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Original Research

In Vitro and *in Vivo* antioxidant activity of ethyl acetate extraction of purple rice

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Abstract: The antioxidant activities of ethyl acetate extraction of purple rice (EAEPR) were evaluated by various methods *in vitro* and *in vivo*. In *in vitro* antioxidant assays, EAEPR was found to have strong 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity hydroxyl radical, reducing power and metal-ion chelating activity. In *in vivo* antioxidant assays, mice were administered with EAEPR *via* gavage for 42 consecutive days. As a result, administration of EAEPR significantly enhanced the activities of glutathione peroxidase in serums and livers of mice. EAEPR could improve the lipid status, especially total cholesterol and low-density lipoprotein cholesterol levels. In addition, total phenolic content of EAEPR was 188.21mg/g. The main phenolic compounds in EAEPR analyzed by ultra-high performance liquid chromatography tandem mass spectrometry were determined as ferulic acid and quercetin. The contents of ferulic acid and quercetin in EAEPR were 14.21mg/g and 35.28mg/g, respectively. The Nrf2 expression was significantly elevated after administration of EAEPR.These results suggested that EAEPR had potent antioxidant activity and could be explored as a novel natural antioxidant.

Key words: Antioxidant activity, purple rice, lipid status, *in vivo, in vitro*.

Introduction

Oxidative stress, resulted from the overproduction and/or insufficient removal of free radicals, usually accompanies many chronic diseases such as cardiovascular disease, aneurysm and type 2diabetes (1-3). Oxygen radical species (ROS) are produced through multiple mechanisms, including enzymatic, non-enzymatic, and mitochondrial pathways (4, 5). Under pathological conditions, ROS production requires protein kinase C-delta (PKCδ) and receptor-interacting protein kinase 3 (RIPK3), critical mediators of cell death and inflammation that have been implicated in multiple vascular diseases (6-8). Through oxidation of protein, lipid, and DNA, ROS can cause cellular damage and lead to disease complications (9). However, ROS can be initially eliminated by essential scavenger enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) (10). Both synthetic and natural antioxidants are used to reduce ROS-induced oxidative damage. Epidemiological studies have shown that regular consumption of antioxidants, such as andrographolide, is associated with reducing risks of various types of chronic diseases such as cardiovascular disease, aneurysm, type 2 diabetes and some cancers (11-16).Thus, it is essential to develop natural nontoxic antioxidants to protect human body from oxidative stress associated with many chronic diseases. In the search of new natural antioxidants, purple rice has been demonstrated to possess potent antioxidant activity and potential applications as a natural antioxidant (17, 18).

Purple rice is mainly produced in Southeast Asia, china and Japan, which is used for food ornamentation purposes (17). It is considered as a valuable health food with high contents of polyphenols, vitamins, minerals andγ-oryzanol (19). Moreover, purple rice has been demonstrated to possess various valuable biological properties including antioxidant, hepatoprotective, antidiabetic and anti-inflammatory effects. Till now, most studies have been focused on the *in vitro* antioxidant activities of purple rice concerning reducing power, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical (19), 2,2-azinobis-(-3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity (20), oxygen radical absorbance capacity (ORAC) (18), and iron chelating capability(18). However, little attention has been paid to other *in vitro* antioxidant activities and especially *in vivo* antioxidant activities of purple rice. In order to utilize this valuable grain resource better, it is desired to evaluate the antioxidant activity of purple rice systematically.

In the present study, the total phenolic content in the extract was determined by Folin-Ciocalteu method. The main phenolic compounds in ethyl acetate extraction were analyzed by ultra-high performance liquid chro-

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matography tandem mass spectrometry (UPLC-MS). Then, *in vitro* antioxidant activities of ethyl acetate extraction of purple rice (EAEPR) including scavenging DPPH radical ability, reducing power and metal chelating activity were investigated. Finally, the potential *in vivo* antioxidant activity of EAEPR was evaluated by measuring the changes in activities of antioxidant enzymes in high-fat diets mice.

Materials and Methods

Chemicals

A total antioxidant capacity (T-AOC) kit, superoxide dismutase (SOD) kit, catalase (CAT) kit, glutathione peroxidase (GSH-Px) kit, malondialdehytde (MDA) kit and bovine serum albumin (BSA) protein assay kit were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Folin-Ciocalteu reagent, p-coumaric acid, ferulic acid, gallic acid, 1,1-di-phenyl-2-picrylhydrazyl (DPPH) and rutin were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), ferrozine and TPTZ (2, 4, 6-tripyridyl-s-triazine) were purchased from J&K (Scientific Co., Ltd., Beijing, China). All other chemicals and solvents were of analytical grade.

Preparation of extraction

Total phenolic compounds were extracted as described (21) with minor modifications. Briefly, purple rice flour was digested with 2mol/L sodium hydroxide at room temperature (25°C) for 1 hour with shaking under nitrogen. The mixture was neutralized with appropriate amount of hydrochloric acid. Petroleum ether was used to extract lipids in the mixture. The ethyl acetate fraction was evaporated to dryness. Phenolic compounds were reconstituted in 80% chilled methanol and stored at -20°C for analysis.

Determination of the total phenolic and total anthocyanin contents

The total phenolic contents of EAEPR were determined using the Folin-Ciocalteu colorimetric method described by Singleton et al with some modifications (22). Gallic acid was used as the standard, and total phenolic content was expressed as mg gallic acid equivalents (GAE)/g EAEPR. Briefly, the appropriate dilutions of extracts were oxidized with Folin-Ciocalteu reagent, and the reaction was neutralized with sodium carbonate. The absorbance of the resulting blue color was measured at 760 nm after 90 min.

The total anthocyanin content (TAC) of EAEPR was measured using a spectophotometric pH differential protocol reported by Lopez-Martinez (23). Absorbance readings at 535 nm were taken and corrected for background absorbance at 700 nm in a photodiode array spectrophotometer. Anthocyanins are expressed as mg of cyanidin-3 glucoside equivalents/100 g, using a molar extinction coefficient of 25,965 $M^{-1}cm^{-1}$ and a molecular weight of 449.2 g/mol.

UPLC-Electrospray Ionization (ESI)/MS Analysis

The UPLC analysis was carried out on a Waters ACQITY UPLC system (Waters, Milford, MA, USA), equipped with a binary solvent delivery system and an auto-sampler. The mass spectrometer was operated in negative electrospray ionization mode (ESI−) using multiple reaction monitoring (MRM) at a capillary voltage of 3kV, cone voltage of 25 V, source temperature of 130°C, desolvation temperature of 350°C, and desolvation gas flow of 650 L/hr. The mass spectrometer was operated in positive electrospray ionization mode (ESI+) using selected ion recording (SIR) at a capillary voltage of 3.5kV, cone voltage of 35 V, source temperature of 140°C, desolvation temperature of 380°C, and desolvation gas flow of 800 L/hr. The analytes of interest were identified by comparison of their mass-to-charge ratio (m/z) and retention time values with those of authentic standards: m/z 194.19 for ferulic acid, m/z 140.09 for p-coumaric acid, m/z 180.15 for caffeic acid, m/z 170.12 for gallic acid, m/z 152.14 for vanillic acid, m/z 308.28 for catechin, m/z 354.31 for chlorogenic acid, m/z 228.24 for resveratrol, m/z 610.51 for rutin,m/z 302 for quercetin.

The column used was Waters Acquity UPLC BEH C18 (2.1mm*100mm, 1.7μm particle size) which was maintained at 30°C. The auto-sampler tray temperature was 4°C and the injection volume was 1.0μL. The phenilic extraction was 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) for phenolic analysis. Solvents were filtered through 0.22 μm filters. The gradient was 5%B from 0 to 2min, 5-10%B from 2 to 4min, 10-15% B from 4 to 6min,15-21% B from 6 to 8min, 21-27%B from 8 to 9min,27-50%B from 9 to 10min,50-0%B from 10 to 11min, 0-5%B from 11 to 12min. The flow rate was 0.3mL/min. MassLynx v. 4.1 software was used to control the UPLC/MS system.

DPPH radical scavenging ability

Antioxidant activity of the obtained extracts was also tested using the 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) as previously described (24) with some modifications. Briefly, the DPPH free radical scavenging activity of grain extracts was determined using a 2×10^{-4}mmol/L DPPH solution. Each sample of whole grains extracts (0.5mL) was mixed with 4 ml 2×10^{-4} mmol/L DPPH in ethanol. The mixture was shaken, and then left to stand for 60 min in the dark. The absorbance was measured at 517nm in a spectrophotometer. The absorbance of the control was obtained by replacing the sample with methanol. The DPPH radical scavenging activity of the sample was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Ferric reducing antioxidant power assay

The Ferric reducing antioxidant power (FRAP) was measured according to the procedures described by Muller with some modifications (25). In our procedure, freshly prepared FRAP reagent (2.5 mL of a 10mmol/l TPTZ solution in 40mmol/l HCl, 2.5 mL of 20mmol/l $FeCl_3$ and 25 mL of 0.1mol/l acetate buffer, pH 3.6) was incubated at 37°C for 10 min. Then, 0.05 ml of rapeseed extracts and 2 mL of FRAP reagent were transferred into a 10ml volumetric flask and made up to volume with redistilled water. The obtained blue solutions were kept at room temperature for 20 min. The absorbance was measured at 593 nm against a reagent blank (2 mL

of FRAP reagent made up to 10mL with redistilled water) using a UV spectrophotometer in a 1cm quartz cell. All solutions were used within the day for preparation. Trolox was used as the positive control, and the results were expressed as A_{593nm}.

Metal Chelating Activity

The metal chelating activities of EAEPR were determined by the method of Liang (26) with minor modifications. A methanol solution of the samples (0.5mL) at various concentrations (0.05, 0.1, 0.15 0.2mg/mL) was mixed with methanol (3mL) $FeCl_2 \cdot 4H_2O$ (2mmol/L, 0.1mL) and ferrozine (5mM, 0.2mL). The mixture stood in the dark for 10 min. The control contained all the reagents without the sample and was used as a blank. The metal chelating activity was determined by measuring the absorbance at 562 nm using a spectrophotometer. The metal chelating activity of EDTA was also determined for comparison.

$$\text{The metal chelating activity } (\%) = \frac{Asample - Acontrol}{Asample} \times 100$$

Animals and experimental design

The license number for using experimental animals was SCXK (SH) 2012-0002. The experimental protocol was developed according to the institution's guideline for the care and use of laboratory animals by Jiangnan University. Animal maintenance and experimental procedures were approved by Animal Ethics Committee of Jiangnan University.

Thirty male mice weighing 20±2g (four weeks old) were used for the present investigation (Shanghai Laboratory Animal Center). Animals were maintained under standard conditions (23±2°C, relative humidity 55±0.5%, 12 h light-dark cycle). Prior to experimental study, animals were fed basal diet for one week for adaptation. Mice in NFD and HFD were fed with physiological saline by gavage. Mice in EAEPR treatment groups were fed with EAEPR in 600 mg/kg BW per day by gavage. The composition of basal diet was showed in Table 1 provided by SLAC Shanghai Laboratory Animals Limited liability Company. All groups were performed once daily for 42 consecutive days.

Biochemical assay

Twenty-four hours after the last drug administration, mice were sacrificed. Blood samples were collected and centrifuged at 4000g at 4°C for 10 min to afford the serums. The livers were removed rapidly, washed and homogenized in ice-cold physiological saline to prepare 10% (w/v) homogenate. Then, the homogenate was centrifuged at 4000g at 4°C for 10 min to remove cellular debris, and the supernatant was collected for analysis.

The biochemical assays were carried out according to the instructions of kits purchased from Nanjing Jiancheng Bioengineering Institute. Briefly, CAT activity was determined by measuring the absorbance of the yellow H_2O_2–ammonium molybdate complex at 405 nm. SOD activity was measured through the inhibition of hydroxylamine oxidation by the superoxide radicals generated in the xanthine–xanthine oxidase system. GSH-Px activity was measured on the basis of the reaction of GSH and 5, 50-dithiobis-(2-nitrobenzoic acid). All above activities were expressed as unit per milliliter (U/ml) in serums or unit per milligram of protein (U/mg protein) in livers. The protein contents in the supernatants of livers were determined by the Lowry method (27) using bovine serum albumin as the standard.

Table 1. Composition of experimental diets.

Ingredient (%, w/w)	Normal-fat diet	High-fat diet
Fish meal	4.00	2.00
Soybean meal	20.00	10.00
Cornmeal	30.00	15.00
Wheat flour	32.80	16.40
Wheat bran	4.00	2.00
NaCl	0.40	0.20
Lysine	0.35	0.34
Methionine	0.30	0.29
Vitamin mixture	0.15	0.14
Mineral mixture	0.20	0.19
Sinkaline	0.20	0.19
Calcium hydrogen phosphate	1.60	1.54
Calcium carbonate	1.50	1.45
Lard	0.00	18.90
Sucrose	0.00	15.00
Casein	0.00	11.30
cholesterol	0.00	1.30
Choline	0.00	0.30
Maltodextrin	0.00	1.20
Protein	20.5	20.5
Carbohydrate	45.82	42.3
Fat	4.62	22.8
Energy(kcal/g)	3.45	4.60

Vitamin mixture (per kg of diet):V_A, 444980.85IU; V_{D3}, 6663.83IU; V_E, 263.78IU; V_{K3},8.33mg; V_{B1}, 22.21mg; VB2, 24.99mg; VB6, 27.77mg; VB12, 0.06mg; D-biotin, 0.49mg; folic acid, 11.11mg; Niacinamide, 124.95mg; D-calcium pantothenate, 55.53mg. Mineral mixture (per kg of diet): Ca, 14.81mg; Se,0.26mg; Mn, 9.81mg; Zn, 35.91mg; Fe, 14.81mg.

The spectrometric method was applied to evaluate T-AOC. In the reaction mixture ferric ion was reduced by antioxidant reducing agents and blue complex Fe^{2+}-TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) is produced. The optical density was measured at 520 nm. One unit (U) of T-AOC was defined as the amount that increased the absorbance by 0.01 at 37°C. Data were expressed as U/ml serum.

The determination of the levels of MDA was performed using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing City, China). The level of lipid peroxidation was indicated by the content of MDA in tissue. Thiobarbituric acid reaction (TBAR) method was used to determine the MDA. MDA content was expressed as nmol per milligram of protein.

Triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL_C) and low-density lipoprotein cholesterol (LDL_C) levels of serum were analyzed on a Mairui Automatic Analyzer (Shen Zhen, China) using commercial enzymatic kits following the

Table 2. Sequences of primers used in quantitative real-time reverse transcription PCR.

Gene	Forward primer	Reverse primer
GAPDH	GGTTGTCTCCTGCGACTTCA	TGGTCCAGGGTTTCTTACTCC
Nrf2	CTCCTATGCGTGAATCCCAATG	GTGTGAGATGAGCCTCTAAGCG
Gpx	TTGAGAAAGGAGATGTGAACGG	CAAAGTTCCAGCGGATGTCA
SOD	AACAATCTCAACGCCACCG	AGTCACGCTTGATAGCCTCCA
HO-1	CCACCAAGTTCAAACAGCTCTAT	'CTGACGAAGTGACGCCATCT
NQO1	CACGAGGATGGGAAAAGGAGTA	CCCGAAGAAAATGGCGAATA
LPL	ATCAACAAGGTCAGAGCCAAGA	CATCCTCAGTCCCAGAAAAGTG

manufacturer's instructions. Al was calculated using the following formula: (TC-HDL_C)/HDL_C.

Gene expression in liver

For determining mRNA expression, total RNA was first extracted from frozen tissues with Trizol reagent. The quantity and quality of the RNA were verified by measuring the A260/A280 ratio and by gel electrophoresis. Total RNA was reverse transcribed to cDNA according to the manufacturer's instructions (AMV First Strand cDNA Synthesis Kit). The mRNA expression was quantified using Real-time Polymerase Chain Reaction (RT-PCR). The primer sequences are listed in Table 2.

RT-PCR was carried out using ABI Stepone plus as the following conditions: 40 cycles of denaturation at 95°C for 10 s, annealing and extension at 60°C for 40s. Porcine primers were designed using Primer Premier 5.0 and synthesized by the Co. Generay Biotechnology (Shanghai, China). The relative expression levels of the target genes were calculated as a ratio to the housekeeping gene GAPDH. A house-keeping gene, GAPDH, was used as internal control to normalize the RT-PCR data. The relative expression level of a gene in a given sample was represented as $2^{-(\Delta\Delta Ct)}$ as compared with NFD group. ΔΔCt=ΔCt (experimental gene in each group)-ΔCt (NFD group), ΔCt=Ct (experimental)-Ct (GAPDH).

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS 17.0, Chicago, IL, USA) was used for all analyses. Analysis of variance (ANOVA) was employed to determine the significance of the main effects. Significant differences (p<0.05) between means were identified using Duncan's multiple range test.

Results

TPC and phenolic compounds

As shown in Figure1, the major phenolic compounds in purple rice were ferulic acid and quercetin. In previous study, cyanidin-3-O-glucoside, apigenin-6/8-C-pentoside-8/6-C-hexoside and quercetin-3-O-glucoside were the major compounds in black-purple rice (28). cyanidin-3-glucoside was the major anthocyanins in purple rice , cyanidin-3-galactoside and peonidin-3-glucoside were also observed(19). The content of cyanidin-3-glucoside (4.26mg/100g DW) and gallic acid (2.88mg/100g DW) were the main phenolic compounds in purple rice reported by Deng (20) .The difference may be the extraction methods and the purple rice varieties.

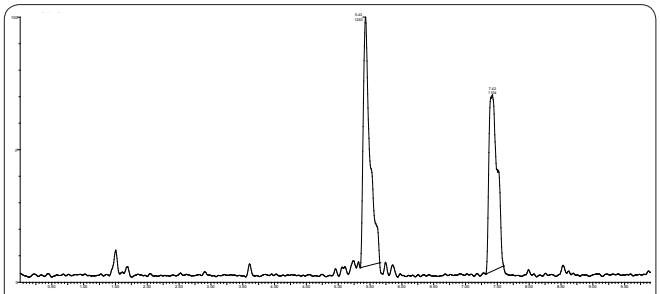

Figure 1. TIC (total ions chromatograph) of EAEPR.5.43min, ferulic acid; 7.42min, quercetin.

In our study, the contents of ferulic acid and quercetin in EAEPR were 14.21mg/g and 35.28mg/g, respectively. Total phenolic content of EAEPR was 188.21mg/g. The results indicated EAEPR was rich in antioxidants.

In vitro antioxidant activity

Scavenging activity on DPPH radical

DPPH method was found to be used mostly for the in vitro antioxidant activity evaluation purpose (29). DPPH is characterized as a stable free radical, owing to the delocalization of the spare electron over the whole molecule, resulting in a deep violet color at 517 nm (30). As shown in Figure 2-A, the scavenging activities on DPPH radical of EAEPR and Trolox increased with the increase of concentrations. They were able to scavenging the DPPH radical in a dose dependent manner. It should be noted that the scavenging activity of Trolox was higher than EAEPR (p<0.05). The IC_{50} of DPPH scavenging activity for EAEPR and Trolox were

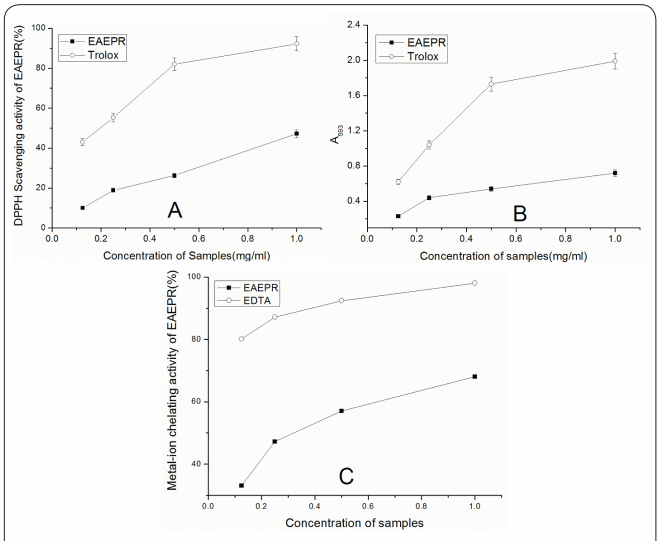

Figure 2. *In vitro* antioxidant activity of EAEPR measured by various methods. A: DPPH radical scavenging ability; B: FRAP method; C: Metal-ion chelating activity.

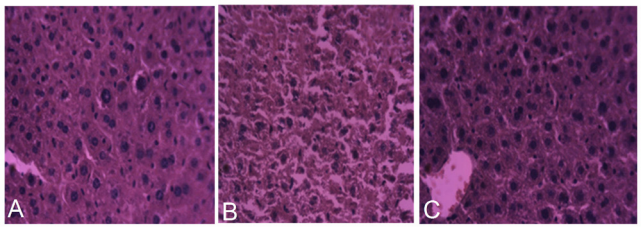

Figure 3. Effect of EAEPR on liver histology in mice fed with a high-fat diet (HE, 400×). A, NFD group; B, HFD group; C, HFD+ EAEPR group

1.46mg/ml and 0.17mg/ml, respectively. The results indicated EAEPR had strong DPPH radical scavenging activity. This was similar with previous studies which showed that purple rice extraction had a potent radical scavenging action (19, 31).

Ferric reducing antioxidant power

FRAP method has also been widely used in the determination of antioxidant capacity in wide range of antioxidants. This test works on the principles of electron donation by an antioxidant. It measures changes in absorbance by the formation of blue iron (II) from colorless oxide of iron (III). Increased absorbance is correlated with the reducing power of the extract. As shown in Figure 3, the ferric reducing power of EAEPR and Trolox were both in a dose dependent manner. Meanwhile, the ferric reducing power of Trolox was higher than EAEPR at the elected concentrations (Figure 2-B). These results indicated that EAEPR had a proper reducing power.

Metal-ion chelating activity

Transition metals have been proposed as the catalysts for the initial formation of radicals. Chelating agents may stabilize transition metals in living systems and inhibit the generation of radicals, consequently reducing free radical-induced damage. Phenolic compound is considered as good chelator for its structure (32). To better estimate the antioxidant potential of the extract, its chelating activity was evaluated against Fe^{2+}. The extract of EAEPR showed chelating activity in a dose dependent manner. The metal chelating activity of positive control ethylenediaminetetraacetic acid (EDTA) was found to be 80.12% (at 0.125 mg/mL). The observed IC_{50} value of EAEPR (0.33mg/mL) was significantly higher than that of the positive control EDTA (0.003 mg/ml). This suggested that the metal-ion chelating activity of EAEPR was much lower than EDTA. However, at concentration of 1mg/ml, the metal-ion chelating activity of EAEPR was 68.04% (Figure 2-C).

In vivo antioxidant activity

Effect of EAEPR on the growth of mice

As shown in Table 3, No changes were found in Daily weight gain, food intake, Feed efficiency, Liver weight and liver index of mice among three groups. The results could be supported by the previous study (33), suggesting that EAEPR did not cause serious toxicity in mice.

Lipid status and atherosclerosis index in serum

As shown in Table 3, serum levels of TG, TC, LDL_C and AI in HFD group significantly increased by 102.32%, 67.15%, 76.77% and 334%, while HDL_C level decreased by 21.08% ($p<0.05$) as compared with NFD group. However, as compared with HFD group, serum levels of TC, LDL_C land AI in HFD+EAEPR group decreased by 14.47%, 22.86% and 23.50% ($p<0.05$), while TG and HDL_C levels were not changed significantly. These results indicated that administration of EAEPR could improve the lipid status, especially TC and LDL_C levels.

Antioxidant activity in serum and liver

As shown in Table 3, serum levels of T-AOC,

Table 3. Effects of EAEPR on body weight grain, food intake, feed efficiency, liver weight, liver index, lipid status in serum, and T-AOC, MDA, SOD, GSH-Px and CAT in serum and liver of mice (n=10).

Group	Body weight grain (g/d)	Food intake (g/d)	Feed efficiency (%)	Liver weight (g)	Liver index (%)
NFD	17.92±4.09[a]	7.54±0.53[a]	5.66±1.29[a]	2.15±0.41[a]	4.76±0.67[a]
HFD	19.54±5.19[a]	6.86±0.55[a]	6.78±1.80[a]	2.18±0.24[a]	4.6±0.49[a]
HFD+ EAEPR	18.34±5.28[a]	6.93±0.63[a]	6.31±1.82[a]	2.17±0.33[a]	4.6±0.35[a]
serum (nmol/L)	TG	TC	HDL_C	LDL_C	AI
NFD	1.29±0.14[b]	2.77±0.11[b]	1.85±0.15[b]	0.99±0.18[b]	0.50±0.09[b]
HFD	2.61±0.14[a]	4.63±0.15[a]	1.46±0.10[a]	1.75±0.22[a]	2.17±0.05[a]
HFD+ EAEPR	2.43±0.11[a]	3.96±0.19[b]	1.49±0.09[a]	1.35±0.13[b]	1.66±0.11[b]
serum(U/mL)	T-AOC	MDA	CAT	SOD	GSH-Px
NFD	11.79±1.22[b]	5.56±0.26[b]	3.39±0.11[b]	243.33±22.11[b]	876.88±20.11[b]
HFD	8.51±0.71[a]	7.98±0.39[a]	1.86±0.09[a]	181.08±20.89[a]	734.73±27.76[a]
HFD+ EAEPR	9.66±0.15[b]	6.67±0.19[a]	2.11±0.17[a]	187.20±12.18[a]	866.26±12.06[b]
Liver(U/mg protein)	T-AOC	MDA	CAT	SOD	GSH-Px
NFD	2.11±0.17[b]	1.56±0.67[b]	37.44±2.65[b]	322.99±28.11[b]	272.29±29.22[b]
HFD	0.99±0.04[a]	2.98±0.75[a]	15.26±1.99[a]	254.78±19.78[a]	144.38±30.09[a]
HFD+ EAEPR	1.03±0.06[a]	1.79±0.28[b]	16.09±1.22[a]	255.02±20.29[a]	211.92±19.09[b]

NFD, normal fat diet; HFD, high fat diet; HFDPR, HFD+600mg/kg BW purple rice

Different letters indicate statistical significance (at least $p<0.05$). Food efficiency (%) = (body weight gain [g]/food intake [g])*100.

CAT, SOD and GSH-Px in HFD group significantly decreased, while MDA levels markedly increased as compared with NFD group. Serum levels of T-AOC and GSH-Px in HFD+ EAEPR group significantly increased by 13.51% and 17.90%, while MDA CAT and SOD levels were no difference as compared with HFD group (p >0.05).

As shown in Table 3, liver levels of T-AOC, CAT, SOD and GSH-Px in HFD group significantly decreased (p <0.05), while MDA levels markedly increased as compared with NFD group (p<0.05). Liver level of MDA in HFD+ EAEPR group decreased by 39.93% (p <0.05), while Liver levels of GSH-Px increased by 46.78% as compared with HFD group (p <0.05).

Hepatic steatosis

To investigate the inhibitory effect of supplementation of different grains on hepatic fat accumulation, we analyzed the histology of liver tissue using hematoxylin-eosin staining. As shown in figure 3, the cell architectures of liver in normal mice showed normal hepatocytes with a central vein (Fig.3A). As compared with the normal liver tissues of mice in NFD group, liver tissue in HDF group revealed liver injury characterized by moderate hypertrophy of hepatocytes, inflammatory cell infiltration, dilated sinusoidal spaces and ballooning degeneration around the central vein (Fig.3B). The hepatocellular steatosis was observed mice of HFD group. However, the hepatic lesions induced by high fat diet were remarkably ameliorated after administration of EAEPR (Fig.3C).

Gene expression in liver

Effects of EAEPR on antioxidant gene expression in liver were shown in Figure 4. As compared with NFD group, the expression of Nrf2, Gpx, SOD HO-1 and LPL in HFD group decreased significantly (p <0.05). However, the Nrf2 expression in HFD+EAEPR group was significantly elevated as compared with HFD group. There were no changes in other antioxidant gene between HFD and HFD+ EAEPR group. Our results are also in accordance with previous studies showed that high fat diet could decrease the expressions of oxidative stress-related genes and supplementation with antioxidants (34-36).

Discussion

Based on our antioxidant experiment results in vitro, EAEPR exhibited obviously high antioxidant activity. Oxidative stress plays an important role in the pathogenesis of many chronic diseases (37, 38). Antioxidant is an important pathway to realize protective effects. As a consequence, antioxidants have been proposed as an adjunct therapy for various chronic diseases. Some natural products with antioxidant activity have been proved to have good protective effects. In the present study, EAEPR exhibited strong DPPH scavenging ability, reducing power and metal chelating activity. However, they were weaker than their positive control.

Results from the serum lipid status of HFD-fed mice for six weeks showed concentrations of serum TC, TG, and LDL increased, whereas HDL decreased. The elevations in serum total TG and TC levels observed on HFD feeding is in agreement with those reported in several studies (39, 40). Treatment of HFD-fed mice with LA and EAEPR showed a considerable restoration of these parameters to that of control levels. The AI, defined as the ratio of TC- HDL-C and HDL-C, is believed to be an important risk factor of atherosclerosis. Our data clearly demonstrate that EAEPR significantly decreased the ratio. High serum levels of LDL-C and low serum levels of HDL-C are associated with an increased risk for atherosclerosis (41). In our study, decreasing the LDL-C concentrations in HFD-fed rats indicates the proper anti-atherogenic property of EAEPR.

Oxidative stress through high fat-induced lipid peroxidation is widely known and has been extensively used in experimental models to understand the oxidative damage and evaluate the therapeutic potential of drugs and dietary antioxidants (39). Elevation in MDA indicated that there was greater production of ROS that increased the rate of lipid peroxidation. Our study found that high-fat-induced mice had significantly increased MDA concentration in both the serum and liver. This agrees with results of previous studies demonstrating increase of lipid peroxidation in serum of mice fed with a high-fat diet (39, 42, 43). However, Administration of EAEPR could ameliorate the lipid peroxidation both in serum and liver in our study.

Antioxidant enzymes such as CAT, SOD and GSH-Px are the first line of defense against biologic macromolecule oxidative injury (44). In order to delineate the mechanisms underlying the protective effect of EAEPR in mice, the activities of CAT, SOD and GSH-Px were observed in our study. Significant lower activities of CAT, SOD and GSH-Px were observed in the high fat diet group than those in normal diet group, which is similar to the previous findings (39). GSH-Px catalyzed the reduction of H_2O_2 and hydroperoxides to non-toxic products. It also quenched lipid peroxidation by eliminating lipid hydroperoxides from the cell membrane (45). Prolonged consumption of EAEPR significantly decreases GSH-Px activity in the liver. However, administration of EAEPR to mice enhanced the activities of GSH-Px, highlighting EAEPR could restore or maintain the activities of antioxidant enzymes in serum and liver of mice fed with a high fat diet.

SOD is the critical antioxidant enzyme defense against ROS, especially superoxide anion radicals (46). In the present study, SOD activity in the liver decreased significantly in high-fat-treated mice compared with the control group. However, administration of EAEPR does not alter SOD activity, similar to previous reports. The most plausible explanation may be the elevated GSH-Px activity in the removal of H_2O_2 due to its location (mitochondria and cytosol) (45). Therefore, hepatic SOD activity did not change in high-fat-induced liver damage.

According to the results of our study, it was clearly indicated that EAEPR had significant antioxidant activity against various antioxidant systems *in vitro*. Furthermore, administration of EAEPR could significantly enhance the activities of GSH-Px in serums and livers of mice. TC and LDL_C levels in serum decreased after administration of EAEPR for 42 days in serum of mice fed with high fat diet. Our study supplied new information on the antioxidant function of purple rice for nutri-

tionists, food policy makers and consumers.

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