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Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract

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Abstract

Pomegranate is an important source of bioactive compounds and has been used for folk medicine for many centuries. Pomegranate juice has been demonstrated to be high in antioxidant activity and is effective in the prevention of atherosclerosis. In a previous study, we found that pomegranate peel had the highest antioxidant activity among the peel, pulp and seed fractions of 28 kinds of fruits commonly consumed in China as determined by FRAP (ferric reducing antioxidant power) assay. In this study, we extracted antioxidants from pomegranate peel, using a mixture of ethanol, methanol and acetone, and the antioxidant properties of the extract were further investigated as compared with the pulp extract. The contents of total phenolics, flavonoids, proathocyanidins and ascorbic acid were also measured. The results showed that pomegranate peel extract had markedly higher antioxidant capacity than the pulp extract in scavenging or preventive capacity against superoxide anion, hydroxyl and peroxyl radicals as well as inhibiting $CuSO_4$-induced LDL oxidation. The contents of total phenolics, flavonoids and proathocyanidins were also higher in peel extract than in pulp extract. The large amount of phenolics contained in peel extract may cause its strong antioxidant ability. We concluded that pomegranate peel extract appeared to have more potential as a health supplement rich in natural antioxidants than the pulp extract and merits further intensive study.

Keywords: Pomegranate peel; antioxidant activity; LDL oxidation

1. Introduction

Antioxidant is defined as any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell, 1995). Epidemiological studies have shown that consumption of fruits and vegetables is negatively associated with the morbidity and mortality of cardio- and cerebro-vascular diseases and certain types of cancers (Johnsen et al., 2003; Rissanen et al., 2003; Temple & Gladwin, 2003), and the antioxidants contained in fruits and vegetables, including ascorbic acid, carotenoids, flavonoids, hydrolysable tannins, are supposed to play an important role in the prevention of these diseases (Huxley & Neil, 2003; Knekt et al., 2002; Lampe, 1999). Evidence from animal and human experiments also reveals that some natural antioxidants other than ascorbic acid, carotenoids and vitamin E could be absorbed significantly and act as potent antioxidants in vivo (Cao, Muccitelli, Sanchez-Moreno, & Prior, 2001; Pataki et al., 2002; Scalbert & Williamson, 2000; Su et al., 2003). These results have been the driving force for many recent studies on the antioxidant activity of different foods of plant origin and the derived products, as well as the effective constituents, responsible for this activity.

Pomegranates (*Punica granatum*) have been used extensively in the folk medicine of many cultures

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(Longtin, 2003). Recently, pomegranate juice, and even fermented pomegranate juice, were demonstrated to be high in antioxidant activity (Gil, Tomas-Barberan, Hess-Pierce, Holcroft, & Kader, 2000; Schubert, Lansky, & Neeman, 1999). Pomegranate juice also displays potent antiatherogenic action in atherosclerotic mice and humans (Aviram et al., 2000; Kaplan et al., 2001). All these activities may be related to diverse phenolic compounds present in pomegranate juice, including punicalagin isomers, ellagic acid derivatives and anthocyanins (delphinidin, cyanidin and pelargonidin 3-glucosides and 3,5-diglucosides). These compounds are known for their properties in scavenging free radicals and inhibiting lipid oxidation in vitro (Gil et al., 2000; Noda, Kaneyuki, Mori, & Packer, 2002).

Previously, we found that pomegranate peel had the highest antioxidant activity among the peel, pulp and seed fractions of 28 kinds of fruits commonly consumed in China, as determined by FRAP (ferric reducing antioxidant power) assay (Guo et al., 2003). It seems, therefore, that pomegranate peel may be a rich source of natural antioxidants and worthy of further study. It is interesting that pomegranate peels have been used since antiquity in the Middle East as colorant for textiles because of their high tannin and phenolic contents. Singh, Murthy, and Jayaprakasha (2002) recently reported that methanol extract of pomegranate peel had much higher antioxidant capacity than that of seeds, as demonstrated by using the β-carotene–linoleate and DPPH model systems. This pomegranate peel extract could effectively protect (after oral administration) against CCl_4^- induced hepatotoxicity, in which ROS damage was intensively involved (Murthy, Jayaprakasha, & Singh, 2002). In the current study, we optimized the antioxidant extracting procedure, based on FRAP value of the extracts. The peel extract, with high FRAP value, that we obtained was further measured for the contents of total phenolic, flavonoids, proanthocyanidins and ascorbic acid and evaluated by using different in vitro models for its antioxidant capacity, including scavenging or preventive capacity against superoxide anion, hydroxyl and peroxyl radicals, as well as inhibiting $CuSO_4$-induced LDL oxidation in comparison with the pulp extract. The objectives of this study were to establish an efficient antioxidant extracting procedure and to explore the possibility of developing a nutraceutical agent rich in natural antioxidants from the pomegranate peel.

2. Materials and methods

2.1. Chemicals

Catechin, rutin, quercetin, 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trolox, ascorbic acid, and disodium fluorescein (FL) were obtained from Aldrich (Milwaukee, WI, USA). The 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) was from Wako Chemicals USA (Richmond, VA). All other chemicals used were of analytical grade.

2.2. Antioxidants extraction

Ripened pomegranates were obtained from Lintong, Shanxi Province of China. The peel and pulp were separated manually. The fresh peels collected were cut into pieces and extracted with different solvents, including methanol, ethanol, acetone and their combinations. The extracts were filtered through Whatman No. 41 filter paper. The residues were re-extracted by the same solvent. All extracts were pooled together and concentrated under vacuum at 60 °C, and the concentrates were powdered and stored in a desiccator. The pulps were similarly extracted.

2.3. Determination of total phenolics, flavonoids, proanthocyanidins and ascorbic acid

The content of phenolic compounds in the extracts was determined according to the method of Jayaprakasha, Singh, and Sakariah (2001). The extracts were dissolved in water. Aliquots of 0.5 ml samples were mixed with 2.5 ml of 10-fold-diluted Folin–Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The mixture was allowed to stand for 30 min at room temperature before the absorbance was measured at 760 nm spectrophotometrically. The final results were expressed as tannic acid equivalents.

The flavonoid content of the extracts was measured using a modified colorimetric method (Jia, Tang, & Wu, 1999). A quantity of 0.5 g of extracts was dissolved in 10 ml water and extracted by 10 ml *n*-butanol, three times. The extracts were pooled and concentrated under vacuum at 60 °C. The residue was re-dissolved in 5 ml of 60% ethanol and washed twice with 5 ml of 30% ethanol. All three parts were pooled together and filtered. The filtrate was diluted, up to 25 ml, with 30% ethanol. A volume of 0.5 ml of the solution was transferred to a test tube containing 4.5 ml of 30% ethanol and mixed with 0.3 ml of 5% sodium nitrite for 5 min. Then, 0.3 ml of 10% aluminium nitrate were added. After 6 min, the reaction was stopped by adding 2 ml of 1 M sodium hydroxide. The mixture was further diluted with 30% ethanol up to 10 ml. The absorbance of the mixture was immediately measured at 510 nm. The flavonoid content was calculated and expressed as rutin equivalents.

Determination of proanthocyanidins was based on the procedure reported by Sun, Ricardo-Da-Silva, and Spranger (1998). A volume of 0.5 ml of 50 mg/l of extract solution was mixed with 3 ml of 4% vanillin–

methanol solution and 1.5 ml hydrochloric acid and the mixture was allowed to stand for 15 min. The absorbance at 500 nm was measured and the final result was expressed as catechin equivalents.

A colorimetric procedure for the determination of total ascorbic acid, including dehydroascorbic acid, in fruits, vegetables and derived products was followed (GB12392-90, 1990). This procedure had been standardized and was approved by the Ministry of Public Health, PR China for national implementation in 1990.

2.4. FRAP assay

The procedure described by Benzie and Strain (1996) was followed. Briefly, the FRAP reagent contained 2.5 ml of 10 mM TPTZ solution in 40 mM HCl plus 2.5 ml of 20 mM $FeCl_3$ and 25 ml of 0.3 M acetate buffer, pH 3.6, and was freshly prepared and warmed at 37 °C prior to use. Aliquots of 40 μl diluted sample solution were mixed with 0.2 ml distilled water and 1.8 ml FRAP reagent. The absorbance of reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37 °C for 10 min. The 1 mM $FeSO_4$ was used as the standard solution. The final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mM $FeSO_4$.

2.5. Superoxide radical ($O_2^{-\bullet}$)-scavenging activity

Superoxide radical-scavenging activity was determined, using a commercial kit (Jiancheng Bioengineering Institute, Nanjing, China). The superoxide radicals were generated by the xanthine/xanthine oxidase system and reacted with 2,4-iodiphenyl-3,4-nitrophenyl-5-phenyltetrazolium chloride to form formazan, a coloured compound which can be spectrophotometrically quantified at 550 nm . The production of formazan is inversely related to the superoxide radical-scavenging activity of the samples tested. The final results were expressed as the inhibition degree of formazan production.

2.6. Hydroxyl radical ($^{\bullet}OH$) prevention activity

Hydroxyl radical prevention activity was measured based on the method reported by Ou et al. (2002). The sample (200 μl), and FL (3.6 ml, 86.1 nM) were pipetted into a test tube and incubated at 37 °C for 15 min. Thereafter, 100 μl of 4% H_2O_2 were added and vortexed, and the initial fluorescence intensity was recorded on a Hitachi spectrofluorophotometer. The excitation and emission wavelengths were 493 and 515 nm, respectively. 100 μl of 9.2 mM $CoSO_4$ were added to initiate the reaction. Fluorescence intensity readings were taken every three minutes until zero fluorescence intensity was reached. The final results were calculated by using a regression equation between the catechin concentration and the net area under the FL decay curve and expressed as catechin equivalents.

2.7. Peroxyl radical ($ROO^{\bullet}$)-scavenging activity

Peroxyl radical-scavenging activity was determined by an improved oxygen radical absorbance capacity (ORAC) assay (Ou, Hampsch-Woodill, & Prior, 2001). The sample (200 μl), phosphate buffer (3.5 ml, 75 mM, pH 7.4), and FL (100 μl, 35 nM) were mixed in a test tube and incubated at 37 °C for 5 min before the initial fluorescence intensity was recorded. AAPH (200 μl, 75 g/l) was added to initiate the reaction. Fluorescence readings were taken every three minutes until zero fluorescence intensity was reached. The excitation and emission wavelengths were 493 and 515 nm, respectively. Trolox was also used as a standard to calibrate the final results.

2.8. Inhibition of low density lipoprotein (LDL) oxidation

Inhibition of LDL oxidation was determined according to the method of Princen, Van Poppel, Vogelezang, Buytenhek, and Kok (1992). Rat serum was collected and diluted by phosphate buffer (50 mM, pH 7.4) to the concentration of 0.6%. Aliquots of 5.0 ml diluted serum were mixed with 10 μl DMSO or 10 μl DMSO containing various concentrations of extracts. The $CuSO_4$ solution (20 μl of 2.5 mM) was added to initiate the reaction. The absorbance at 234 nm was recorded immediately and was taken every 20 min thereafter for 200 min at room temperature. The net area under the curve was calculated and treated as the final result.

3. Results and discussion

Both pomegranate pulp and peel contain many different kinds of antioxidants, including those possibly not so far well characterized . Gil et al. (2000) identified several phenolic compounds from pomegranate juice, such as anthocyanins, punicalagins, ellagic acids, and hydrolysable tannins, Noda et al. (2002) reported that three major anthocyanidins found in pomegranate juice were delphinidin, cyanidin and pelargonidin. Pomegranate peel, also, had been shown to be rich in polyphenols (Ben, Ayed, & Metche, 1996). It is time-consuming to purify all antioxidants, one by one, from pomegranate peel. From the practical point of view, a suitable extracting procedure should be developed to recover as many antioxidants as possible before an extract rich in natural antioxidants could be further explored for possible application in health-promoting supplements for the food industry. Singh et al. (2002) extracted antioxidants from pomegranate peel and seed with the use of

methanol, acetone or water and found that methanol gave maximum antioxidant yield. We consider that a combination of different solvents may be more efficient for extracting antioxidants because antioxidants may differ in their solubility in different solvents. In the present study, the peel extract obtained by use of a mixture, composed of methanol, ethanol, acetone and water, was significantly higher in FRAP value than those obtained using individual solvents, namely using methanol, ethanol or acetone (Fig. 1). This result indicates that the mixture of different solvents is more powerful in recovering antioxidants than are individual solvents. Based on fresh weight, the yields of dried extracts from peel and pulp were 31.5 ± 3.1% and 14.5 ± 1.7%, respectively, by the extracting procedure developed in this study. A document based on this extracting procedure is being prepared to be submitted for Chinese Patent application.

We measured some antioxidant fractions present in the peel and pulp extracts we obtained. As shown in Table 1, the total phenolics content of peel extract was nearly 10-fold as high as that of pulp extract. The contents of flavonoids and proanthocyanidins were also higher in peel extract than in pulp extract. This result clearly indicates that peel extract contains more antioxidants than does the pulp extract. This is consistent with the data reported by Tomas-Barberan et al. (2001), who found that peel tissues usually contained larger amount of phenolics, anthocyanins and flavonols than did flesh tissues in nectarines, peaches and plums. However, flavonoids or proanthocyanidins account for only a small part of total phenolics present in the peel extract. In addition, both peel and pulp extracts contained a small amount of ascorbic acid. Therefore, ascorbic acid could not be an important antioxidant, either in the peel or pulp extract that we obtained.

The FRAP assay treats the antioxidants contained in the samples as reductants in a redox-linked colorimetric reaction and the value reflects the reducing power of the antioxidants. The procedure is relatively simple and easy to standardize. Thus, it has been used frequently in the assessment of antioxidant activity of various fruits and vegetables and some biological samples, though we understand that it has some limitations (Guo et al., 2003; Halvorsen et al., 2002; Pulido, Bravo, & Saura-Calixo, 2000). Based on FRAP value, the peel extract was much stronger than the pulp extract in reducing power in a dose-dependent manner (Fig. 2), indicating that peel extract has more potential antioxidant activity.

We further compared the scavenging or preventive capacity of peel and pulp extracts against several common free radicals in vitro. The superoxide anion is a well-recognized free radical species and is generated continuously by several cellular processes, including the

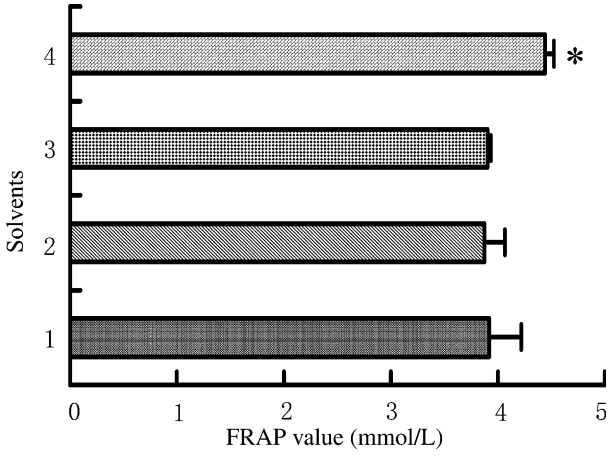

Fig. 1. Comparison of antioxidant extracting efficiency from pomegranate peel by different solvents based on FRAP value, $n = 4$. Solvent 1, methanol; solvent 2, ethanol; solvent 3, acetone; solvent 4, mixture of methanol, ethanol, acetone and water. Data were analyzed by one way analysis of variance. $^*P < 0.05$, solvent 4 vs. solvents 1, 2 or 3.

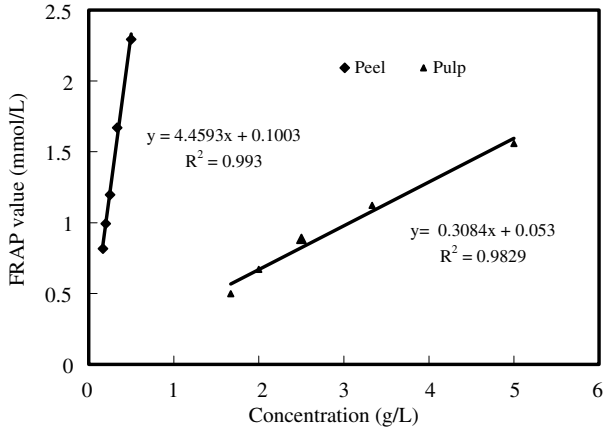

Fig. 2. Antioxidant activity of pomegranate peel and pulp extracts as measured by FRAP assay. The result is expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1.0 mM $FeSO_4$.

Table 1
Analysis of main antioxidant fractions contained in pomegranate peel and pulp extracts ($\bar{x} \pm s$, $n = 4$)

Extract	Yield (%)	Phenolics (mg/g)	Flavonoids (mg/g)	Proanthocyanidins(mg/g)	Ascorbic acid (mg/g)	Water (%)
Pulp	14.5 ± 1.7	24.4 ± 2.7	17.2 ± 3.3	5.3 ± 0.7	0.85 ± 0.02	10.9 ± 1.1
Peel	31.5 ± 3.0	249.4 ± 17.2	59.1 ± 4.8	10.9 ± 0.5	0.99 ± 0.02	8.0 ± 0.8

Phenolics, tannic acid equivalents; flavonoids, rutin equivalents; proanthocyanin, catechin equivalents.

microsomal and mitochondrial electron transport systems. Although the superoxide anion is limited in activity, it may combine with other reactive species, such as nitric oxide, produced by macrophages, to yield a more reactive species (Fridovich, 1995). The results of our study showed that the peel extract presented rather more superoxide radical-scavenging ability than the pulp extract (Fig. 3), based on the inhibition of superoxide radical-related formazan production. At a concentration of 50 g/l, the inhibition activities were 43.0% and 37.7% for the peel and pulp extracts, respectively.

The hydroxyl radical is a highly reactive free radical species and capable of damaging almost every molecule found in living cells. It can be generated in vivo in the presence of both superoxide radicals and transition cations, such as iron or copper via the Haber–Weiss reaction (Castro & Freeman, 2001). We used the hydroxyl radical prevention capacity assay, as developed by Ou et al. (2002), to compare the preventive capacity of peel and pulp extracts against hydroxyl radicals. The results revealed that the peel extract possessed about 25 times higher activity than the pulp extract (Fig. 4). Since the procedure is based on the metal-chelating property of the antioxidants, the so-called preventive capacity against hydroxyl radicals is actually related to the metal-chelating capability of the samples tested (Ou et al., 2002).

The peroxyl radicals occur during oxidation of lipids in oxidative stress. They may diffuse a considerable distance and can react avidly with sulfhydryl groups (Thomas, 1999). The improved ORAC assay used in this study is basically similar to the hydroxyl radical prevention capacity assay in principle, in which the fluorescein is employed as the sensitive probe for free radical attack. However, AAPH is used, instead of the H_2O_2–$CoSO_4$ system, to generate peroxyl radicals in this procedure. Again, the peel extract appeared to be more effective than the pulp extract in scavenging peroxyl radicals (Fig. 5).

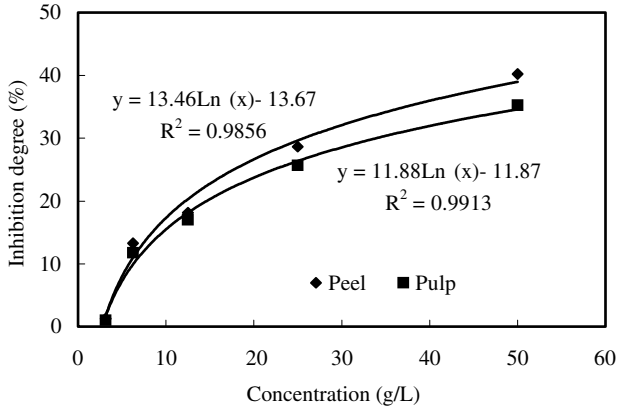

Fig. 3. Dose-dependent superoxide radical scavenging ability of pomegranate peel or pulp extract as determined by xanthine/xanthine oxidase method. The result is expressed as the inhibition degree of formazan production. The curves were simulated logarithmically.

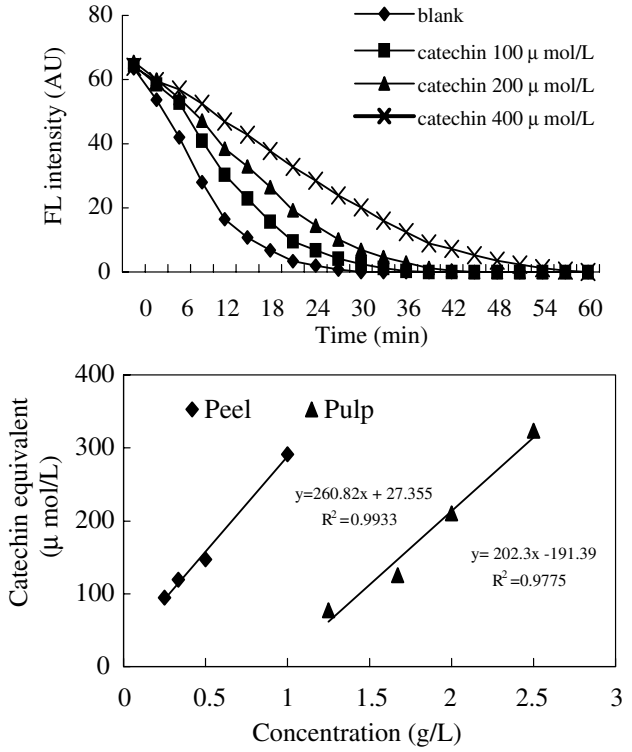

Fig. 4. Hydroxyl radical prevention activity of pomegranate peel and pulp extracts as determined by hydroxyl radical prevention capacity assay. The upper is the decay curve of fluorescein (FL) in the presence or absence of catechin. The below is the dose-dependent hydroxyl radical prevention activity of peel or pulp extract calculated as catechin equivalents.

The "oxidative modification of lipoproteins" hypothesis proposes that LDL oxidation plays a key role in early atherosclerosis. The oxidized LDL (Ox-LDL) is atherogenic because it is cytotoxic toward arterial cells and stimulates the monocytes to be adhesive to the endothelium. The uptake of Ox-LDL, via scavenger receptors, by the monocytes promotes cholesterol accumulation and foam cell formation, which leads to the development of atheromatous plaques. Thereby, inhibition of LDL oxidation is supposed to be one of the crucial steps in retarding the foam cell formation and development of aortic lesions (Chisolm & Steinberg, 2000). Aviram et al. (2000) reported that pomegranate juice could effectively protect LDL against oxidation in vitro, which was attributed to the polyphenols and ascorbic acid contained in the juice. Further studies from the same laboratory demonstrated that pomegranate juice consumption reduced the LDL susceptibility to macrophage-mediated oxidation in atherosclerotic E^0 mice and healthy human subjects (Aviram et al., 2000; Kaplan et al., 2001). In the current study, we also confirmed the inhibitive action of pomegranate pulp extract against $CuSO_4$-induced LDL oxidation, as evidenced by decreased conjugated dienes production in a dose-dependent fashion. As compared to the pulp extract,

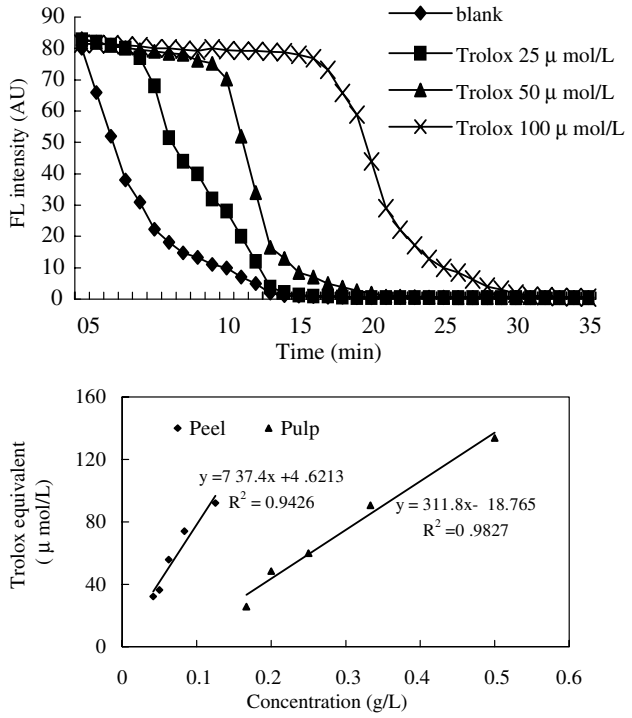

Fig. 5. ROO· scavenging capacity of pomegranate peel and pulp extracts as determined by improved ORAC assay. The upper is the decay curve of fluorescein (FL) in the presence or absence of Trolox. The below is the dose-dependent ROO· scavenging capacity of peel or pulp extract expressed as Trolox equivalents.

the peel extract acted more dramatically in protecting LDL against oxidation (Fig. 6), indicating a possibility that pomegranate peel extract may be more promising in the prevention of atherosclerosis by inhibiting LDL oxidation.

Phenolic compounds, or polyphenols, constitute one of the most numerous and widely distributed groups of substances in the plant kingdom. They can range from simple molecules, such as phenolic acids, to highly polymerized compounds, such as tannins. Flavonoids

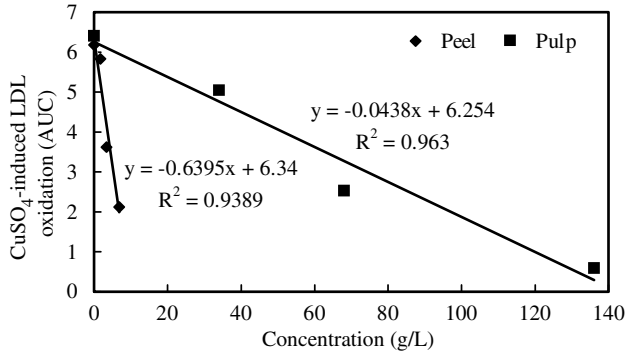

Fig. 6. Dose-dependent inhibition of $CuSO_4$-induced LDL oxidation in vitro by pomegranate peel or pulp extracts. The conjugated dienes formation was monitored kinetically as the absorbance at 234 nm and the result is expressed as the area under the curve (AUC).

are reported to be the most abundant polyphenols in human diets. The reasons for recent renewed interest in phenolics are that most phenolics possess strong antioxidant capacity in vitro and some of them have been demonstrated to be significantly bioavailable in vivo (Bravo, 1998; Jialal & Devaraj, 1996; Rice-Evans, Miller, & Paganga, 1996; Scalbert & Williamson, 2000). Prior et al. (1998) reported that the content of total phenolics was well correlated with the antioxidant capacity of fruits. It has also been shown that phenolics from red wine, green tea and chocolate could inhibit LDL oxidation significantly in vitro (Teissedre, Frankel, Waterhouse, Peleg, & German, 1996; Waterhouse, Shirley, & Donovan, 1996; Yoshida et al., 1999). Given the higher amount of phenolics contained in peel extract, it is not surprising that peel extract displays higher activity, in scavenging or preventive capacity against free radicals and inhibiting LDL oxidation, than the pulp extract. Although both peel and pulp extracts contain certain amount of flavonoids and proanthocyanidins, we are not sure how much they may contribute to the antioxidant activity presented by the peel or pulp extract. Moreover, the possible synergistic action among different antioxidants contained in the extracts cannot currently be ruled out . Further study should be carried out to identify the predominant phenolics responsible for the antioxidant activity of peel extract.

In conclusion, we used a mixture of methanol, ethanol, acetone and water to extract antioxidants from the pomegranate peel and the extract we obtained possessed stronger antioxidant properties than the pulp extract, including scavenging or preventive capability against several reactive oxygen species and inhibiting LDL oxidation. The high antioxidant activity of the peel extract appeared to be attributed to its high phenolics content. We consider that this peel extract deserves more intensive study, including its antioxidant composition, bioavailability and possible protection against cardiovascular diseases.

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