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Studies on the Antioxidant Activity of Pomegranate (*Punica granatum*) Peel and Seed Extracts Using in Vitro Models

R. P. SINGH,*,† K. N. CHIDAMBARA MURTHY,‡ AND G. K. JAYAPRAKASHA†

Human Resource Development, Central Food Technological Research Institute, Mysore 570 013, India, and Government College of Pharmacy, Bangalore 560 027, India

Antioxidant-rich fractions were extracted from pomegranate (*Punica granatum*) peels and seeds using ethyl acetate, methanol, and water. The extracts were screened for their potential as antioxidants using various in vitro models, such as β-carotene−linoleate and 1,1-diphenyl-2-picryl hydrazyl (DPPH) model systems. The methanol extract of peels showed 83 and 81% antioxidant activity at 50 ppm using the β-carotene-linoleate and DPPH model systems, respectively. Similarly, the methanol extract of seeds showed 22.6 and 23.2% antioxidant activity at 100 ppm using the β-carotene−linoleate and DPPH model systems, respectively. As the methanol extract of pomegranate peel showed the highest antioxidant activity among all of the extracts, it was selected for testing of its effect on lipid peroxidation, hydroxyl radical scavenging activity, and human low-density lipoprotein (LDL) oxidation. The methanol extract showed 56, 58, and 93.7% inhibition using the thiobarbituric acid method, hydroxyl radical scavenging activity, and LDL oxidation, respectively, at 100 ppm. This is the first report on the antioxidant properties of the extracts from pomegranate peel and seeds. Owing to this property, the studies can be further extended to exploit them for their possible application for the preservation of food products as well as their use as health supplements and neutraceuticals.

KEYWORDS: ***Punica granatum*; antioxidant activity; β-carotene−linoleate model system; DPPH; LDL; free radicals**

INTRODUCTION

Antioxidants are the compounds that when added to food products, especially to lipids and lipid-containing foods, can increase the shelf life by retarding the process of lipid peroxidation, which is one of the major reasons for deterioration of food products during processing and storage. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytolune (BHT), have restricted use in foods as these synthetic antioxidants are suspected to be carcinogenic (*1*). Therefore, the importance of the search for and exploitation of natural antioxidants, especially of plant origin, has greatly increased in recent years (*2*).

A cursory survey of the literature reveals that the tannins from the pericarp of pomegranate exhibit antiviral activity against the genital herpes virus (*3*). The pomegranate rind extract is also shown to be a potent virucidal agent (*4*) and has been used as a constituent of antifungal and antiviral preparations (*5*). Pomegranate is also used as a part of a fungicidal preparations (*6*). There are reports of the use of a water decoction of pomegranate peel powder as a multifunctional vaginal suppository (*7*) for contraception and for the prevention and cure of venereal disease. Pomegranate peel is reported as a part of a preparation used for treating the infection of male or female sexual organs, mastitis, acne, folliculitis, pile, allergic dermatitis, tympanitis, and scald for curing diarrhea and dysentery (*8*) and as part of the medicine for the treatment of oral diseases (*9*). The pomegranate peel extract, when introduced into juice, improves the process intensity due to acceleration of deposit precipitation of the haze-forming substance (*10*). The presence of antioxidants has been reported from pomegranate juice (*11*); however, no literature was found reporting the antioxidant activity in the pomegranate peels and seeds. The objectives of this study were to prepare antioxidant-rich fractions from pomegranate peels and seed extracts and to evaluate their antioxidant activity using various in vitro models.

MATERIALS AND METHODS

Materials. β-Carotene, catechin, epicatechin, linoleic acid, 1,1-diphenyl-2-picryl hydrazyl (DPPH), and BHA were obtained from Sigma Chemical Co. (St. Louis, MO). All solvents/chemicals used were of analytical grade and obtained from Merck, Mumbai, India. A Millipore Swinnex type filter (pore size = 0.45 μm) was obtained from Millipore (Bangalore, India). UV−visible spectra measurements was done using a Genesys-5 UV−visible spectrophotometer (Milton Roy, New York).

Extraction. Ripened pomegranates *(Punica granatum* cv. Ganesha) were obtained from local markets. The peels and seeds were manually separated. The seeds were washed with excess water for the removal of sugars and adhering materials and sun-dried. Peels were directly sun-dried. Both parts were powdered in a grinder to get 40-mesh size

† Central Food Technological Research Institute.
‡ Government College of Pharmacy.

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powder. The moisture contents of peel and seed powder were found to be 15.5 and 21.1%, respectively.

The peel powder (25 g) was extracted by mixing using a magnetic stirrer with 100 mL of EtOAc at 30 °C for 1 h. The extract was filtered through Whatman No. 41 filter paper for removal of peel particles. The residue was re-extracted with the same solvent. The extracts were pooled and concentrated under vacuum at 40 °C. The same procedure was followed for other solvents such as methanol and water for the extraction of antioxidant fractions (*12*).

The seed powder (25 g) was extracted in a Soxhlet with hexane (100 mL) for 6 h for the removal of fatty matter. The hexane extract was discarded, and residues were successively extracted with EtOAc, MeOH, and water (80 mL each) for 8 h each. The extracts were filtered and concentrated under vacuum (Buchi, Switzerland) to get concentrate, which was dried in a vacuum oven and stored in a desiccator.

HPLC Analysis. The liquid chromatographic system consisted of a Shimadzu LC-6A model (Shimadzu, Tokyo, Japan), fitted with a Waters μ-Bondapack (Waters Corp., Milford, MA) C_{18} column (250 × 4.6 mm i.d.) and an SCL-6A system controller. The injection system used was a 20 μL sample loop. Detection was done by a UV−visible spectrophotometer SPD- 6AV set at a sensitivity of 0.04 AUFS and a wavelength of 280 nm. Elution was carried out at a flow rate of 0.7 mL/min under a linear gradient of acetonitrile (solvent A) and 0.3% phosphoric acid (solvent B) from 10% A to 20% A in 45 min and then to 60% A in 20 min. The pomegranate peel extracts were dissolved in a mixture of methanol and water (6:4 v/v), and 20 μL was injected into the HPLC. The compounds were quantified using a Shimadzu C-R4A Chromatopak data processor at a chart speed of 2.5 mm/min.

Determination of Total Phenolics. The concentration of phenolic compounds in the extracts was determined according to the method of Jayaprakasha et al. (*13*), and results were expressed as tannic acid equivalents. The pomegranate peel extracts were dissolved in a mixture of methanol and water (6:4 v/v). Samples (0.2 mL) were mixed with 1.0 mL of 10-fold-diluted Folin−Ciocalteu reagent and 0.8 mL of 7.5% sodium carbonate solution. After the mixture had been allowed to stand for 30 min at room temperature, the absorbance was measured at 765 nm using a Genesys-5 UV−visible spectrophotometer. The estimation of phenolic compounds in the fractions was carried out in triplicate, and the results were averaged.

Antioxidant Assay Using β-Carotene—Linoleate Model System. The antioxidant activity of pomegranate peel and seed extracts was evaluated according to the method of Jayaprakasha et al. (*13*). β-Carotene (0.2 mg) in 0.2 mL of chloroform, linoleic acid (20 mg), and Tween-40 (polyoxyethylene sorbitan monopalmitate) (200 mg) were mixed. Chloroform was removed at 40 °C under vacuum, and the resulting mixture was diluted with 10 mL of water and mixed well. To this emulsion was added 40 mL of oxygenated water. Four milliliter aliquots of the emulsion were pipetted into different test tubes containing 0.2 mL of pomegranate peel and seed extracts (50 and 100 ppm) and BHA (25 and 50 ppm) in ethanol. BHA was used for comparative purposes. A control containing 0.2 mL of ethanol and 4 mL of the above emulsion was prepared. The tubes were placed at 50 °C in a water bath, and the absorbance at 470 nm were taken at zero time (t = 0). Measurement of absorbance was continued until the color of β-carotene disappeared in the control tubes (t = 180 min) at an interval of 15 min. A mixture prepared as above without β-carotene served as blank. All determinations were carried out in triplicate. The antioxidant activity (AA) of the extracts was evaluated in terms of bleaching of the β-carotene using the following formula, $AA = 100[1 - (A_0 - A_t)/(A°_0 - A°_t)]$, where A_0 and $A°_0$ are the absorbance values measured at zero time of the incubation for test sample and control, respectively, and A_t and $A°_t$ are the absorbances measured in the test sample and control, respectively, after incubation for 180 min.

Radical Scavenging Activity Using DPPH Method. Different concentrations (50 and 100 μL equivalent to 50 and 100 ppm) of pomegranate peel and seed extracts and BHA (25 and 50 ppm) were taken in different test tubes. The volume was adjusted to 100 μL by adding MeOH. Five milliliters of a 0.1 mM methanolic solution of DPPH was added to these tubes and shaken vigorously. The tubes were allowed to stand at 27 °C for 20 min (*14*). The control was prepared as above without any extract, and MeOH was used for the baseline correction. Changes in the absorbance of the samples were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula: % radical scavenging activity = (control OD − sample OD/control OD) × 100.

On the basis of the results of the above two experiments, the methanol extract of pomegranate peel, which showed significant activity with both methods, was selected for further studies.

Measurement of Lipid Peroxidation by Thiobarbituric Acid (TBA) Assay. Thiobarbituric acid reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm as per Halliwell and Gutteridge (*15*). Normal albino rats of the Wister strain were used for the preparation of liver homogenate. The perfused liver was isolated, and 10% (w/v) homogenate was prepared using a Potter Elvehjem homogenizer under ice-cold (0−4 °C) conditions. The homogenate was centrifuged at 1500*g* for 15 min, and clear supernatant was taken for the analysis. Cell-free (800 g) supernatants of albino rat liver homogenate were used for the study of in vitro lipid peroxidation. Different concentrations (25, 50, and 100 ppm) of extract (dissolved in EtOH) were taken in test tubes and evaporated to dryness. One milliliter of 0.15 M potassium chloride and 0.5 mL of rat liver homogenates were added to the test tubes. Peroxidation was initiated by adding 100 μL of 0.2 mM ferric chloride. After incubation at 37 °C for 30 min, the reaction was stopped by adding 2 mL of ice-cold HCl (0.25 N) containing 15% trichloroacetic acid (TCA), 0.38% TBA, and 0.5% BHT. The reaction mixtures were heated at 80 °C for 60 min. The samples were cooled and centrifuged, and the absorbance of the supernatants was measured at 532 nm. An identical experiment was performed in the absence of extract to determine the amount of lipid peroxidation obtained in the presence of inducing agents without any extract. The percentage of antilipid peroxidative activity (% ALP) is calculated by the following formula: antilipid peroxidation (%) = 1− (sample OD/blank OD) × 100.

Hydroxyl Radical Scavenging Activity. The hydroxyl radical scavenging activity was determined according to the method of Klein et al. (*16*). Various concentrations (25, 50, and 100 ppm) of extracts in EtOH were taken in different test tubes and evaporated to dryness. One milliliter of iron−EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 mL of EDTA (0.018%), and 1 mL of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) were added to these tubes, and the reaction was initiated by adding 0.5 mL of 0.22% ascorbic acid. Test tubes were capped tightly and heated on a water bath at 80−90 °C for 15 min. The reaction was terminated by the addition of 1 mL of ice-cold TCA (17.5% w/v). Three milliliters of Nash reagent (75.0 g of ammonium acetate, 3 mL of glacial acetic acid, and 2 mL of acetyl acetone were mixed and raised to 1 L with distilled water) was added to all of the tubes and left at room temperature for 15 min for color development. The intensity of the yellow color formed was measured spectrophotometrically at 412 nm against reagent blank. The percentage hydroxyl radical scavenging is calculated by the following formula: % hydroxyl radical scavenging activity = 1 − (difference in absorbance of sample/difference in absorbance of blank) × 100.

Antioxidant Activity on Human Low-Density Lipoprotein (LDL) Oxidation. Plasma was prepared from blood drawn from human volunteers. The plasma collected was stored at 0−4 °C. The LDL was prepared from the plasma according to the method of Princen et al. (*17*) using a differential ultracentrifugation method. Various concentrations (25, 50, and 100 ppm) of extract were taken in test tubes, 40 μL of copper sulfate (2 mM) was added, and the volume was made to 1.5 mL in all test tubes with phosphate buffer (50 mM, pH 7.4). A tube without extract and copper sulfate served as negative control, and another tube without copper sulfate served as positive control. All tubes were incubated at 37 °C. Aliquots of 0.5 mL from each tube were drawn at 2 h intervals, and 0.25 mL of TBA (1% in 50 mM NaOH) was added followed by 0.25 mL of TCA (2.8%). The tubes were incubated for 45 min at 95 °C. A pink chromogen was extracted after the mixture had cooled to room temperature by centrifugation (at 2000 rpm for 10 min). Thiobarbituric acid-reactive species in the pink chromogen were detected by fluorescence at 515 nm excitation and 553 nm emission. Data were expressed in terms of MDA equivalent, which was estimated by comparison with the standard graph drawn for 1,1,3,3-tetramethoxypropane (which was used as standard), which gave the amount of

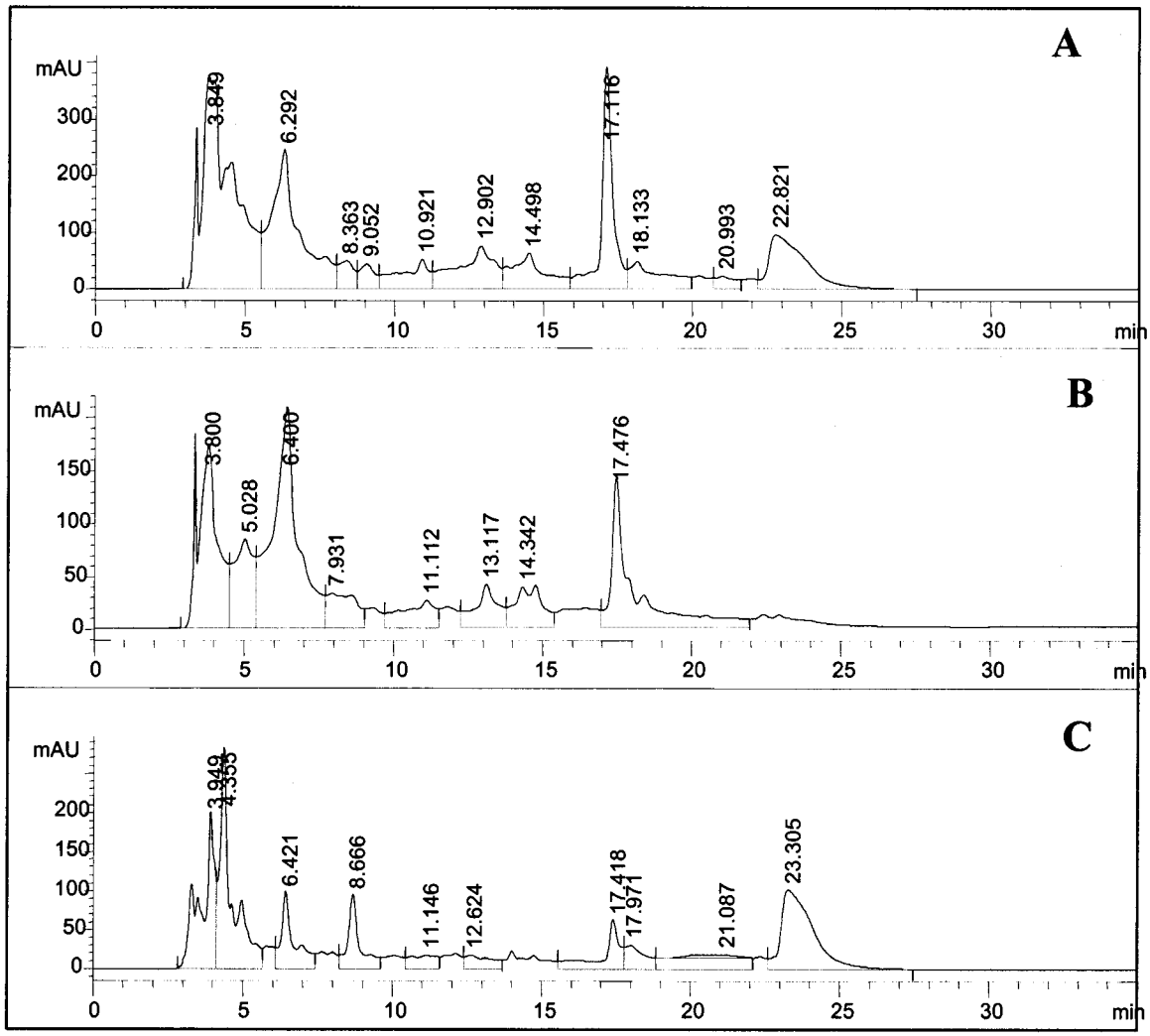

Figure 1. HPLC chromatograms of pomegranate peel (A) EtOAc extract and (B) MeOH extract and (C) water extract recorded at 280 nm.

Table 1. Yield and Phenolic Content of Pomegranate Peel and Seed Extracts[a]

pomegranate	extracts	yield (%, w/w)	phenolics (%, w/w)
peel	EtOAc	1.04	18.0
	MeOH	9.38	44.0
	Water	7.53	03.0
seed	EtOAc	2.32	02.1
	MeOH	8.62	02.6
	Water	7.53	03.0

[a] Values expressed are the mean of three replications.

oxidation. The amount of protein was estimated by using the Folin−phenol method (*18*), and the results were expressed as protection per unit of protein concentration. Using the amount of MDA, the percentage protection was calculated using the following formula: (oxidation in control − oxidation in experimental/oxidation in control) × 100.

Statistical Analysis. Student's *t* test was used to compare the data, and all tests were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The yields of extracts obtained from pomegranate peel and seeds using various solvents are shown in Table 1. Pomegranate peel extracted with MeOH gave maximum yield of extract followed by water and EtOAc extracts. The phenolic content of the MeOH extract was maximum; the phenolic content was very low in the water extract, despite high extract yield than the phenolic content of EtOAc extract. Similarly, in the case of seeds, the yield of extracts obtained by extraction with MeOH was highest followed by water and EtOAc. The water extract of seeds possesses high phenolic content followed by the MeOH and EtOAc extracts. The antioxidant activity may be directly correlated to the phenolic content of various peel extracts; thus, the MeOH extract of peel showed higher activity as compared to the other extracts. However, in the case of seed extracts, the phenolic content is quite low and there may not be any direct correlation between phenolic content and antioxidant activity.

The HPLC pattern of the of EtOAc, methanol, and water extracts of pomegranate peel are shown in Figure 1. The presence of gallic acid is shown to be the major component. In Figure 1, the HPLC patterns of the three extracts are comparable, but the methanolic extract has a greater concentration of peaks at retention times of 6.4 and 17.4 min: 30.5 and 18%. Also, the peak at 23 min was not found in the MeOH extract. The presence of ellagic acid, gallic acid, and tertgallic acid has been reported in the pomegranate juice (*11*).

The antioxidant activity of pomegranate peel and seed extracts as measured by the bleaching of β-carotene is presented in Figures 2 and 3, respectively. It can be seen that pomegranate peel and seed extracts prepared by different solvents exhibited various degrees of antioxidant activity. At 50 ppm concentration, EtOAc, MeOH, and water extracts of peel were shown to exhibit 53, 82, and 64% antioxidant activity, respectively. At 100 ppm concentration, EtOAc, MeOH, and water extracts of seed exhibit

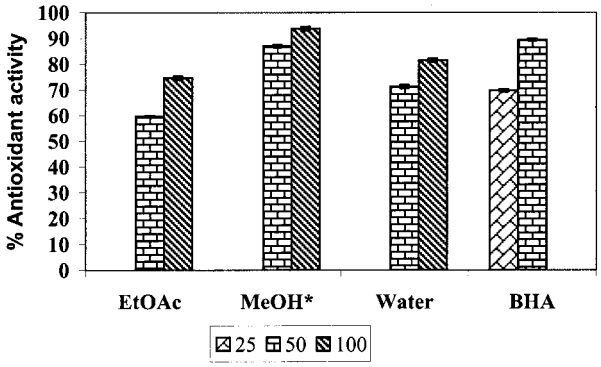

Figure 2. Antioxidant activity of pomegranate peel extracts and BHA by β-carotene–linoleate model system at different concentrations (ppm). *, Significant when compared to BHA.

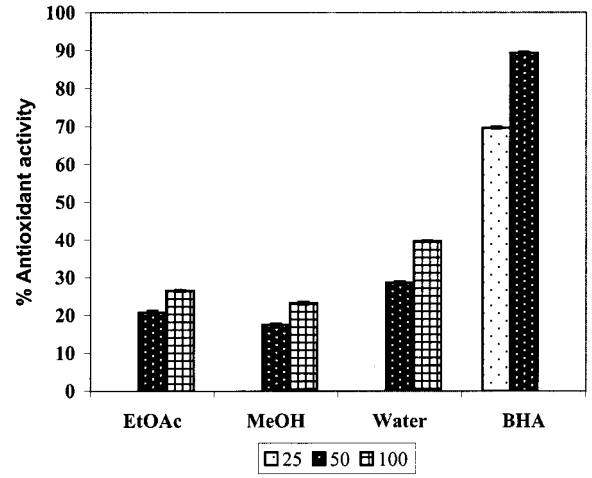

Figure 3. Antioxidant activity of pomegranate seed extracts and BHA by β-carotene–linoleate model system at different concentrations (ppm).

39, 22, and 57% antioxidant activity, respectively. The mechanism of bleaching of β-carotene is a free radical mediated phenomenon resulting from the hydroperoxides formed from linoleic acid. β-Carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β-carotene molecules. As β-carotene molecules lose their double bonds by oxidation, the compound loses its chromophore and characteristic orange color, which can be monitored spectrophotometrically. The presence of different extracts can hinder the extent of β-carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system.

Free radical scavenging potentials of pomegranate peel and seed extracts at different concentrations were tested by the DPPH method, and the results are shown in Figures 4 and 5, respectively. Antioxidant reacts with DPPH, which is a stable free radical, and convert it to α,α-diphenyl-β-picryl hydrazine. The degree of discoloration indicates the scavenging potentials of the antioxidant extract. At 50 ppm, EtOAc, MeOH, and water extracts of pomegranate peel exhibit 46, 81, and 43% free radical scavenging activity, respectively, according to this method. At 100 ppm, EtOAc, MeOH, and water extracts of pomegranate seed exhibit 26.5, 23.2, and 39.6% free radical scavenging activity, respectively. The activity of the extracts is attributed to their hydrogen donating ability (*19*). It is well-known that free radicals cause autoxidation of unsaturated lipids in food (*20*). On the other hand, antioxidants are believed to intercept

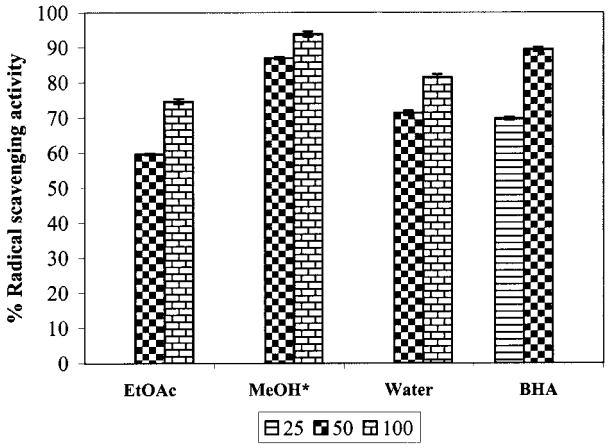

Figure 4. Radical scavenging activity of pomegranate peel extracts and BHA by DPPH method at different concentrations (ppm). *, Significant when compared to BHA.

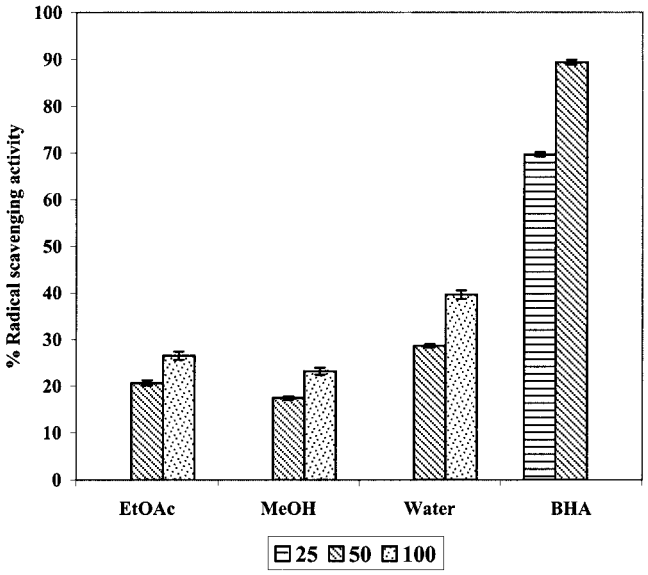

Figure 5. Radical scavenging activity of pomegranate seed extracts and BHA by DPPH method at different concentrations (ppm).

the free radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups, thereby forming a stable end-product, which does not initiate or propagate further oxidation of the lipid (*21*). The data obtained reveal that the extracts are free radical inhibitors and primary antioxidants that react with free radicals.

The results of the effect of MeOH extract of pomegranate peel to prevent lipid peroxidation are shown in Figure 6. At 100 ppm, the extract shows 56% scavenging activity by this method. In biological systems, MDA is a very reactive species and takes part in cross-linking of DNA with proteins and also damaging the liver cells (*22*). Lipid peroxidation has been broadly defined as the oxidative deterioration of polyunsaturated lipids. Initiation of a peroxidation sequence in a membrane or polyunsaturated fatty acid is due to abstraction of a hydrogen atom from the double bond in the fatty acid. The free radical tends to be stabilized by a molecular rearrangement to produce a conjugated diene, which then easily reacts with an oxygen molecule to give a peroxy radical (*23*). Peroxy radicals can abstract a hydrogen atom from another molecule or they can abstract a hydrogen atom to give a lipid hydroperoxide, R−OOH. A probable alternative fate of peroxy radicals is to form cyclic peroxides; these cyclic peroxides, lipid peroxides,

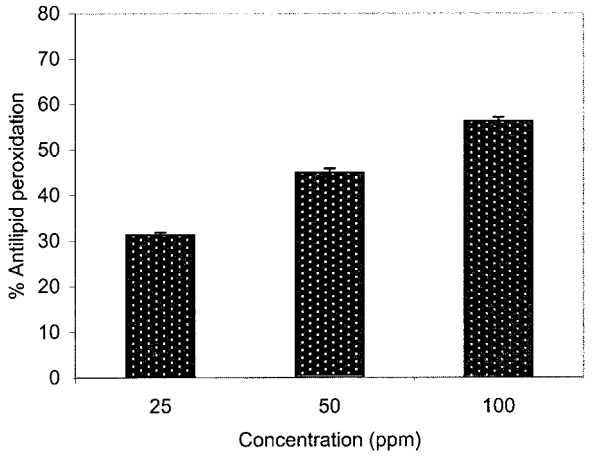

Figure 6. Antilipid peroxidation activity of pomegranate peel MeOH extract at different concentrations by TBA method.

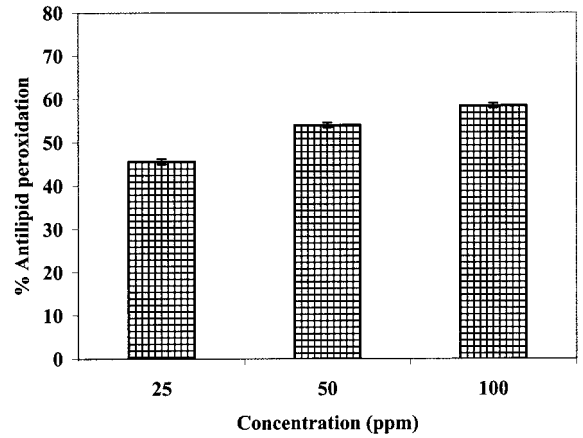

Figure 7. Hydroxyl radical scavenging activity of pomegranate peel MeOH extract at different concentrations.

and cyclic endoperoxides fragment to aldehydes including MDA and polymerization products. MDA is the major product of lipid peroxidation and is used to study the lipid peroxidation process in rat liver homogenate. Determination of the lipid peroxide content was carried out indirectly by means of derivatizing MDA with TBA at high temperature and acidic conditions (*15*).

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells (*24*, *25*). This radical has the capacity to join nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis, and cytotoxicity. In addition, this species is considered to be one of the quick initiators of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids (*26*). Hydroxyl radical scavenging activity was estimated by generating the hydroxyl radicals using ascorbic acid−iron EDTA. The hydroxyl radicals formed by the oxidation react with dimethyl sulfoxide (DMSO) to yield formaldehyde, which provides a convenient method to detect hydroxyl radicals by treatment with Nash reagent. The hydroxyl radical scavenging activity of the MeOH extract of pomegranate peel is shown in Figure 7. In the present investigation, the methanol extract of pomegranate exhibits 58% hydroxyl radical scavenging activity at 100 ppm. The ability of the pomegranate extract to quench hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and seems to be a good scavenger of active oxygen species, thus reducing the rate of chain reaction.

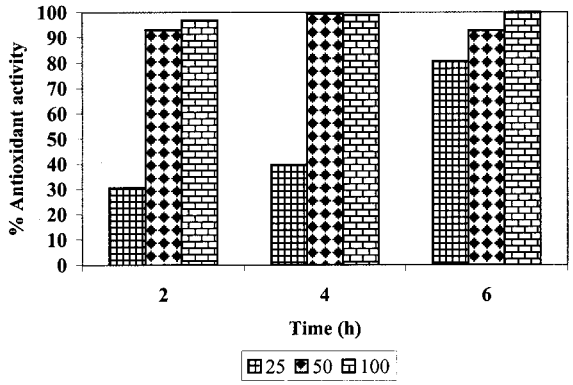

Figure 8. Antioxidant activity of pomegranate peel methanol extract on human LDL oxidation at different concentrations (ppm).

Oxidative modification is known to play an important role in the pathogenesis of atherosclerosis and coronary heart diseases (*27*), and the dietary antioxidants that protect LDL from oxidation may therefore reduce atherogenesis and coronary heart diseases (*28*). The antioxidant activity of methanol pomegranate extracts against human LDL oxidation is shown in Figure 8. The polyunsaturated fatty acids (PUFA) of human LDL were oxidized, and the MDA formed have been estimated by using the TBA method. The average induction time for copper-mediated LDL oxidation was ~20 min without the addition of phenolic extracts. The MeOH extracts strongly protected LDL from oxidation as measured by the prolongation of this induction time of the formation of conjugated dienes. The methanol extract of pomegranate peel exhibits 31, 93, and 96% protection at 25, 50, and 100 ppm at the end of 2 h, indicating a dose-dependent antioxidant effect against LDL oxidation with respect to the concentration of phenolics.

The reducing properties are generally associated with the presence of reductones (*29*). Gordon (*25*) reported that the antioxidant action of reductones is based on the breaking of the free radical chain by the donation of a hydrogen atom. Reductones also react with certain precursors of peroxide, thus preventing peroxide formation. The data presented here indicated that the marked antioxidant activity of pomegranate extracts seems to be due to the presence of polyphenols, which may act in a similar fashion as reductones by donating the electrons and reacting with free radicals to convert them to more stable product and terminate free radical chain reaction.

The results shown above indicate that the extraction with methanol not only gives high yield of the extract but also gives high antioxidant activity, which was confirmed by various methods used for the antioxidant assay. Thus, the results of the present work indicate that the selective extraction of antioxidant from natural sources by appropriate solvent is very important in obtaining fractions with high antioxidant activity.

The results of the present work indicate the presence of compounds possessing antioxidant activity from peel and seeds of pomegranate with peel as an enriched source of the antioxidants exhibiting higher activity as compared to seeds. The difference in the antioxidant activity of the peel and seed may be ascribed to their different phenolic compositions. Because this is the first report on the description of antioxidants from pomegranante, further studies are needed on the isolation and characterization of individual phenolic compounds to elucidate their different antioxidant mechanisms and the existence of possible synergism, if any, among the compounds.

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