2+}/H_2O_2 and Fe^{3+}-ADP/NADPH in a concentration-dependent manner with 90.3 and 87 % inhibition of lipid peroxidation at 50 mg/L, respectively, which were similar to that of butylated hydroxytoluene (BHT) at 20 mg/L. The antioxidant potential of EHWB at a concentration ranging from 10 to 20 mg/L in the nonenzymatic system was more effective than in the enzymatic system. EHWB strongly inhibited *in vitro* copper- and AAPH-mediated oxidation of LDL in a concentration- and time-dependent manner with 52.41 and 63.03 % inhibition at 20 mg/L, respectively, which were similar to that of ascorbate at 10 mg/L. EHWB significantly decreased the level of thiobarbituric acid reactive substances (TBARS) and increased the activities of glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) in serum and liver of alloxan-treated mice compared with the control. These results demonstrated that EHWB might be efficient in the protection of food products and humans against free radical-induced oxidative damage.

Key words: wheat bran, enzymatic hydrolysates, alloxan, antioxidative enzyme, lipid peroxidation, liver microsome, low-density lipoprotein

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Introduction

Lipid peroxidation was first studied as a mechanism of deterioration of dietary oils and fats, reflecting the interaction between molecular oxygen and polyunsaturated fatty acids with two or more double bonds because it leads to the development of undesirable off-flavours and potentially toxic reaction products. As an example, the long-chain polyunsaturated fatty acids are oxidized to form lipid hydroperoxides, which are decomposed into different kinds of aldehydes such as *n*-alkanals, related α,β-unsaturated aldehydes including *trans*-2-alkenals or 2,4-alkadienal, and 4-hydroxy-2-alkenals (*1*). These aldehydes cause the characteristic off-flavour and odours of rancid fats and oils (*2*).

In recent years, it has become evident that lipid peroxidation occurs in aerobic cells, both under pathological and physiological conditions. Lipid peroxidation can cause injury directly to biomolecules such as nucleic acids, proteins, structural carbohydrates, and lipids, and induce disturbance of membrane organization, functional loss and modification of proteins and DNA bases. The major products of lipid peroxidation including hydroperoxides, cleavage products such as aldehydes, and polymeric materials, are substrates for various enzymes and exert cytotoxic and genotoxic effects *in vivo* (*3*). Lipid peroxidation has been implicated as the underlying mechanism in various diseases and ageing, including atherosclerosis, cataract, rheumatoid arthritis, and neurodegenerative disorders (*4*,*5*). Various kinds of antioxidants with different functions inhibit lipid peroxidation and the deleterious effects caused by the lipid peroxidation products (*6*). Phenolics, an integral part of the diet, are widely distributed in the plant kingdom. They can inhibit lipid peroxidation by scavenging free radicals that form a low-energy radical, whose energy is insufficient to promote lipid peroxidation at biologically significant rates (*7*).

Wheat contains numerous phenolic compounds including ferulic, *p*-coumaric, vanillic, caffeic, and chlorogenic acids, which are concentrated in the bran and aleurone portions of wheat kernels (*8*). The phenolic acids are primarily in an insoluble bound form, and esterified to the C-5 hydroxyl group of α-L-arabinofuranosyl substituents, which are linked to C-2 or C-3 on the xylopyranosyl backbone (*9*). Wheat bran as an important by-product of the cereal industry is produced worldwide in enormous quantities and recognized as a good source of dietary fibre. It has been shown to protect against early biomarkers of colon cancer, which is ascribed to the dietary fibre and components such as phytic acid and various phenolics in the cell contents (*10*). The phenolic compounds are ester-linked to the main polymers in the plant cell wall and cannot be absorbed in this complex form (*11*). Several investigations have been conducted to study antioxidant properties of wheat bran extracts and acid- and alkali-hydrolysed wheat bran fractions (*12*–*14*). Garcia-Conesa *et al.* (*15*) demonstrated that 8-O-4-diferulic acid purified from destarched wheat bran after alkaline hydrolysis was better antioxidant than ferulic acid in both lipid and aqueous phases. Moore *et al.* (*16*) reported that solid-state enzyme treatments could significantly accelerate the conversion of insoluble, bound phenolics from wheat bran to soluble, free phenolics and improve the bioaccessible antioxidant properties and the potential bioavailability of antioxidants in wheat bran. The feruloylated oligosaccharides isolated from wheat bran by treatment with xylanases showed good antioxidant activity (*17*,*18*). However, to the best of our knowledge, little is known about lipid peroxidation inhibition by EHWB in biological systems.

In our previous study, EHWB exhibited effective antioxidant activities in the employed *in vitro* experiments, including metal chelation analysis, reducing power test, DDPH radical and reactive oxygen species (H_2O_2, $O_2^{\cdot-}$, $^{\cdot}OH$) scavenging activity assays (*19*). In this study, the inhibition of lipid peroxidation by EHWB was further investigated in various biochemical assays, namely, enzymatic and non-enzymatic lipid peroxidation *in vitro* induced by Fe^{3+}-ADP/NADPH and Fe^{2+}/H_2O_2 in rat liver microsomes, *in vitro* copper- and AAPH-induced human LDL oxidation systems, and *in vivo* alloxan-induced lipid peroxidation in mice.

Materials and Methods

Materials

EHWB used in this study was prepared according to our previous methods (*19*). Test kits for the activities of CAT, SOD, GSH-Px and the level of malondialdehyde (MDA) in the experiments for alloxan-induced lipid peroxidation and the loss of antioxidant enzymes in mice were purchased from the Nanjing Jianchen Bioengineering Institute (Jiangsu, PR China). Alloxan, AAPH, NADPH, ADP, bovine serum albumin (BSA, fraction V), butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP) and ascorbate were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents used in this study were of analytical grade.

Animals and treatments

Female Wistar rats weighing (250±20) g and healthy Kunming mice (25 females, 25 males) weighing (20±2) g were obtained from Beijing Vitalriver Laboratory Animal Co., Ltd. (Beijing, PR China). The handling of the animals was approved by the local ethical committee for the care and use of laboratory animals.

Preparation of rat liver microsomes

Rat liver microsomes were prepared according to the method of Wu *et al.* (*20*). Briefly, the liver tissue was homogenized with an ice-cold Tris-HCl buffer (pH=7.4) in a motor-driven glass homogenizer. The prepared homogenate was centrifuged at 4 °C for 20 min to obtain the postmitochondrial fraction, which was then centrifuged in a Hitachi 55P-72 ultracentrifuge (Hitachi, Ltd, Tokyo, Japan) at 105 000×*g* for 60 min to obtain the microsomal fraction. The microsomes were washed twice with 0.15 mol/L KCl at 105 000×*g* for 30 min. The washed microsomes were suspended in 0.15 mol/L Tris-HCl buffer (pH=7.4) containing 20 % glycerol and stored in a deep freezer at –80 °C until use. The stored microsomes were diluted with 0.05 mol/L Tris-HCl buffer (pH=7.4) to ob-

tain an appropriate protein concentration when used for the measurement of lipid peroxidation. Microsomal protein was determined by the Lowry method using BSA as a standard (*21*).

Microsomal membrane peroxidation induced by enzymatic and nonenzymatic systems

Fe^{2+}/H_2O_2 and Fe^{3+}-ADP/NADPH-induced liver microsomal oxidations were carried out according to the method described by Yen and Hsieh (*22*). The inhibition of lipid peroxidation by EHWB was calculated by measuring the concentration of TBARS. TBARS results were expressed as MDA equivalents using TEP as standard. MDA concentration was expressed as μmol of TBARS per mg of protein. BHT was used as reference material. The inhibition of rat liver microsome oxidation was calculated as follows:

$$\text{Inhibition}_{\text{TBARS}} = [1-(\text{MDA}_{\text{with antioxidant}}/\text{MDA}_{\text{no antioxidant}})]\times 100 \quad /1/$$

Preparation of human LDL

Blood was collected in tubes from three healthy normolipidemic volunteers after an overnight fast and human LDL was immediately prepared from plasma by the discontinuous density gradient centrifugation procedure as described by Chung *et al.* (*23*). The purity of LDL fraction evaluated by agarose gel electrophoresis was 97 %. The LDL protein concentration was measured by the method of Lowry *et al.* (*21*).

Inhibitory effect of EHWB on human LDL oxidation induced by copper ion

Copper-induced LDL oxidation was performed according to the protocol described by Yoon *et al.* (*24*) with minor modifications. The formation of conjugated dienes was measured every 15 min over a 3-hour period by monitoring the absorbance at 234 nm using a UV-visible recording spectrophotometer (Rayleigh Analytical Instruments, Beijing, PR China). Ascorbate was used for reference material. The inhibition of LDL oxidation by EHWB was calculated as follows:

$$\text{Inhibition}_{\text{conjugated dienes}} = [1-(\text{lag time}_{\text{no antioxidant}}/\text{lag time}_{\text{with antioxidant}})]\times 100 \quad /2/$$

Inhibitory effect of EHWB on human LDL oxidation induced by AAPH

The oxidation of LDL by peroxyl radicals was performed according to the method described by Lee (*25*). The oxidation of LDL, quantified as the generation of MDA equivalents, was measured using the TBARS method as described by Yoon *et al.* (*24*) with minor modifications. Briefly, after the oxidation, the LDL was mixed with 1.0 mL of 0.67 % (by mass per volume) TBA and 1.0 mL of 20 % (by mass per volume) trichloroacetic acid (TCA). The reaction product was maintained at 100 °C for 15 min and then cooled. After centrifugation at 1500×*g* for 15 min to remove the precipitated proteins, the absorbance of the supernatant was measured at 532 nm. The concentration of TBARS was expressed as MDA equivalents using TEP as the standard. Results are expressed as nmol of MDA per mg of protein. Ascorbate was used as reference material. Analyses were performed in duplicate. The inhibition of LDL oxidation was calculated using Eq. 1.

Alloxan-induced lipid peroxidation and the loss of antioxidant enzymes in mice

Healthy Kunming mice were maintained in plastic cages under environmentally controlled conditions with a 12-hour light/dark cycle at a temperature of 22–24 °C and a relative humidity of 75 %. All mice were fed with a standard chow diet and water *ad libitum*. The mice were randomly divided into five groups (ten mice per group): group 1: normal, group 2: control, group 3: EHWB at a low dose of 50 mg/kg of body mass per day, group 4: EHWB at a high dose of 100 mg/kg of body mass per day, and group 5: ascorbate at a dose of 50 mg/kg of body mass per day, which was used as a positive control group. Groups 1 and 2, groups 3 and 4, and group 5 were fed with physiological saline solution of 1 mL/day, EHWB, and ascorbate by intragastric incubation for 20 days, respectively. On the 16th day, groups 2, 3, 4 and 5 were injected into the vein with alloxan at a dose of 75 mg/kg, dissolved in normal saline solution. On the 21st day, the anesthetized mice (by inhaling diethyl ether) were fixed on an experimental desk. Blood was drawn by cardiac puncture into tubes, and serum was separated by centrifugation at 2000×*g* for 10 min at 4 °C. Liver was excised immediately, blood was blotted off, then it was rinsed in PBS (pH=7.4), vacuum packed, and stored at –80 °C until analysis. The liver was cut into pieces and milled to paste, normal saline was added to prepare 10 % (by mass per volume) solution of tissue homogenate, and then the tissue homogenate was centrifuged at 2000×*g* for 10 min and the supernatant was kept. The sample of liver was used to measure enzyme activities of CAT, SOD, GSH-Px, and the values of TBARS.

The protein content in each sample was measured by the method of Lowry *et al.* (*21*) using BSA as the standard. The values of TBARS were determined with MDA according to the method of Asakawa and Matsushita (*26*) using Kit A003 (Nanjing Jianchen Bioengineering Institute, Jiangsu, PR China); CAT activity was determined by the colourimetric method of Sumner and Dounce (*27*) using Kit A007 (Nanjing Jianchen Bioengineering Institute); SOD activity was determined according to Oyanagui's method (*28*) using Kit A001 (Nanjing Jianchen Bioengineering Institute); and GSH-Px activity was determined on the basis of the method of Rotruck *et al.* (*29*) using Kit A005 (Nanjing Jianchen Bioengineering Institute).

Statistical analysis

Data were reported as the mean value±standard deviation (S.D.) of triplicate determinations. Data were analyzed by an analysis of variance ($p<0.05$) and the means were separated by Duncan's multiple range test. Results were processed by STATISTICA software (v. 6.0; StatSoft, Tulsa, OK, USA).

Results and Discussion

Effect of EHWB on enzymatically and nonenzymatically induced microsomal lipid peroxidation

Microsomes, especially smooth surfaced endoplasmic reticulum, are particularly susceptible to oxidative stress, not only because of the high content of polyunsaturated fatty acids but also because of their association in the cell membrane with enzymatic and nonenzymatic systems capable of generating free radical species. They have been widely used as a model for oxidative stress and antioxidant studies (*30*). In the present study, two *in vitro* models of lipid peroxidation including nonenzymatic system induced by Fe^{2+}/H_2O_2 and enzymatic system induced by Fe^{3+}-ADP/NADPH were used. Many lipid peroxidation products can react with TBA, therefore, the TBA assay was commonly used to measure the relative degree of lipid oxidation (*31*). MDA, one of the products of lipid peroxidation, has been studied widely as an index of lipid peroxidation and as a marker of oxidative stress (*32*). The amount of MDA formed in rat liver microsomes upon Fe^{2+}/H_2O_2 or Fe^{3+}-ADP/NADPH oxidation increased over incubation time, reached a maximum around 1 h, and then was constant. Therefore, 1--hour incubation time was used for further experiment on microsomes. Table 1 shows the antioxidative activity of EHWB measured in a rat liver microsome system oxidized by Fe^{2+}/H_2O_2 and Fe^{3+}-ADP/NADPH. The results show that EHWB inhibited lipid peroxidation in a concentration-dependent manner in the enzymatic and nonenzymatic systems. In the enzymatic system, EHWB at 10 mg/L hardly inhibited the formation of MDA in rat liver microsomes compared to the control. Maximal inhibition values reached 90.3 and 87.0 % at 50 mg/L of EHWB in the nonenzymatic system induced by Fe^{2+}/H_2O_2 and the enzymatic system induced by Fe^{3+}-ADP/NADPH, respectively, which were similar to that of BHT at 20 mg/L with no statistical difference. Interestingly, the antioxidant capacity of EHWB at a concentration ranging from 10 to 20 mg/L in the nonenzymatic system was more effective ($p<0.05$) than that in the enzymatic system. This might be attributed to the antioxidant action of phenolic compounds in EHWB. The total phenolics were 0.3712 g of ferulic acid equivalents per gram of EHWB (*19*). Verma *et al*. (*33*) investigated the phenolic acid composition of acid- and alkali-hydrolysed wheat bran fractions and demonstrated that ferulic acid was the dominant phenolic acid in the studied wheat bran. Nyström *et al*. (*12*) demonstrated that steryl ferulate extracts from wheat bran by treatement with 6 mol/L of hydrochloric acid showed good antioxidant activities in both bulk and emulsion lipid systems, principally owing to the radical scavenging activity of the ferulic acid moiety of the molecule.

In the enzymatic system, NADPH cytochrome P450 reductase is involved in NADPH-dependent enzymatic lipid peroxidation in rat liver microsomes (*34*). The enzymatic lipid peroxidation is initiated by the reduction of a trace amount of Fe^{3+} or Fe^{3+}-ADP complex by NADPH cytochrome P450 reductase with NADPH. We have shown that EHWB exhibited effective antioxidant activities in the employed *in vitro* experiments, including metal chelation analysis, reducing power test, DDPH radical and reactive oxygen species (H_2O_2, $O_2^{\cdot-}$, $^{\cdot}OH$) scavenging activity assays (*19*). The inhibition effect of EHWB on the enzymatic lipid peroxidation in rat liver microsomes might be related to its antioxidant property and/or the supply of electrons derived from NADPH to the Fe^{3+}--ADP complex in liver microsomes. In the microsome system oxidized by Fe^{2+}/H_2O_2, initiation of lipid peroxidation by Fe^{2+} takes place either through ferryl-perferryl complex or through hydroxyl radical generation (*35*). Therefore, it might be possible that EHWB inhibited the lipid peroxidation in rat liver microsomes by the absence of ferryl-perferryl complex, by chelating the iron ions, by suppressing the generation of free radicals or by scavenging free radicals generated by the Fenton reaction in the nonenzymatic system.

Oxidation of polyunsaturated fatty acids in food generally occurs during raw material storage, processing, heat treatment and further storage of final products. It is one of the basic processes resulting in losses of fatty food quality by forming the products that have negative effect on the aroma and nutritional value of the food, and leading to its deterioration. Lipid oxidation can be effectively prevented by using antioxidants. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and BHT have been used in stabilization of food. However, the use of these chemical compounds has begun to be restricted due to their side effects (*36*). Therefore, there is great interest in finding new and safe antioxidants from natural sources. Plants containing high levels of phe-

Table 1. The effect of EHWB on lipid peroxidation in rat liver microsomes

Sample	Nonenzymatically induced lipid peroxidation		Enzymatically induced lipid peroxidation	
	n(TBARS)/*m*(protein)	Inhibition	*n*(TBARS)/*m*(protein)	Inhibition
	nmol/mg	%	nmol/mg	%
control	(23.6±1.8)^a		(49.8±2.5)^a	
γ(EHWB)=10 mg/L	(16.7±1.1)^b	(29.2±2.7)^a	(43.9±1.5)^a	(11.7±1.6)^a
γ(EHWB)=20 mg/L	(9.5±0.9)^c	(59.9±1.7)^b	(28.1±1.2)^b	(43.5±1.5)^b
γ(EHWB)=50 mg/L	(2.3±0.4)^d	(90.3±1.0)^c	(6.5±1.1)^c	(87.0±2.3)^c
γ(BHT)=20 mg/L	(1.9±0.4)^d	(92.1±1.1)^c	(4.3±0.6)^c	(91.4±0.9)^c

TBARS production was measured in microsomes exposed to nonenzymatic system induced by Fe^{2+}/H_2O_2 and enzymatic system induced by NADPH/ADP/Fe^{3+}; values are mean±S.D. of at least three duplicate, independent measurements
$^{a-d}$mean values within a column with different superscripts are significantly different ($p<0.05$)

nolics have a great importance as natural antioxidants. Wheat is the staple dietary cereal in many regions of the world and its bran is rich in hydroxycinnamic acids. Our previous study demonstrated that the phenolic content of EHWB was 0.3712 g of ferulic acid equivalents per gram of EHWB and showed good scavenging activity, indicating that EHWB may be considered as a potent antioxidant for food preservation.

Inhibitory activity of EHWB against LDL oxidation induced by Cu^{2+} and AAPH

LDL is the major cholesterol carrier in the blood and is exposed to a highly oxygenated and lipid-enriched environment, making it susceptible to free radical-mediated lipid peroxidation, which can result in the formation of oxidized LDL (oxLDL). OxLDL is associated with the pathogenesis of atherosclerosis (*37*). It has been widely accepted that antioxidants may inhibit LDL oxidation, and eventually may reduce the risk of cardiovascular diseases (*38*). In the studies of antioxidant activity of hydroxycinnamates towards human LDL, Cu^{2+}, metmyoglobin and AAPH have been employed to induce oxidation (*39*). AAPH is an artificial hydrophilic Azo initiator that is widely used in quantitative studies of oxidative kinetics because it has the advantage of generating localized radicals by its decomposition at the physiological temperature without biotransformation or enzymatic degradation and the rate of radical generation is easily controlled by adjusting the concentration of initiators (*40*). Although the use of copper as an initiator for *in vivo* oxidation is debated, copper-induced LDL oxidation is the most intensively studied procedure *in vitro* (*39*).

In this study, we examined the inhibitory activity of EHWB against the LDL oxidation mediated by Cu^{2+} and AAPH. Fig. 1 shows the effects of EHWB on the formation of conjugated diene from LDL oxidation induced by Cu^{2+}. The oxidative modification of LDL was suppressed when EHWB was added, and the lag time was also significantly extended as shown in Table 2. The lag time was increased with the increase of EHWB concentration and the inhibition at 20 mg/L was increased about 3.7--fold compared to that at 5 mg/L. The lag time for EHWB at 20 mg/L was comparable to that of ascorbate with no statistical difference. Ascorbate, a major water-soluble, chain-breaking antioxidant, significantly inhibited Cu^{2+}-

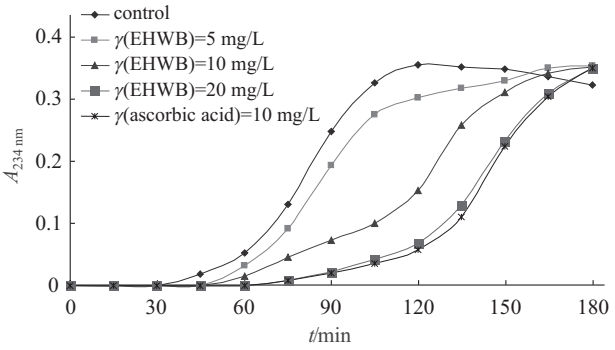

Fig. 1. Time course of the formation of conjugated diene in LDL mediated by Cu^{2+} in the presence or absence of EHWB. LDL solution (0.1 mg/mL) in PBS was incubated with 10 μmol/L of $CuSO_4$ at 37 °C in the presence or absence of the tested compounds. Conjugated diene formation was measured by determining the absorbance at 234 nm every 15 min for 3 h and the results are expressed as relative absorbance at 234 nm

-induced oxidative modification of LDL with 97 % decrease of TBARS at 40 μmol/L and helped to preserve its endogenous lipid-soluble antioxidants (*41*). The generation of TBARS during AAPH-initiated human LDL oxidation is shown in Table 2. It was observed that the addition of EHWB retarded the LDL oxidation. EHWB inhibited human LDL oxidation in a dose-dependent manner with inhibition of 63.03 % at 20 mg/L, which was similar to that of ascorbate at 10 mg/L with no statistical difference.

The obtained results confirmed that EHWB exhibited significant antioxidant activity *in vitro* against LDL oxidation by copper- and AAPH-initiated systems. It has been reported that wheat bran extracts significantly reduced lipid peroxidation in LDL induced by Cu^{2+} *in vitro*, which was rich in hydroxycinnamates with ferulic acid predominating (*42*). Phenolic compounds may act as antioxidants by several different mechanisms, including free radical scavenging, metal chelation and protein binding (*39*). Cu^{2+}-induced LDL oxidation is generally believed to involve direct reduction of Cu^{2+} to Cu^{+} by LDL itself. The Cu^{+} is a strong prooxidant and can rapidly generate the ultimate initiating radicals to initiate LDL oxidation (*43*). In the Cu^{2+}-induced LDL oxidation system, EHWB may inhibit LDL oxidation by the following

Table 2. Inhibitory effect of EHWB on human LDL oxidation induced by Cu^{2+} and AAPH

Sample	Cu^{2+}-mediated LDL oxidation		AAPH-mediated LDL oxidation	
	Lag time	Inhibition	*n*(TBARS)/*m*(protein)	Inhibition
	min	%	nmol/mg	%
control	(60.8±2.0)[a]		(35.8±1.3)[a]	
γ(EHWB)=5 mg/L	(70.6±3.7)[b]	(14.1±1.8)[a]	(26.6±1.4)[b]	(25.7±2.2)[a]
γ(EHWB)=10 mg/L	(115.5±4.6)[c]	(47.3±3.5)[b]	(17.9±1.2)[c]	(50.0±3.3)[b]
γ(EHWB)=20 mg/L	(127.9±7.2)[d]	(52.4±2.4)[c]	(13.2±1.1)[d]	(63.0±4.6)[c]
γ(ascorbate)=10 mg/L	(130.4±9.8)[d]	(53.2±4.5)[c]	(13.4±1.7)[d]	(62.7±3.4)[c]

LDL (0.1 mg/mL) was incubated at 37 °C with 10 μmol/L of $CuSO_4$ for 3 h for determination of lag time in the absence (control) or presence of the tested compounds; LDL (0.2 mg/mL) was incubated at 37 °C with 45 mmol/L of AAPH for 2 h for determination of TBARS in the absence (control) or presence of the tested compounds; data are expressed as mean values±S.D. (*N*=3)
[a–d]mean values within a column with different superscripts are significantly different ($p<0.05$)

probable mechanisms. First, EHWB is diffused to either lipid or protein fractions of LDL and directly reacts with and quenches free radicals in the system. Second, EHWB can chelate Cu^{2+}, which may act as a catalyst to generate the first few radicals that initiate the oxidative chain reaction. Third, EHWB directly reacts with and converts the peroxides in the system to less reactive compounds (*40*). In the case of AAPH, it decomposes at physiological temperature producing alkyl radicals (R) followed by fast reaction with oxygen to give alkyl peroxyl radicals (ROO) to initiate LDL peroxidation (LOO). In our previous study, EHWB was rich in phenolics which possess the capability of donating electrons (*19*). In the presence of EHWB, either the initiating peroxyl radical and/or the propagating lipid peroxyl radical could be trapped, thereby inhibiting the LDL oxidation induced by AAPH (*44,45*).

Loss of antioxidant enzymes in mice caused by alloxan-induced lipid peroxidation

Alloxan (2,4,5,6-tetraoxypyrimidine; 5,6-dioxyuracil), a mildly oxidizing agent that exhibits the most potent toxicity, can act as a generator of reactive oxygen species as long as sufficient suitable reducing agents (*e.g.* reduced glutathione) and oxygen are available. These alloxan-induced reactive oxygen species can interact with cellular membranes to cause lipid peroxidation, and thereby drastically alter the structure and function of membranes and generate highly toxic by-products (*46*). In this study, alloxan was employed to induce lipid peroxidation after which the loss of antioxidant enzymes in mice and the antioxidant potential of EHWB were evaluated. The TBARS concentration and the levels of such key antioxidants as SOD, CAT and GSH-Px were determined to examine the level of lipid peroxidation. Tables 3 and 4 show the TBARS level and the levels of CAT, SOD and GSH-Px activities in the serum and liver of normal and experimental mice. The content of TBARS was significantly increased, whereas CAT, SOD and GSH-Px activities were significantly decreased in the serum and liver of alloxan-treated mice compared to normal mice. However, EHWB and ascorbate significantly decreased the level of TBARS and were associated with significantly increased CAT, SOD and GSH-Px activities in the serum and liver of alloxan-treated mice compared to the control. EHWB at a low dose of 50 mg/kg of body mass also significantly increased the levels of CAT, SOD and GSH-Px activities in the serum of alloxan-treated mice compared to normal mice. EHWB and ascorbate maintained similar levels of TBARS and CAT, SOD and GSH-Px activities in the liver of alloxan-treated mice compared to normal mice. Interestingly, treatment with EHWB at a low dose of 50 mg/kg of body mass was found to be more effective in restoring the antioxidant status in the serum of alloxan-treated mice than of those treated with EHWB at a high dose of 100 mg/kg of body mass or with ascorbate at a dose of 50 mg/kg of body mass. Treatment with EHWB at a low or high dose was comparable to that of ascorbate at 50 mg/kg of body mass in decreasing the level of TBARS. Ascorbate treatment with a dose of 60 mg/kg of body mass decreased significantly the TBARS level in the

Table 3. The content of TBARS and the activities of SOD, GSH-Px and CAT in the serum of alloxan-treated mice

Group	*c*(TBARS) / nmol/mL	SOD activity / U/mL	GSH-Px activitiy / U/mL	CAT activity / U/mL
normal	(1.3±0.9)[a]	(146.3±9.7)[a]	(281.2±17.4)[a]	(14.5±1.6)[a]
alloxan control	(6.5±0.7)[b]	(121.2±11.4)[b]	(217.1±16.9)[b]	(8.6±1.0)[b]
w(EHWB)=50 mg/kg	(2.3±0.6)[c]	(156.3±15.0)[c]	(324.8±21.6)[c]	(16.5±1.6)[c]
w(EHWB)=100 mg/kg	(2.1±0.5)[c]	(149.4±12.9)[a]	(304.7±20.8)[a]	(14.9±1.5)[a]
w(ascorbate)=50 mg/kg	(2.2±0.5)[c]	(152.1±13.5)[a]	(301.2±19.5)[a]	(15.4±1.5)[a]

data are expressed as mean values±S.D. (*N*=10)
[a–c]mean values within a column with different superscripts are significantly different ($p<0.05$)

Table 4. The content of TBARS and the activities of SOD, GSH-Px and CAT in the liver of alloxan-treated mice

Group	*n*(TBARS)/*m*(protein) / nmol/mg	SOD activity / U/mg	GSH-Px activity / U/mg	CAT activity / U/mg
normal	(1.4±0.2)[a]	(12.6±0.8)[a]	(9.6±0.2)[a]	(116.0±9.9)[a]
alloxan control	(3.1±0.4)[b]	(5.7±0.4)[b]	(4.7±0.8)[b]	(68.8±8.0)[b]
w(EHWB)=50 mg/kg	(1.2±0.2)[a]	(12.9±0.8)[a]	(10.1±0.7)[a]	(116.6±4.9)[a]
w(EHWB)=100 mg/kg	(1.1±0.3)[a]	(11.7±0.5)[a]	(9.8±0.5)[a]	(109.6±5.4)[a]
w(ascorbate)=50 mg/kg	(1.3±0.2)[a]	(12.7±0.5)[a]	(9.9±0.6)[a]	(114.7±5.3)[a]

data are expressed as mean values±S.D. (*N*=10)
[a–c]mean values within a column with different superscripts are significantly different ($p<0.05$)

liver of rats fed either standard diet or a diet with high cholesterol and high saturated fatty acid content (Nath diet) (*47*). Wang *et al*. (*48*) reported that tartary buckwheat bran extract could increase the levels of SOD and GSH-Px, and decrease the MDA content in the serum of rats fed a high-fat diet, which might be due to the antioxidant effects of phenolics in the bran. The feruloylated oligosaccharides, which were isolated from wheat bran by treatment with xylanases, could decrease the level of MDA, and increase the activities of antioxidant enzymes such as CAT, SOD and GSH-Px in the plasma of rats (*18*). The antioxidant potential of ferulic acid is expressed within the colon to protect the cells from oxidative radicals released by bacteria or from other sources (*49*). The improvement of antioxidant status in the serum and liver of the alloxan-treated mice indicated that EHWB might be beneficial for human health.

In the alloxan-treated mice, the significantly elevated TBARS indicated the increase of lipid peroxidation, thereby decreasing the antioxidant defense in biological system including SOD, CAT and GSH-Px. The antioxidant enzymes can destroy the peroxides and play a significant role in providing antioxidant defense to an organism. GSH-Px and CAT are involved in the elimination of H_2O_2. SOD acts to dismutate superoxide radical to H_2O_2, which is then acted upon by GSH-Px. In the presence of EHWB rich in phenolics, the observed decrease in TBARS indicated that EHWB could inhibit the lipid peroxidation *in vivo*, thus clearly protecting or improving the activity of GSH-Px, SOD and CAT. The increase of the activity of these biological antioxidant enzymes in the serum and liver of alloxan-treated mice might directly scavenge free radicals or prevent their conversion to toxic products, thereby, *in vivo* they play an important role in the removal of damaging effects caused by oxidative stress in living systems.

Besides pre-hydrolysis, fermentation of wheat bran by the microflora may be another way of rendering phenolic acids more bio-accessible. Several *in vivo* studies demonstrated that there was significant plasma uptake and urinary excretion of phenolics in humans after consumption of wheat bran and the plasma total antioxidant status was significantly increased (*50–52*). It has been reported that wheat bran phenolics were relatively well absorbed and might enhance antioxidant status in humans, compared to the other phenolics-rich food (*52*). Therefore, the nutritional and health potential of wheat bran deserves to be nutritionally valorized for humans.

Conclusions

EHWB exhibited significant inhibitory activity towards lipid peroxidation by different *ex vivo* and *in vivo* biological systems. The biochemical anti-lipid peroxidative activities of EHWB might be related to the free radical scavenging and antioxidative activities of the phenolics in them. The results demonstrated that the EHWB could be used as natural antioxidant agents in the food preservation and for human health. The exact protection mechanism of EHWB against lipid peroxidation will be further investigated.

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