

Full Length Research Paper

***In vitro* antioxidant activity and inhibitory hepatic steatosis effect on oleic acid-induced fatty liver model of consecutive extracts from *Rosa davurica* Pall.**

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The nutrient and phytochemical composition of *Rosa davurica* Pall. fruit (RDF) from China were determined, including sugar, reducing sugar, ascorbic acid, carotenoids and phenolics. RDF was successively extracted with chloroform, ethyl acetate (EA), water-saturated *n*-butanol, ethanol and distilled water, respectively. Among the five fractions, EA fraction revealed the highest total phenolic content and the strongest antioxidant ability in cyclic voltammograms (CV) and oxygen radical absorbance capacity (ORAC) assays. Moreover, the strongest inhibitory activity against oleic acid-induced (OA-induced) fatty liver *in vitro* was also the EA fraction. Further, it was separated by a semi-preparative high performance liquid chromatography (HPLC) on C₁₈ column. The most active fraction (EA-FII) of EA in all assays above, which mainly included catechin, quercetin-sedoheptulose and quercetin-94, was identified by LC/MS, HPLC and ultraviolet (UV)/V spectrum analysis.

Key words: RDF extracts, composition properties, antioxidant ability, inhibitory activity, oleic acid-induced, HPLC, LC/MS.

INTRODUCTION

In today's modern lifestyle, an increase in dietary fat intake, particularly saturated fat increases the prevalence of obesity and diseases caused by metabolic syndrome, diabetes and steatosis (Damjanovic and Barton, 2008; Cerf, 2007). Numerous studies suggest that obesity, diabetes, and the metabolic syndrome are closely associated with the disease progression of nonalcoholic fatty liver disease (NAFLD) (Malhi et al., 2006; Utzschneider and Kahn, 2006). NAFLD is the most common liver disease and affects millions of people worldwide, and has emerged as a major public health concern. NAFLD has two stages: Nonalcoholic steatohepatitis (NASH), which is the inflammatory form of NAFLD, and end-stage liver disease (Adams and Lindor, 2007).

NASH has frequently been encountered in the general population and the biological mechanism of underlying steatosis occurrence and progression to NASH is not entirely understood. In this complex pathogenesis, a two-hit hypothesis proposed by Day and James (1998) has been advanced. It is well accepted that oxidative stress and macrovesicular steatosis play a pivotal role in the pathogenesis of NASH (Sheth et al., 1997). Recently, natural antioxidant properties in dietary are used as regulatory factor to counteract liver damage.

In this paper, attention is focused on rose hips, as the small wild fruit, belong to Rosaceae family which is the polymorphic group of scrambling rose species. Enriching the high nutrient compositions, such as vitamin C,

carotene and phenolic compounds (Olsson et al., 2005; Wenzig et al., 2008), rose hips are commonly used in fruit tea infusions, jam and laxative (Orhan et al., 2007). Some researches showed that rose hips had high antioxidant ability evaluated by DPPH radical scavenging, ferric reducing antioxidant power (FRAP), β -carotene linoleic acid system, hydroxyl radical scavenging assays (Barros et al., 2010) and cyclic voltammograms (CV) assay (Žegarac et al., 2010).

Rosa davurica Pall. (Chinese name: Ci-mei-guo) fruit (RDF), as a species of wild rose hips, is a traditional Chinese medicine (tonic) and distributed mainly in the northeast of China. RDF has also been applied in food and medicine industries as a raw material due to its valuable function for a long time, such as treatment of dyspepsia, nursing the blood, anti-aging and anti-fatigue. Recently, the research about natural products as antioxidants has become a significant field in food industry, and epidemiological studies have shown that wild fruits possess high antioxidant activity because of phenolic compounds (Cieslik et al., 2006; Yang et al., 2010). With antioxidants protection, consumptions of wild rose hips can reduce the risk of chronic diseases related to oxidative stress, such as aging, fatigue, cardiovascular and cerebrovascular diseases (Halliwell, 1997).

There were a lot of studies focused on structure, composition, phenolic antioxidants and anti-inflammatory factor in the crude extracts of rose hips in Europe (Demir and Ozcan, 2001; Barros et al., 2010). However, the detailed composition, antioxidant ability and inhibitory activity against hepatic steatosis of the wild RDF in China were scarcely reported except the exploratory research of Kuang et al. (1989), which did not research the properties of RDF but confirmed fourteen phenolic compounds in RDF extracted by chloroform and butanol. Therefore, the aims of this study were to present chemical compositions, identify the main antioxidant properties and evaluate the inhibitory hepatic steatosis effect of RDF. Nutrient and phytochemical compositions of RDF were analyzed and the RDF was successively extracted by five solvents of different polarity, respectively. The antioxidant activities of different extracts were conducted on cyclic voltammograms (CV) and oxygen radical absorbance capacity (ORAC) assays. The inhibitory hepatic steatosis effect of different extracts was evaluated with OA-induced fatty liver model *in vitro*. Furthermore, the relationship of antioxidant activity and inhibitory activity was discussed, and the fraction with the strongest antioxidant activity and inhibitory effect was analyzed by HPLC and HPLC/MS.

MATERIALS AND METHODS

Reagent

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid (Trolox), disodium fluorescein (FL), Dimethyl Sulphoxide (DMSO), 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) and 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) were

purchased from Sigma Chemicals Co. (St. Louis, MO). HepG2 cells were purchased from Peking Union Medical College Hospital (Beijing, China). HPLC grade acetonitrile was from Mallinckrodt Baker (Phillipsburg, New Jersey, U.S.A.). Other chemicals of analytical grade were all purchased from Beijing Chemical Reagent Co., Ltd. (Beijing, China).

Plant materials

Rosa davurica Pall. fruit was harvested in 2008 from Daxing'an Mountain range of Heilong Jiang province (China). After being dried naturally, RDF was vacuumized every 200 g and stored at -20°C.

Samples

Five solvents having different polarity, including chloroform, ethyl acetate (EA), *n*-butanol, ethanol, and distilled water were used to extract RDF consecutively. RDF (500 g) was ground into powder with pulverizer and the powder was sifted by 60-mesh sieve. The powder was subsequently extracted with 4 L chloroform (4 L \times 2 times) in ultrasonic bath at 30°C for 30 min. The residue was further extracted with ethyl acetate, *n*-butanol and distilled water, respectively in the same way. The water fraction extract was removed protein and sugars with a macroporous resin ADS-8 column (30 \times 3.0 cm i.d., NanKai University, China). All of five solutions were evaporated under reduced pressure and dried under vacuum to obtain the chloroform, ethyl acetate, *n*-butanol, ethanol, and water extract, respectively.

Chemical composition

Total soluble solids and protein contents

Total soluble solids content (TSS) was determined by a digital abbe-refractometer (Shanghai, China) at 25°C. The crude protein content of the samples was estimated by the method of coomassie brilliant blue.

Sugars

RDF powder (10 g) was extracted with distilled water and 2 ml extract solution was mixed with 1.0 mL of phenol (15%). The mixture was vortexed and added immediately to 5 mL of strong H₂SO₄. After 10 min, the tubes were left at room temperature for 20 min and then the absorbance was determined at 490 nm. Glucose was used to calculate the standard curve ($y=0.0115x-0.0001$; $R^2 = 0.9977$), and the results were expressed as mg of glucose equivalents per 100 g of extract. Reducing sugar was determined by the titrimetry method of dinitro salicylic acid (DNS) (Miller, 1959). Fine powder (10 g) of RDF was accurately weighed and extracted with 90 mL of distilled water at 90°C for 50 min. Then, the mixture was filtered through filter paper and made up to 100 mL with distilled water as sample solution. A certain sample solution (< 2 mL) was made up to 2 mL with distilled water and mixed with 1.5 mL of DNS reagent. The tubes were mixed adequately and put in water bath at 100°C for 5 min. After being cooled to room temperature, the mixture was added to distilled water to make the final volume of 10 mL and vortexed. Absorbances were taken at 540 nm. Glucose was used to calculate the standard curve ($y=0.9615x-0.1011$; $R^2 = 0.9981$).

Fatty acid

Fat was extracted by soxhlet extraction. RDF (10 g) was accurately

weighed and ground using mortar and pestle. The fine powder was extracted in a Soxhlet apparatus with 250 mL of a mixture of *n*-hexane for 7 h at the boiling point. The solvent was evaporated on a rotary evaporator at 40°C, and then the extract was dried under vacuum at 75°C for 120 min and cooled to room temperature. Fatty acids were determined by gas-liquid chromatography equipped with flame ionization detection (Agilent 6890-FID) and a HP-88/capillary column (100 m × 0.25 mm ID × 0.20 μm) from USA. The initial temperature of the column was held at 50°C, increased from 50 to 100°C at 5°C min⁻¹ for 5 min, from 100 to 180°C at 5°C min⁻¹ for 5 min, from 180 to 210°C at 1°C min⁻¹ for 15 min, and from 210 to 230°C at 5°C min⁻¹ for 5 min. The temperatures of both injector and detector were 260°C. The flow rate of hydrogen carrier gas was 1.0 mL min⁻¹, the injection volume was 1.0 μL, and the split ratio was 1:20.

Phytochemicals composition

Ascorbic acid

Vitamin C was determined by titrimetry method with 2,6-dichloroindophenol. RDF (100 g) was accurately weighed and ground using miller with an additional 100 mL of oxalic acid (2%). The slurry (10 to 40 g) was made up to 100 mL with oxalic acid (2%). 2,6-Dichloroindophenol (5 mL) was pipetted into a 50 mL Erlenmeyer flask and followed by 30 mL of distilled water. The dye solution was titrated separately with the RDF extract solution until a light rose pink persisted for 5 s.

Carotenoids

β-Carotene and lycopene were determined according to the method of Nagata and Yamashita (1992). Dried RDF powder was extracted by acetone-hexane mixture (4:6) for 1 min under ultrasonication and then filtered. The absorbance of the filtrate was measured at 453, 505, 645 and 663 nm, respectively. The content of β-Carotene and lycopene were calculated as the following equation: β-carotene (mg 100 mL⁻¹) = 0.216 × A₆₆₃ - 1.220 × A₆₄₅ - 0.304 × A₅₀₅ + 0.452 × A₄₅₃; lycopene (mg 100 mL⁻¹) = -0.0458 × A₆₆₃ + 0.204 × A₆₄₅ + 0.372 × A₅₀₅ - 0.0806 × A₄₅₃. The results were expressed as mg of carotene per g dry weight.

Total phenolic content (TPC)

The method of Folin-Ciocalteu (Singleton et al., 1965) was used to measure the TPC in RDF with GBC UV-visible 6 spectrometer (GBC scientific equipment Pty Ltd, Australia). The absorbances of five extracts were determined at 765 nm. Various concentrations of gallic acid were used as standards for calibration curve ($y = 0.0963x - 0.0062$; $R^2 = 0.9971$), and the results were expressed as gallic acid.

In vitro evaluation of the antioxidant properties

Cyclic voltammograms determination of antioxidant capacity

Electrochemical measurements were carried out on a Model CHI 620C electrochemical analyzer (CHENHUA, Shanghai, China). Cyclic voltammetric measurements were carried out by three electrode systems. The working electrode was a glassy carbon electrode (3.0 mm diameter) and an Ag/AgCl (KCl 3 M) electrode was used as reference. A platinum foil served as the auxiliary electrode. The glassy carbon working electrode was polished successively on 1, 0.3 and 0.05 alumina powder before each scan-

ning. The supporting electrolytes were 0.2 M Britton-Robinson buffered solutions (BRS) at pH=3.0 plus 0.2 M KCl. RDF extracts and Trolox standard solutions were prepared for the measurement by a mixing solvents of BRS and DMSO (70:30, V:V). Prior to each run, RDF solutions were removed, dissolved oxygen bubbled with N₂ for about 15 min. Voltammetric scans were carried out in the potential interval of -0.4 V to +1.2 V versus Ag/AgCl with a scan rate of 400 mV s⁻¹ at room temperature (25°C). The total charge below the anodic wave curve of the voltammogram was measured, the current response due to blank (solvent + supporting electrode) was then subtracted. The solution of Trolox within the concentration range of 50- to 400 μM was used for construction of the calibration curve.

Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was carried out on SpectraMax M2^e plate reader (Molecular Devices, USA). The procedures were based on the method of Ou et al. (2001). AAPH, Trolox and fluorescein were used as the peroxy radical generator, the standard and the fluorescent probe, respectively. Trolox and five RDF extracts were directly dissolved in DMSO and diluted to proper concentrations with 75 mM potassium phosphate buffer (pH=7.4) for analysis. Following this, 25 μL of diluted samples were mixed with 100 μL of 48 nM fluorescein and incubated for 15 min at 37°C. Then, 75 μL AAPH solution (173 mM) was injected, and the fluorescence was taken immediately every 2 min for 2 h at 37°C. The final ORAC values were calculated by the differences of area under the decay curves between a blank and a sample. The unit was expressed as millimoles of trolox equivalents per gram RDF extract fractions (μM Trolox g⁻¹). Various concentrations of Trolox (50-200 μM) were used as standards for calibration curve.

OA-induced fatty liver model in vitro

Cell culture

HepG2 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 units mL⁻¹) and streptomycin (100 μg mL⁻¹) in a humidified incubator (model MCO-20AIC, Sanyo Electric Biomedical Co., Japan) with 5% CO₂ in air at 37°C. The cells with 10 to 20 passages were used in this study.

Cytotoxicity of samples assay

Five extracts and subfractions of EA fraction from RDF were dissolved in DMSO and diluted with DMEM medium to different concentrations and incubated with HepG2 cells for 24 h. The cytotoxicity of samples was tested using MTT assay (Mossmann, 1983). The cells were washed twice with PBS carefully and incubated with 200 μL serum-free DMEM medium containing MTT (500 μg mL⁻¹). After 4 h, the culture medium was replaced with 150 μL DMSO to solubilize the formed formazan. The absorbance of each well was measured at 570 nm with microplate reader. Results were compared with the controls.

Inhibitory effect on OA-induced fatty liver model in vitro

Cells were plated in 24-well Costar plates (Corning Life Sciences, Lowell, MA), at a seeding density of 1 × 10⁶ cells per well in maintenance medium. After 24 h in culture, FBS-containing medium was changed to the experimental medium which was 1.0 mM oleic acid-bovine serum albumin (OA-BSA) complex (molar ratio of OA/BSA was 4/1) with the absence or presence of samples (50 μg

Table 1. Nutrient and phytochemical compositions of RDF.

Parameter	Total sugar ^a	Reducing sugar ^a	Ascorbic acid ^b	β -carotene ^b	lycopene ^b
content	64.17±0.63	16.92±0.58	4300.60±444.99	9.52±0.16	2.37±0.08

Results are expressed as means \pm standard deviation (n = 3); ^aThe contents are calculated as gram composition per 100 g dry weight of RDF; ^bThe contents are calculated as milligram composition per 100 g dry weight of RDF

mL⁻¹). And then the cells were incubated for another 24 h (Lin et al., 1995). The medium with only BSA and only OA-BSA were selected as the control and the standard, respectively. Finally, the supernatants of different samples were collected to determine the triglyceride (TG) levels in the cell lysates with Multiskan MK3 (Thermo Electron co., USA).

Separation of EA fraction by semi-preparative HPLC

The separation was performed on a HPLC system (Model LC-10ATvp, Shimadzu, Kyoto, Japan) equipped with two pumps and a diode array detector (DAD). The column for separation was a Kromasil C₁₈ column (10 \times 250 mm, 5 μ m, SE-445 80 Bohus, Sweden). HPLC conditions were as follows: Eluent A, acetonitrile, and eluent B, 0.5% formic acid in water. The gradient elution condition was 0 to 5 min, 20 to 60% A; 5 to 20 min, 60 to 65% A; 20 to 25 min, 65 to 20% A. The flow rate was 1.5 mL min⁻¹, temperature was at 30°C and the detection was performed at 280 nm. Sample solution was filtered through a 0.22 μ m syringe filter before HPLC analysis and each injection volume was 500 μ L.

Identification of antioxidants from EA fraction

UV-vis spectrophotometric analysis

The absorbance of sample solution (EA fraction) was scanned with the wavelength range from 200 to 800 nm with a micro plate reader (model SpectraMax M2^e, Molecular Devices, USA). The characteristic absorption peak of EA fraction was detected at 280 nm.

HPLC/DAD analysis

In the same system with the semi-preparative HPLC, analytical HPLC was performed with a ZORBAX SB-C₁₈ column (4.6 mm \times 250 mm, 5 μ m, Agilent Technologies, USA). Eluent A was acetonitrile and eluent B was 0.5% formic acid in water. The elution condition was 0 to 5 min, 20 to 50 % A; 5 to 10 min, 50 to 60% A; 10 to 20 min, 60 to 61% A; 20 to 25 min, 61 to 62% A, 25 to 30 min 62 to 20% A. The flow rate was 0.3 mL min⁻¹, temperature was at 30°C and detection was carried out at 280 nm. A loop of 20 μ L was prepared for the injection.

HPLC/ESI/MS analysis

The Agilent 1100 series LC/MS Trap used in this study was equipped with an ion trap MS detector with electrospray ionization (ESI) interface. The system contained the same column and HPLC conditions as that in the HPLC-DAD analysis. The flow splitting of the HPLC eluate introduced into the ESI interface was 3:1. The ESI voltage was 3.5 kV, and a mass range (*m/z*) of 50 to 800 was scanned in positive full ion monitoring mode. The nebulizing gas

(nitrogen) pressure was set at 35 psi and the drying gas (nitrogen) was held at 6 L min⁻¹ for ionization.

Statistical analysis

Each experiment was carried out with three replications. All data were expressed as means \pm SD. Statistical analysis was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Differences at *p* < 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Nutrient compositions

The nutrient compositions of RDF are shown in Table 1. The total soluble solids content was 22.13%, which was significantly lower than the results of Ercisli (from 29.42 to 37.33%) (2007). RDF contained higher sugar content (64.17% dry weight) than other compositions. Reducing sugar was 16.92 g/100 g of dry weight and a part of total sugar (26.37%), which was similar to *Rosa canina* sl. in Portugal (26.90%) (Barros et al., 2010). Protein of the samples was considerably low (1.35%), the Turkish of rose hips (6.71 to 8.44%, Demir and Özcan, 2001) had higher protein level. RDF presented the highest fat content (2.85%) among all the reported rose species. In the study of Ercisli (2007), the total fat contents of the rose species were found to be 1.52% (*Rosa villosa*) and 1.85% (*Rosa dumalis* subsp. *boissieri*), respectively. These different results could be ascribed to the various growth conditions, environmental factors, and fruit sizes. Fifteen fatty acid constituents were identified from RDF extract, and the fatty acids composition from RDF are shown in Table 2. The main fatty acids were mono-unsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), amounting to 36.85 and 37.57% of the total acid fraction, while the content of saturated fatty acids (SFA) was 25.58%. RDF was characterized by high content of *cis*-11-Eicosenoic acid methyl ester (28.13%), as the major compound followed by palmitic acid (20.08%), linoleic acid (19.97%), α -Linolenic acid (14.77%) and oleic acid (8.12%), respectively. In agreement with the study of Ercisli (2007) and Wenzig (2008), palmitic acid, linoleic acid and α -linolenic acid were the main free fatty acids presented in the lipophilic rose hips extract. RDF presented higher MUFA, which belonged to ω -3 and ω -6 fatty acids known as the most

Table 2. Fatty acids composition of RDF. The results are expressed in relative percentage of each fatty acid.

Fatty acids	Content	Fatty acids	Content
C12:0	1.69 ± 0.00	C20:0	0.88 ± 0.00
C14:0	1.53 ± 0.00	C20:1	28.13 ± 0.06
C15:0	0.25 ± 0.00	C20:4n6	1.76 ± 0.00
C16:0	20.08 ± 0.04	C20:5n3	1.07 ± 0.00
C16:1n7	0.60 ± 0.00	C22:0	0.41 ± 0.00
C17:0	0.35 ± 0.00		
C18:0	0.39 ± 0.00		
C18:1n9	8.12 ± 0.02	PUFA	37.57
C18:2n6	19.97 ± 0.02	MUFA	36.85
C18:3n3	14.77 ± 0.03	SFA	29.32

Results are expressed as means ± standard deviation (n = 3).

effective natural compounds for decrease LDL cholesterol, inhibition cardiovascular disease and cancer (Morise et al., 2004; Watababe et al., 1989). In addition, linoleic acid, α -Linolenic acid, arachidonic acid and eicosapentaenoic acid (EPA) are essential fatty acids, which cannot be synthesized *in vivo* as lack of precursor, elongation and desaturation enzymes. Therefore, these essential fatty acids must be ingestion from diet and play an important role in transporting substances in and out of the cell by its own metabolic path (Chow, 2000; O'Keefe, 2000).

Phytochemicals composition

Rose hips contained higher level of ascorbic acid than apple, orange, lemon and kiwi fruit (Jacoba, 1999), which can effectively prevent scurvy for many years (Kharazmi, 2008). The ascorbic acid, β -carotenoids and lycopene contents of wild RDF were determined and given in Table 1. The results reveal that the content of ascorbic acid in wild RDF (4300.60 mg per 100 g of dry weight) was much higher than the values described in study of Nojavan et al. (417.57 mg per 100 g) (2008) and Barros et al. (68.04 mg per 100 g) (2010). The reason of difference is that the fruit used in our study is dry while the fruit used in the other two reports is fresh. Moreover, high concentration ascorbic acid in rose fruits (27.12 mg Kg⁻¹ dry weight) was also reported in the study of Demir and Ozcan (2001). There are little β -carotenoids and lycopene in most fruits, and β -carotenoids content was 1.29 mg 100 g⁻¹ and lycopene content was 0.51 mg 100 g⁻¹ in rose hips reported by Barros et al. (2010). However, β -carotenoids and lycopene contents of RDF were found to be 9.52 mg per 100 g and 2.37 mg per 100 g in our study. Moreover, previous study by Hodisan et al. (1997) reported that total carotenoids of *R. canina* fruits was 7.85 mg per 100 g dry weight, the little difference between two studies might be the different species of rose

hips or fresh/dry raw material.

Total phenolic assay by Folin-Ciocalteu reagent was commonly used as total phenolic content measurement assay. Actually, the results of total phenolic assay also reflect a sample's reducing capacity because the reaction occurs through electron transfer mechanism between reductants and Mo (VI). As shown in Table 3, total phenolics of five extract fractions varied from 24.46 to 752.92 mg GAE per g extract. Among the five fractions, the EA fraction had the highest value ($p < 0.01$) of phenolic content, followed by *n*-butanol, ethanol-solute, and water fractions, while the chloroform fraction had the lowest GAE value ($p < 0.01$). The results indicated that total phenolic concentrations in different polar solvent extracts varied widely and the EA fraction may have higher value of phenolic antioxidants than in other extract fractions.

Antioxidant activities of extracts

Two methods was used to measure the antioxidant activities of RDF extracts: CV and ORAC assays.

Firstly, the CV assay has been widely used to determine reducing capacity of antioxidant compounds to reflect its antioxidant activity. As shown in Table 3, the reducing capacity of the five fractions of RDF extract was determined by the total charge under the anodic waveform. The results were expressed as millimoles of trolox equivalents per gram RDF extract fractions ($\mu\text{M Trolox g}^{-1}$), which was based on the function the area of the anodic wave (S) vs set of Trolox solutions.

The area of the anodic wave (S), related to the total charge, could be applied as a good parameter reflecting the total antioxidant capacity of a sample (Chevion et al., 2000), and the larger area of the anodic wave (S) means the higher antioxidant capacity of the sample. According to the results of this study, the antioxidant activities of all extracts were in the order: EA > water > *n*-butanol > chloroform > ethanol. The EA fraction, which contained the most TPC, presented the highest antioxidant properties. All of the extract fractions had significantly higher values compared to Trolox ($p < 0.01$) and the antioxidant values correlated well ($r = 0.9848$, $p < 0.05$) with total phenolic content. Secondly, the ORAC assay measures the ability of antioxidants to scavenge peroxy radicals (ROO[•]) by hydrogen atom transfer (HAT) reactions. In the assay, antioxidant and substrate compete for thermally generated peroxy radicals which can simulate inhibition process of lipid oxidation chain reactions by antioxidants *in vivo*. The ORAC values for RDF extract are listed in Table 3. The EA fraction behaved significantly higher chain-breaking antioxidant capacity than other four extract fractions, and chloroform and ethanol fractions showed lower antioxidant activities.

All of the extract fractions had significantly higher values compared to Trolox ($p < 0.01$), and the results are in agreement with their phenolic content ranking in total

Table 3. Total phenolic content and antioxidant activities in five extracts of RDF.

Samples	Yield ^a	TPC ^b	CV ^c	ORAC ^c
chloroform	1.70 ± 0.04	24.46 ± 2.19	139.5±25.70	338.81±29.32
EA	0.31 ± 0.002	752.92± 11.43	854.3±42.81	8392.26±229.85
<i>n</i> -butanol	0.33 ± 0.02	240.30 ± 8.12	400.5±18.09	3097.09±96.76
ethanol	1.90 ± 0.05	36.50 ± 0.39	103.11±1.63	388.92±25.62
water	17.80 ± 0.06	366.43 ± 2.50	405.1±21.38	8208.53±240.56

^aYields of extracts were calculated as percentage (w/w) of dry material of RDF; ^bTPC, Total phenolic content. Total phenolic contents are expressed as mg gallic acid equivalents per gram dry weight of RDF; ^cCV, Cyclic voltammograms assay. The results of CV assay are expressed as μM Trolox equivalents per gram extract; ^dORAC, Oxygen radical absorbance capacity assay. The results of ORAC assay are expressed as μM Trolox equivalents per gram extract.

phenolic assay ($r = 0.9074$, $p < 0.05$). Although, ORAC assay and CV assay focused on different aspects in antioxidant capacity assessment (H⁺ atom donating capacity and reducing capacity), there was still an obvious linear correlation coefficient ($r = 0.834$, $p < 0.05$) between the two assays

Inhibitory hepatic steatosis effect

The potential cytotoxicity of RDF extract was tested at different concentrations (10 to 200 $\mu\text{g}/\text{mL}$). Results demonstrate that no toxicity on HepG2 as tested up to the 50 $\mu\text{g}/\text{mL}$ concentration. Therefore, 50 $\mu\text{g}/\text{mL}$ concentration of sample can be used in our study.

The accumulation of TG in the liver was a hallmark of hepatic steatosis, as it is correlated with disease severity in NAFLD and inhibition of FFA-associated hepatic toxicity represents a potential therapeutic strategy (Nehra, 2001). As shown in Figure 1, the intracellular TG levels increased significantly ($p < 0.01$) by the addition of 1.0 mM OA-BSA complex and reached similar levels of maximal intracellular lipid accumulation as found in human liver with steatosis.

Compared to the model group, the addition of EA fraction (50 $\mu\text{g mL}^{-1}$) showed the strongest inhibitory effect ($p < 0.01$) on the increase of TG levels, followed by *n*-butanol fraction ($p < 0.01$), water fraction, ethanol fraction and chloroform fraction. The inhibition percentages were 23.34, 19.59, 11.86, 4.15 and 2.34%, respectively, while the ethanol fraction and the chloroform fraction showed no inhibitory effect on the increase of TG levels at such concentration.

The statistical analysis revealed that there was a significant correlation between the TG levels and the TPC ($r = 0.8569$, $p < 0.05$). And TG levels correlated well with CV assay ($r = 0.9012$) and ORAC assay ($r = 0.8229$). The result exhibited that there were an obvious correlation among TPC, antioxidant activity and inhibitory effect on the increase of TG levels.

Identification of main components from EA-FII

According to antioxidant assays and inhibitory hepatic steatosis effect, EA fraction possessed the greatest antioxidant activities and inhibitory effect among the five fractions. It also suggested that EA fraction contained a lot of active components responsible for the antioxidant activity. The fractions of EA were mainly divided into three fractions (EA-FI, EA-FII and EA-FIII) with a semi-preparative HPLC, and the amount of EA-FI, EA-FII and EA-FIII was quantified to be 8.10, 57.10 and 15.95% (Figure 2). Furthermore, the antioxidant activities of EA-FI, EA-FII and EA-FIII were evaluated by ORAC assay, the results showed that the antioxidant activity of EA-FII (18256.34 μM Trolox/g extract) was significantly better than EA-FIII (15130.91 μM Trolox per g extract) and EA-FI (5014.37 μM Trolox per g extract). As well as the results of antioxidant activities, EA-FII (28.91%) presented the most obvious inhibitory effect on the increase of TG level, followed by EA-FIII (20.75%) and EA-FI (10.70%).

In order to get information about the molecular masses of phenolic compound, HPLC-DAD and HPLC-ESI-MS analysis of EAFII was carried out. The characteristic absorption peak of EA-FII fraction was detected at 280 nm. The main m/z values observed from negative ion experiments with HPLC-ESI-MS for the EAFII of EA are shown in Table 4. Peak 1 gave the main ions at m/z 289, 271 and 245. Data inspection enabled identification of m/z 271 and 245 as $[\text{M}-\text{H}-\text{H}_2\text{O}]^-$ and $[\text{M}-\text{H}-\text{CO}_2]^-$. Product ion spectrum and retention time of standard identified that the peak 1 was catechin. Peak 2 showed five main ions at m/z 301, 283, 273, 255 and, and the peak 3 gave similar mass spectrum (m/z 301, 283, 273, 255 and 163) to peak 2. Peak 2 and peak 3 gave one dominant fragment ions at m/z 301, and the fragment ions at 283 and 255 were identified as $[\text{M}-\text{H}-\text{H}_2\text{O}]^-$ and $[\text{M}-\text{H}-\text{H}_2\text{O}-\text{CO}]^-$. The fragment ions at m/z 118 and 163 were identified as a product ion at m/z 301. The fragment ion at m/z 301 was identified as quercetin by comparison of

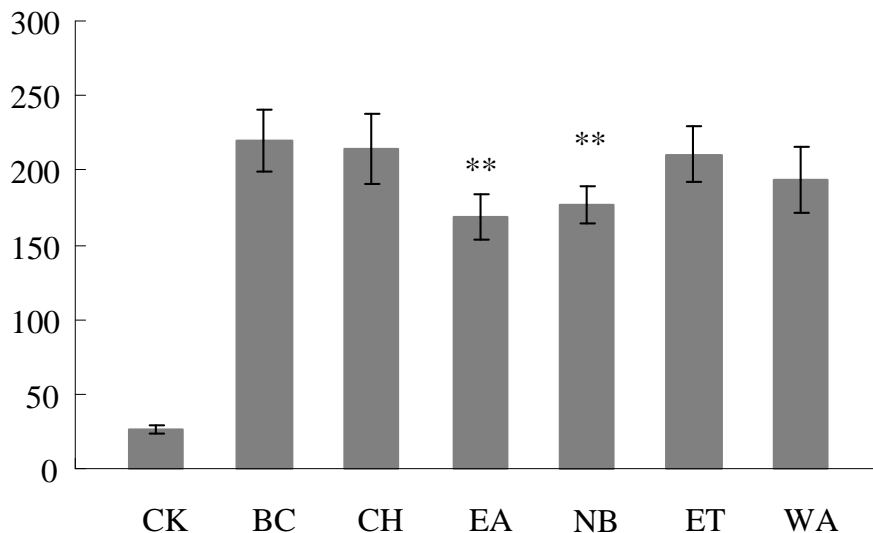


Figure 1. Effect of 1.0 mM OA-BSA complex with five extracts from RDF on the TG level of HepG2 cells. CK, control group. BC, model group. CH, chloroform group. EA, ethyl acetate group. *n*-Hexane group. ET, ethanol group. WA, water group. ** $p < 0.01$ means significantly different from the model.

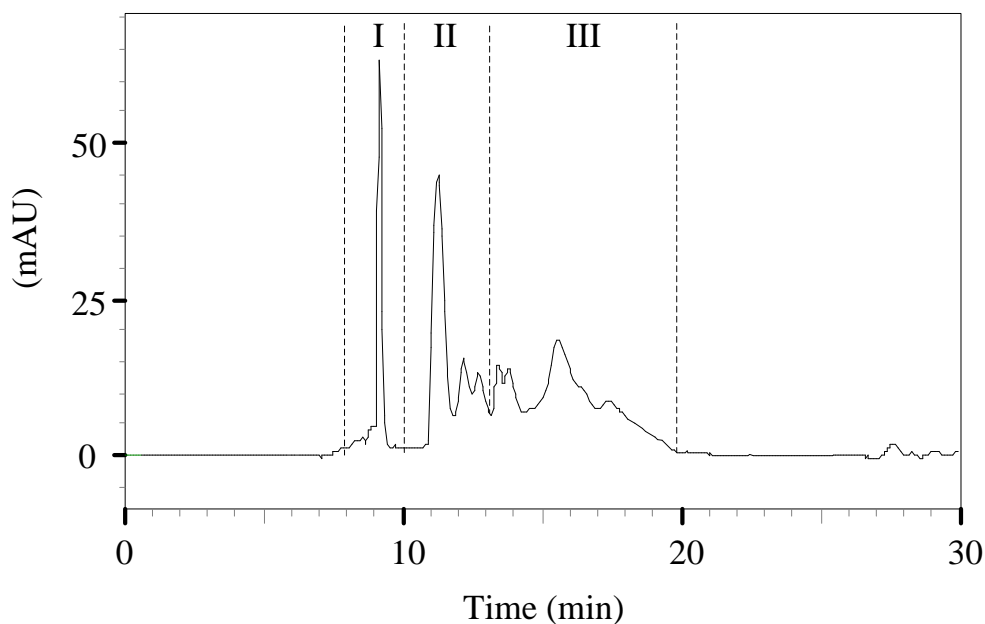


Figure 2. Semi-preparative HPLC chromatogram at 280 nm of EA. Chromatographic conditions are described in the materials and methods section.

Table 4. Identification of phenolic compounds in RDF by HPLC–DAD–ESI–MS.

Peak no.	t_r (min) ^a	[M-H] ⁻	Products of [M-H] ⁻	Tentative name
1	11.8	289	271, 245	Catechin
2	12.5	475	301, 283, 255, 191, 118	Quercetin-sedoheptulose
3	12.8	395	301, 283, 255, 163, 79	Quercetin-94

^a t_r , Retention time.

the standard. Therefore, peak 2 exhibited the molecular ion $[M-H]^-$ at m/z 475 and fragment ions at m/z 301 $[M-H-174]^-$, which could correspond to a possible sedoheptulosan conjugates quercetin. For peak 3, the loss of 94 Da (m/z 395 to 301) gives, no indication of the nature of the compound associated with the flavanol. Further confirmation of the component structure and additional assay are required.

Based on the results of LC/MS and UV/Vis spectrum analysis, three compounds of EAFII were identified as catechin, quercetin-sedoheptulose and Quercetin-94, similar to the results of Hvattum (2002). Although RDF after extraction was simplified, there were still unidentified peaks which made the analysis complex. The main reasons were the complex character of plants and the inherent structural similarity of most flavonoids, such as quercetin with a large variety of sugars, position isomer and structural isomer. Owing to their similar structures, it was not easy to separate these compounds. In addition, some reports presented that catechin and quercetin were great antioxidant in plants, so catechin, quercetin-sedoheptulose and quercetin-94 would be main compounds in EA-FII.

Conclusion

In conclusion, the results indicated that wild RDF was rich in vitamin C, β -Carotene, lycopene and phenolic compounds, which are known as the effective antioxidant components. Moreover, there was compact correlation among the phenolic contents, the antioxidant activities and the inhibitory hepatic steatosis effect of five fractions from wild RDF. The EA fraction exhibited the strongest activities evaluated by CV assay, ORAC assay and OA-induced fatty liver model, and EA-FII from EA was proved to be the most active in the ORAC assay and OA-induced fatty liver model *in vitro*. According to HPLC and HPLC-ESI-MS analysis, catechin, quercetin-sedoheptulose and quercetin-94 were identified from EA-FII as the main components. In future, other unidentified components will be separated and purified and their specific relationship of structure-bioactivity will be studied.

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