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Studies on Antioxidant Activity of Pomegranate (*Punica granatum*) Peel Extract Using in Vivo Models

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Pomegranate (*Punica granatum*) peel extracts have been shown to possess significant antioxidant activity in various in vitro models. Dried pomegranate peels were powdered and extracted with methanol for 4 h. The dried methanolic extract was fed to albino rats of the Wistar strain, followed by carbon tetrachloride (CCl_4), and the levels of various enzymes, such as catalase, peroxidase, and superoxide dismutase (SOD), and lipid peroxidation were studied. Treatment of rats with a single dose of CCl_4 at 2.0 g/kg of body weight decreases the levels of catalase, SOD, and peroxidase by 81, 49, and 89% respectively, whereas the lipid peroxidation value increased nearly 3-fold. Pretreatment of the rats with a methanolic extract of pomegranate peel at 50 mg/kg (in terms of catechin equivalents) followed by CCl_4 treatment causes preservation of catalase, peroxidase, and SOD to values comparable with control values, wheres lipid peroxidation was brought back by 54% as compared to control. Histopathological studies of the liver were also carried out to determine the hepatoprotection effect exhibited by the pomegranate peel extract against the toxic effects of CCl_4. Histopathological studies of the liver of different groups also support the protective effects exhibited by the MeOH extract of pomegranate peel by restoring the normal hepatic architecture.

KEYWORDS: ***Punica granatum*; reactive oxygen species; antioxidant activity; lipid peroxidation; SOD; peroxidase; catalase**

INTRODUCTION

Oxidation is a metabolic process that leads to energy production necessary for essential cell activities. However, metabolism of oxygen in living cells also leads to the unavoidable production of oxygen-derived free radicals, commonly known as reactive oxygen species (ROS) (*1*, *2*), which are involved in the onset of many diseases (*3*). These free radicals attack the unsaturated fatty acids of biomembranes, which results in lipid peroxidation and the destruction of proteins and DNA, which causes a series of deteriorative changes in the biological systems leading to cell inactivation. Thus, the identification of antioxidants, which can retard the process of lipid peroxidation by blocking the generation of free radical chain reaction, has gained importance in recent years. In living systems, varieties of antioxidant mechanisms play an important role in combating ROS (*4*, *5*). The antioxidants may act by raising the levels of endogenous defenses by up-regulating the expression of genes encoding the enzymes such as superoxide dismutase (SOD), catalase, or glutathione peroxidase (*4*, *6*).

Pomegranate (*Punica granatum* L.) is native to the Mediterranean region and has been used extensively in the folk medicine of many countries. In India, it is used in the form of juice, concentrate, canned beverage, wine, jam, and jelly (*7*). Fresh juice contains a small amount of pectin, ascorbic acid, and flavonoids. The soluble polyphenolic content of pomegranate juice (0.2−1.0%) includes anthocyanins, catechins, ellagic tannins, and gallic and ellagic acids (*8*). Previous work carried out in this laboratory showed high antioxidant activities of the methanolic extracts of pomegranate peel in various in vitro models (*9*). The objective of the present study was to determine the protective effect of feeding the methanolic extract of pomegranate peel to albino rats of the Wistar strain against the toxic effects of carbon tetrachloride (CCl_4) by biochemical and histopathological methods. The level of various ROS-combating enzymes in the liver, namely, catalase, peroxidase, and SOD, and lipid peroxidation were estimated in liver homogenates to assess the protection provided by pomegranate peel methanolic extract against CCl_4 toxicity.

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MATERIALS AND METHODS

Materials. All solvents/chemicals used were of analytical/HPLC grade and obtained from Merck, Mumbai, India. UV−visible spectra measurements was carried out using a Genesys-5 UV−visible spectrophotometer (Milton Roy, Ivyland, PA). Gallic acid was obtained from Sigma Chemical Co. (St. Louis, MO). Ellagic acid was generously provided by Dr. Takuro Koga, Kikkoman Corp., Biochemicals Division, Noda, Japan.

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Extraction. Pomegranates (*P. granatum* var. Ganesha) were purchased from a local market. The peels were manually removed, sun-dried, and powdered to get 60 mesh size. The peel powder (100 g) was extracted by stirring using a magnetic stirrer with 600 mL of MeOH at 30 °C for 4 h. The extract was filtered through Whatman no. 41 filter paper for removal of peel particles. The residue was re-extracted with 500 mL of MeOH and filtered. The extracts were pooled and concentrated under vacuum at 40 °C (*10*).

Determination of Total Phenolics. The concentration of total phenolics in the methanolic extract was determined according to the method of Jayaprakasha et al. (*11*), and results were expressed as (+)-catechin equivalents. Methanolic extract (0.2 mg) in MeOH was mixed with 1.0 mL of 10-fold diluted Folin−Ciocalteu reagent and 0.8 mL of a 7.5% sodium carbonate solution. After the mixture had been allowed to stand for 30 min at 30 °C, the absorbance was measured at 765 nm using a Genesys-5 UV−visible spectrophotometer. Estimation of the phenolic compounds was carried out in triplicate and averaged.

HPLC Analysis. The high-performance liquid chromatographic system consisted of a Hewlett-Packard HPLC model HP 1100 series (Hewlett-Packard, Palo Alto, CA) equipped witha quaternary pump, fitted with a Zorbax C_{18} (Hewlett-Packard) analytical column (25 cm × 4.6 mm I.D, 5 μm particle size). The injection system (Rheodyne) used was a 20 μL sample loop. Detection was done by an HP 1100 series variable-wavelength detector at wavelength of 280 nm. The gradient mobile phase consists of (A) MeOH and (B) 2% AcOH with the flow rate of 0.7 mL/min. The elution program involved a linear gradient from 95 to 50% B in A for 0−25 min and 95% B in A by 30 min followed by 5 min of equilibrium with 95% B. The compounds were quantified using HP Chemstation software. The pomegranate peel methanolic extract was dissolved in methanol, and 20 μL was injected to the HPLC.

Experimental Procedure. Male albino rats of the Wistar strain, weighing 180−220 g, were used for the studies. The animals were grouped into three groups containing six animals in each group. The first group served as control, the second group was administered CCl_4 (negative control), and the third group was administered the methanolic extract of pomegranate peel. The extract was suspended in 0.5% sodium carboxymethylcellulose and was fed to third-group rats via oral route at 50 mg (in terms of catechin equivalents)/kg of body weight for 14 days. The dose was selected on the basis of the LD_{50} value of polyphenols (*12*). The animals of the first and second groups were simultaneously administered saline until the 14th day. The animals of the second and third groups were given a single oral dose of CCl_4 (1:1 in olive oil) at 2.0 g/kg of body weight 6 h after the last dose of administration of extract/saline on the 14th day. After 24 h, animals were sacrificed, and the liver was isolated to prepare the liver homogenate.

Five percent liver homogenate was prepared with 0.15 M KCl and centrifuged at 800*g* for 10 min. The cell-free supernatant was used for the estimation of lipid peroxidation, peroxidase, catalase, and SOD.

Catalase Assay. The catalase assay was carried out per the method of Aebi (*13*). One milliliter of liver homogenate from groups 1−3 was taken with 1.9 mL of phosphate buffer in different test tubes (50 mM, pH 7.4). The reaction was initiated by the addition of 1 mL of hydrogen peroxide (30 mM). Blank without liver homogenate was prepared with 2.9 mL of phosphate buffer and 1 mL of hydrogen peroxide. The decrease in optical density due to decomposition of hydrogen peroxide was measured at the end of 1 min against the blank at 240 nm. Units of catalase were expressed as the amount of enzyme that decomposes 1 μM H_2O_2 per minute at 25 °C. The specific activity was expressed in terms of units per milligram of proteins.

Estimation of SOD. The assay of SOD was based on the reduction of nitro blue tetrazolium (NBT) to water insoluble blue formazan, per the method of Beauchamp and Fridovich (*14*). Liver homogenate (0.5 mL) was taken, and 1 mL of 50 mM sodium carbonate, 0.4 mL of 24 μM NBT, and 0.2 mL of 0.1 mM EDTA were added. The reaction was initiated by adding 0.4 mL of 1 mM hydroxylamine hydrochloride. Zero time absorbance was taken at 560 nm followed by recording the absorbance after 5 min at 25 °C. The control was simultaneously run without liver homogenate. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50%. The specific activity was expressed in terms of units per milligram of proteins.

Estimation of Peroxidase. The peroxidase assay was carried out per the method of Nicholos (*15*). Liver homogenate (0.5 mL) was taken, and to this were added 1 mL of 10 mM KI solution and 1 mL of 40 mM sodium acetate solution. The absorbance of potassium periodide was read at 353 nm, which indicates the amount of peroxidase. Twenty microliters of hydrogen peroxide (15 mM) was added, and the change in the absorbance in 5 min was recorded. Units of peroxidase activity were expressed as the amount of enzyme required to change the OD by 1 unit per minute. The specific activity was expressed in terms of units per milligram of proteins.

Lipid Peroxidation Activity. Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm per the method of Buege and Aust (*16*). Liver homogenate (0.5 mL) and 1 mL of 0.15 M KCl were taken. Peroxidation was initiated by adding 100 μL of 0.2 mM ferric chloride. The reaction was run at 37 °C for 30 min. The reaction was stopped by adding 2 mL of an ice-cold mixture of 0.25 N HCl containing 15% trichloroacetic acid, 0.30% TBA, and 0.05% BHT and was heated at 80 °C for 60 min. The samples were cooled and centrifuged, and the absorbance of the supernatant was measured. The results were expressed as MDA equivalents, which was calculated by using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. One unit of lipid peroxidation activity was defined as the amount of TBA that converts to TBARS. The specific activity was expressed in terms of units per milligram of protein.

Determination of Proteins. Protein was determined using the method of Lowry et al. (*17*).

Histopathological Studies. Histopathological studies of the livers of the different groups of rats were carried out to determine the effect of protection offered by feeding the methanolic extract of pomegranate peel against the toxic effects of CCl_4.

Statistical Analysis. The experiments were done in triplicate. Data are expressed as mean ± SD. One-way analysis of variance (ANOVA) was used and the *t* test was used for comparison of mean values. All tests were considered to be statistically significant at $p < 0.001$.

RESULTS AND DISCUSSION

The yield of extract obtained from pomegranate peel using MeOH was found to be 10.38 ± 0.89% (w/w). The total phenolic content of the extract [(+)-catechin equivalent] was found to be 42% (w/w). The HPLC pattern of the methanolic extract of pomegranate peel showed the presence of gallic acid and ellagic acid as the major components along with other components. The identities of these two peaks were confirmed by determination of relative retention times and by spiking with corresponding standards. The percentages of gallic and ellagic acids were found to be 7.5 and 54.6%, respectively, with respect to MeOH extract. The presence of ellagic acid, gallic acid, and *tert*-gallic acid has been reported in pomegranate juice (*18*).

Recently, the antioxidant activity of EtOAc, MeOH, and water extracts of pomegranate peels and seeds has been reported in various in vitro models (*9*). Among all of the extracts, pomegranate peel MeOH extract was reported to possess high antioxidant activity in various models. Hence, in the present study, only the methanolic extract of pomegranate peels has been used. **Figure 1** depicts the effect of treatment of rats with CCl_4 and pretreatment with pomegranate peel extracts followed by CCl_4 treatment on the levels of catalase, SOD, and peroxidase enzymes and lipid peroxidation activity in liver homogenates. Treatment of rats with a single dose of CCl_4 at 2.0 g/kg of body weight significantly reduces the levels of catalase, peroxidase, and SOD by 81, 89, and 49%, respectively. On the other hand, lipid peroxidation increases ∼3-fold as compared to control due to CCl_4 treatment. However, pretreatment of the rats with the

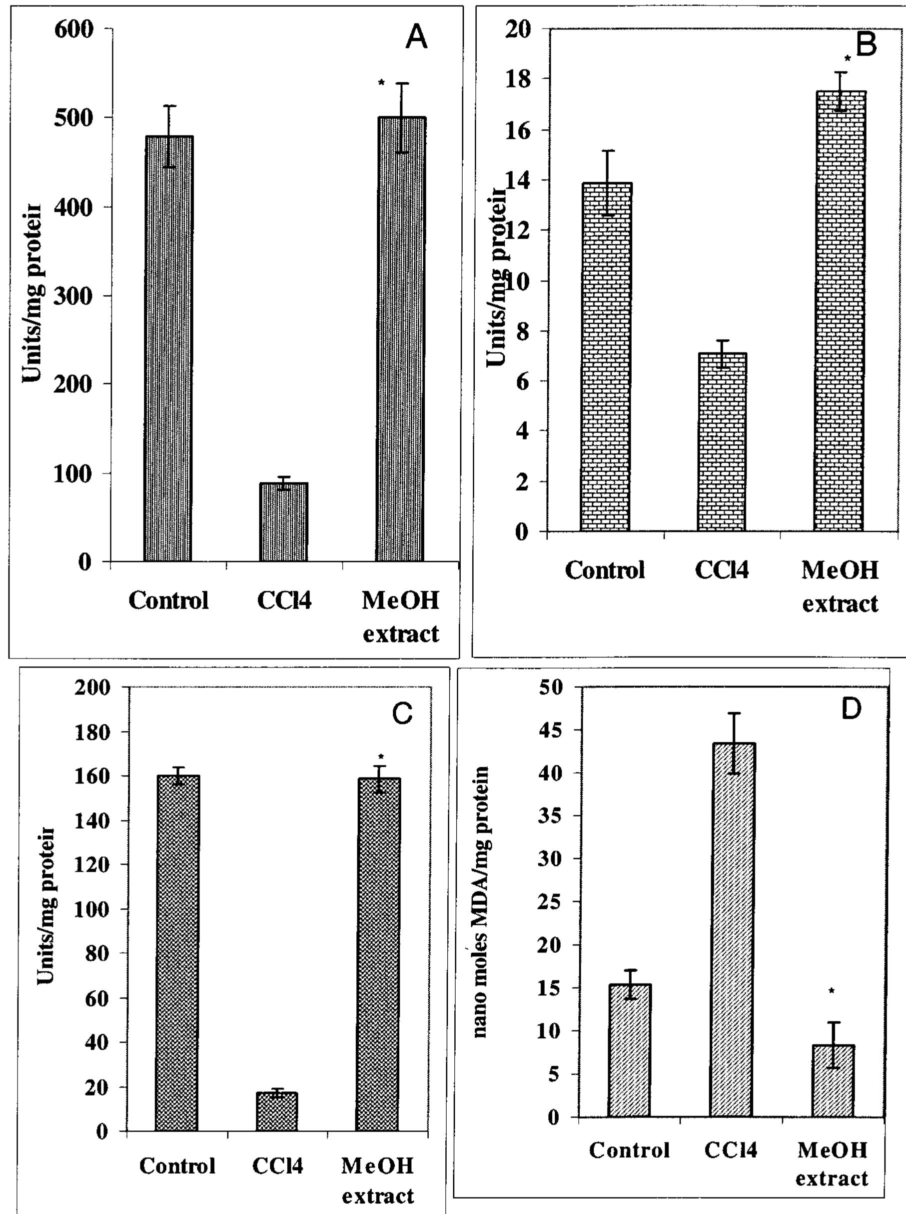

Figure 1. Effect of treatment of rats with MeOH extract of pomegranate peel followed by feeding carbon tetrachloride on the levels of various enzymes and lipid peroxidation of liver: (A) catalase; (B) SOD; (C) peroxidase; (D) inhibition of lipid peroxidation. Each data bar represents the mean ± SD of three replicates. An asterisk (*) indicates high significance when compared with carbon tetrachloride treatment ($p < 0.01$).

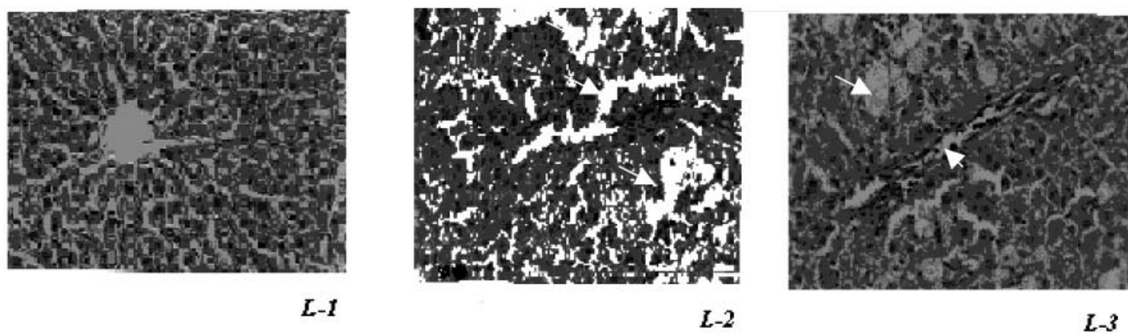

Figure 2. (L-1) control: normal hepatocytes showing normal architecture with portal tried, showing portal veins, hepatic artery, and vein. (L-2) Carbon tetrachloride: total loss of hepatic architecture, areas of hemorrhage, and necrosis are seen. (L-3) Pomegranate peel methanol extract: retains normal hepatic architecture with few areas of hemorrhage between the columns of hepatocytes.

methanolic extract of pomegranate peel at 50 mg/kg (in terms of catechin equivalents) preserves catalase, peroxidase, and SOD activities, which are comparable with the control values of the enzymes. This shows the protection provided by feeding pomegranate peel extract to the rats by maintaining the levels of these enzymes even after CCl_4 treatment. The lipid peroxidation values have been shown to be restored by 54% as compared to control showing antilipid peroxidative effects of

the components of pomegranate peel extract. The effect of free radical on the mean liver detoxificant enzymes (catalase, SOD, and peroxidase) reduced the enzyme activity, mainly catalase and peroxidase, due to enzyme inactivation during the catalytic cycle. In these conditions, the pomegranate extract containing ellagic acid and gallic acid acts as a potent free radical scavenger, reducing the levels of hydrogen peroxide and superoxide anion and, consequently, lipid peroxidation and enzyme inactivation, restoring enzyme activity. This may also point toward the possible de novo synthesis of these enzymes induced by the components of pomegranate peel extract (*4*, *6*).

CCl_4 has been extensively studied as a liver toxicant, and its metabolites such as trichloromethyl radical ($CCl_3^•$) and trichloromethyl peroxy radical ($CCl_3O_2^•$) are involved in the pathogenesis of liver (*19*) and kidney damage (*20*). As shown by Lin et al. (*21*) CCl_4 causes changes around the liver central vein in the liver and other oxidative damages with the leakage of marker enzymes such as GOT and GPT in the serum. Histopathological studies carried out for the liver of control, carbon tetrachloride treated, and pomegranate peel methanolic extract treated have are shown in **Figure 2** (arrows indicate the area of necrosis and hemorrhage). The massive generation of free radical in the CCl_4-induced liver damage provokes a sharp increase of lipid peroxidation in liver. When free radical generation is massive, in the CCl_4-induced liver damage, the cytotoxic effect is not localized but can be propagated intracellularly, increasing the interaction of these radicals with phospholipid structures and inducing peroxidation processes that destroy organ structure. The studies showed that in the case of the control, hepatocytes having normal architecture with portal triad, portal veins, and hepatic artery and vein were visible. However, in case of CCl_{4-t}reated rats, total loss of hepatic architecture and areas of hemorrhage and necrosis were seen. In the case of rats pretreated with pomegranate peel extract followed by exposure to CCl_4, the liver was shown to retain normal hepatic architecture with few areas of hemorrhage between the columns of hepatocytes. These results clearly indicate the protection provided by the pomegranate peel extract. Lin et al. (*21*) indicated that the liver protective and antioxidative effects of certain plant extracts against CCl_4-induced liver injury possibly involve mechanisms related to free radical scavenging effects. Singh et al. (*18*) also described the hepatoprotective and antilipid peroxidative effects of ellagic acid against CCl_4-induced hepatotoxicity in rat liver. The effects of various phenolic compounds such as caffeic acid, chlorogenic acid, cyanarin, and cyanaroside in protecting rat hepatocytes against *tert*-butyl hydroperoxide toxicity have been demonstrated (*22*).

The results of the present work indicate that the pomegranate peel MeOH extract is capable of enhancing/maintaining the activity of hepatic enzymes which are involved in combating ROS. Also, feeding of the pomegranate peel extract provides protection against CCl_4 toxicity as shown by histopathological studies conducted on the liver. Pomegranate peel is rich in phenolic compounds, and different activities of the pomegranate extract can be ascribed to their different phenolic composition. Further studies are needed, however, with individual phenolic compounds of pomegranate peel to elucidate the mechanisms involved in the enhancement of enzyme activity and the protection provided to liver and also to explore the possible synergism, if any, that may potentiate the protective effects against ROS.

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