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In Vitro Studies on the Binding, Antioxidant, and Cytotoxic Actions of Punicalagin

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The protective bioactivity of punicalagin, a high molecular weight polyphenol isolated from pomegranate fruit pith and carpellary membrane, against oxidative damages to lipids, amino acids constituting the proteins, and guanosine as a model for DNA has been investigated. The $ABTS^{\bullet-}$, guanosine, and tryptophan radical generated pulse radiolytically were repaired by punicalagin, $k = (0.9-15) \times 10^7$ $dm^3\ mol^{-1}\ s^{-1}$. The results are rationalized on the basis of the scavenging activity of punicalagin against various one-electron oxidizing radicals, namely, $^{\bullet}OH$, $N_3^{\bullet}$, and $NO_2^{\bullet}$. The formation of the transient species in these reactions and the rate constants of the scavenging reactions have been probed using a time-resolved kinetic spectrophotometric technique. The antioxidant action of punicalagin is expressed not only through its scavenging reactions but also by its ability to form metal chelates. Binding of punicalagin with bovine serum albumin and metal ions such as iron and copper revealed different binding affinities, whereas its binding with DNA was very weak and nonspecific. In vitro cytotoxic studies against three cell lines, namely, Vero (normal African green monkey kidney cell line), Hep-2 (human larynx epithelial cancer cell line), and A-549 (human small cell lung carcinoma cell line) showed that this polyphenol is toxic only at higher concentration.

KEYWORDS: Punicalagin; antioxidant; cytotoxicity; binding; pomegranate; metal chelates

INTRODUCTION

Free radicals such as reactive oxygen species (ROS) are endogenously formed as a result of cellular functions. Under normal conditions, a balance exists in the rate of formation of ROS and their scavenging by endogenously present antioxidants. However, under oxidative stress conditions there occurs a shift in the delicate balance between pro-oxidants and antioxidants in favor of the former. It has been now experimentally proved that oxidative stress and lipid peroxidation play important roles in chronic diseases such as cancer, atherosclerosis, and arthritis (*1*−*3*). Exogenous dietary antioxidants, called nutraceuticals (*4*), are capable of scavenging free radicals and have shown promise in preventing certain diseased conditions. Fruits and vegetables, in general, are a rich source of polyphenols, flavonoids, and cinnamic acid derivatives, some of which are lipid-soluble and others water-soluble. Hence, there is a wide interest to characterize their antioxidant and radical scavenging activities.

Pomegranate fruit has long been used for salad dressing and in desserts. The juice is known to possess medicinal and nutritional properties. Grenadine, a pomegranate juice product, is used as a flavoring agent for alcoholic drinks. Punicalagin (**Figure 1**) is a high molecular weight (MW = 1108) water-soluble polyphenolic antioxidant molecule isolated from pomegranate fruit waste, namely, pith and carpellary membrane (CM), the structure of which has been determined previously (*5*). It has shown remarkable pharmacological activities including anti-inflammatory (*6*) hepatoprotective (*7*, *8*) and antigenotoxic activities (*9*). One of the important factors responsible for all of the above activities of punicalagin could be ascribed to its antioxidant activity (*5*, *7*, *8*), which has been attributed to the presence of 16 dissociable −OH groups. A study on the bioavailability of punicalagin in rats showed that punicalagin and its metabolites were observed in feces and urine and also in plasma (*10*). A similar study on the toxicity of punicalagin revealed that repeated oral administration of high doses of punicalagin to rats for 37 days showed no evidence of toxicity (*11*). The health beneficial properties, coupled with its bioavailability and its nontoxic nature, render punicalagin as a promising multifunctional molecule.

Alhough a lot of research work on different physiological, pharmacological, antiproliforative, and apopotic (*12*) activities of punicalagin has been carried out, its free radical scavenging properties as well as its repair reactions need to be fully assessed. We have in the past studied the free radical reactions with various antioxidants and of other superoxide mimetics (*13*, *14*). The present study on the antioxidant activity of punicalagin may explain its wide-spectrum health benefits. We envisaged that

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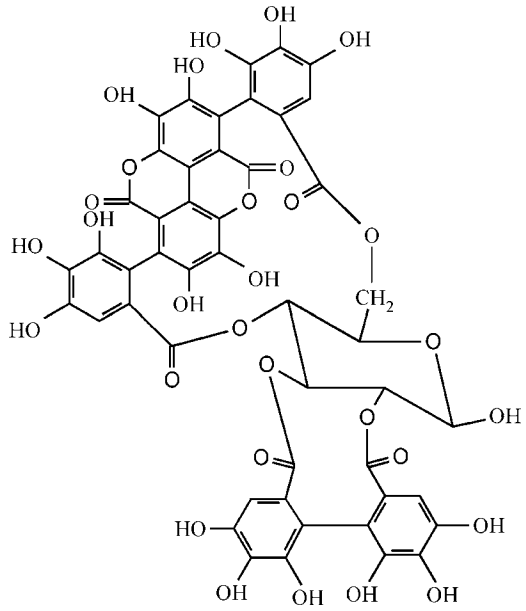

Figure 1. Structure of punicalagin.

the presence of many dissociable phenolic groups as well as other substituents might lead to its interaction with proteins. With this aim, the present study has been undertaken. A pulse radiolysis technique was used to study the reactions of punicalagin with various oxidizing radicals at near physiological pH in aqueous media, and the resultant transient intermediates were characterized. In addition to the radical scavenging ability of punicalagin, the repair reaction for radiolytically produced tryptophan and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet-}$) and guanosine radicals was also studied, and their rate constants were determined. In vitro studies have also been carried out to ascertain the ability of punicalagin to inhibit lipid peroxidation in the rat liver microsomes induced by iron and ascorbic acid. The interaction of punicalagin with metal ions, bovine serum albumin (BSA), and DNA and other studies on its cytotoxicity, using three cell lines, namely, Vero (normal African green monkey kidney cell line), Hep-2 (human larynx epithelial cancer cell line), and A-549 (human small cell lung carcinoma cell line), are also discussed.

EXPERIMENTAL PROCEDURES

Materials. Punicalagin used in the present study was isolated and purified from the pith and carpellary membrane of pomegranate fruit as explained in an earlier paper (*5*). Ferrous ammonium sulfate, cupric chloride, sodium nitrite, and perchloric acid were of GR grade (Merck). Diammonium salt of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{2-}$), bovine serum albumin (BSA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, sulforhodamine B, guanosine, and tryptophan were obtained from Sigma. DNA sodium salt from herring sperm was purchased from HiMedia. All other chemicals used in this study were of the highest purity. Distilled water was further purified by passage through Thermolyne's nanopure water filtering assembly (specific conductivity $< 0.1\ \mu s\ cm^{-1}$). All of the solutions were freshly prepared prior to experiment using this water. Sample solutions were bubbled by either N_2O, N_2, or O_2 gas (Indian Oxygen, IOLAR grade) using a syringe bubbling technique prior to pulse irradiation.

Methods. Fluorescence experiments were carried out using a Shimadzu RF-5000 fluorescence spectrometer with built-in stirrer and thermostat. The UV−vis spectra were recorded between 300 and 900 nm on a Chemito spectrascan UV-2600 spectrophotometer.

Lipid Peroxidation of Liver Microsomes. Rat liver (1 g) was homogenized in 5 mL of 2×10^{-3} mol dm^{-3} Tris buffer (pH 7.4), and the microsomes were isolated by using the calcium aggregation method. The pellet was resuspended in 10^{-1} mol dm^{-3} phosphate buffer. Microsomal lipid peroxidation was assayed according to the thiobarbituric acid method (*15*). Lipid peroxidation was initiated by the addition of known aliquots of 10^{-4} mol dm^{-3} ferrous sulfate and 10^{-4} mol dm^{-3} ascorbic acid to 100 μL of microsomes, in the absence and presence of $(0.1-2) \times 10^{-4}$ mol dm^{-3}/mL punicalagin in 10^{-1} mol dm^{-3} phosphate buffer (pH 7.4). This solution was incubated at 37 °C for 1 h, followed by the addition of 20% trichloroacetic acid (2 mL) and 1% thiobarbituric acid (2 mL). The mixture was heated in a boiling water bath for 10 min, cooled, and centrifuged for 20 min. The absorbance of the supernatant was measured spectrophotometerically at 535 nm, and the IC_{50} values were calculated for punicalagin and also butylated hydroxyanisole (BHA), a reference antioxidant.

ABTS Radical Scavenging Activity. The diammonium salt of ABTS exists in the anionic form as $ABTS^{2-}$ at neutral pH. It undergoes oxidation to form the $ABTS^{\bullet-}$ radical. Scavenging of this radical by punicalagin resulted in its discoloration, which was determined according to the method of Re et al. (*16*).

Pulse Radiolysis Studies. Pulse radiolysis experiments were carried out on a 7 MeV linear electron accelerator at BARC (Forward Industries Ltd.) providing 50 or 500 ns single electron pulses. The pulse irradiates the sample contained in a 1 cm × 1 cm suprasil quartz cuvette. An optical detection system comprising a 450 W xenon arc lamp, lenses, mirrors, and a monochromator monitors the transient changes in absorbance of the solution following electron pulse. The output from the PMT is fed through a DC offset circuit to the "Y" input of an L&T digital oscilloscope (model 4072).

An on-line computer is used for data analysis. Other details of the linear electron accelerator have been given elsewhere (*17*). The absorbed dose was usually ∼12 Gy/pulse as determined by pulsing an aerated 10^{-2} mol dm^{-3} KSCN solution. The formation of $(SCN)_2^{\bullet-}$ radical was monitored at 475 nm. The absorbed dose per pulse was calculated (*18*) by assuming $G\epsilon\ [(SCN)_2^{\bullet-}] = 2.6 \times 10^{-4}\ m^2\ J^{-1}$ at 475 nm.

Water, when subjected to ionizing radiations from a linear electron accelerator, generates e^-_{aq}, $^\bullet OH$, and $^\bullet H$ as the three main primary species besides small amounts of molecular products, the yield of these primary species being $G(^\bullet OH^\bullet) \cong G(e_{aq}^-) = 2.9 \times 10^{-7}$ mol J^{-1} and $G(H^{\bullet\bullet}) = 0.6 \times 10^{-7}$ mol J^{-1} (*19*). The yield of hydroxyl radicals was doubled by irradiating a nitrous oxide (N_2O) saturated aqueous solution, where e_{aq}^- was quantitatively converted to the $^\bullet OH$ radical (reaction 2). Generation of different oxidizing radicals has been extensively covered in the literature (*20*), and hence only a brief mention is made here.

$$H_2O \rightsquigarrow e_{aq}^-,\ ^\bullet OH,\ ^\bullet H,\ H_3O^+,\ H_2, \text{ and } H_2O_2 \qquad (1)$$

$$e_{aq}^- + N_2O + H_2O \rightarrow {}^\bullet OH + OH^- + N_2 \qquad (2)$$

Azide radical ($N_3^\bullet$) was generated by irradiating a N_2O-saturated aqueous solution containing sodium azide, where all of the $^\bullet OH$ radicals were exclusively converted to $N_3^\bullet$ radical, $G(N^\bullet_3) = 0.58 \times 10^{-7}$ mol J^{-1} (reaction 3).

$$^\bullet OH + N_3^- \rightarrow N_3^\bullet + OH^- \qquad (3)$$

$$^\bullet OH + NO_2^- \rightarrow NO_2^\bullet + OH^- \qquad (4)$$

Nitrogen dioxide radical ($NO_2^\bullet$) was generated by the reaction of $^\bullet OH$ radical with nitrite ions (reaction 4).

Cyclic Voltammetric Studies. The reduction potential of punicalagin radical was determined in aqueous solutions by cyclic voltammetric and differential pulse voltammetric techniques. The experiments were carried out using an Eco Chemie make Potentiostat/Galvanostat (Autolab 100). Data acquisition and analysis were made by Autolab-GPES software. Aqueous buffered solutions of samples were placed in a glass cell containing a glassy carbon electrode, a saturated calomel reference electrode, and a platinum auxiliary electrode. The experiments were carried out at 28 °C. Preconditioning of the glassy carbon electrode was carried out prior to every measurement by polishing the surface of the electrode using very fine alumina powder and then rinsing it

thoroughly before use. Aqueous solutions containing 5×10^{-4} mol dm^{-3} punicalagin, 0.1 mol dm^{-3} KCl, and 10^{-3} mol dm^{-3} of phosphate buffer were bubbled with pure N_2 gas prior to each measurement. The sweep rate was 20 mV/s. The electrochemical system was calibrated by carrying out a cyclic voltammetric scan of a Cd(II) solution and a standard potassium ferricyanide solution. All of the potential values were measured with respect to the saturated calomel electrode (SCE) and converted to normal hydrogen electrode (NHE) by suitable corrections.

Cell Lines and Culture Medium. The cell lines, namely, Vero (normal African green monkey kidney cell line), Hep-2 (human larynx epithelial cancer cell line), and A-549 (human small cell lung carcinoma cell line), were obtained from Pasteur Institute of India (Coonoor, India), were cultured with Dulbecco's modified Eagle's medium (DMEM) (HiMedia), and supplemented with 10% heat-inactivated newborn calf serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin maintained at 37 °C in a 5% CO_2 atmosphere with 95% humidity.

Microculture Tetrazolium Assay. Cellular viability in the presence and absence of punicalagin was determined using the 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (*21*). The principle involved is the cleavage of yellow MTT to a blue formazan by mitochondrial enzyme succinate dehydrogenase. The formation of formazan is found to be proportional to the number of viable cells. For the assay, in brief, a monolayer cell culture was trypsinized and the cell count adjusted to 1.0×10^5 cells/mL using a medium containing 10% newborn calf serum. To each of the 96-well microtiter plates was added 0.1 mL of the diluted cell suspension (containing ~10000 cells). After 24 h, the supernatant was flicked off and washed, and 0.1 mL of the test sample (variable concentration) was added to the cell in the microtiter plate. The plates were then incubated at 37 °C for 3 days in a 5% CO_2 atmosphere. After 72 h, the solutions in the wells were discarded, and 50 μL of MTT was added to each well. The plates were gently shaken and incubated for 3 h at 37 °C in a 5% CO_2 atmosphere. The supernatant was removed, 50 μL of propanol was added, and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader, at 540 nm. The absorbance recorded from cells grown in the absence of punicalagin was taken as 100% viable cells (control). The viability was plotted against punicalagin concentration, and the IC_{50} (concentration required to reduce viability by 50%) value against the cell lines was calculated.

Sulforhodamine B (SRB) Assay. The antiproliferative SRB assay (*22*) was performed to assess growth inhibition, which measures the cell number indirectly by staining total cellular protein with dye SRB. In brief, treated and control cells were fixed by layering 0.1 mL of ice-cold 40% trichloroacetic acid (TCA) on the surface of the growth medium. Cells were incubated at 4 °C for 1 h, after which the plates were washed five times with cold water. Excess water was drained off, and the plates were left to dry in the air. SRB stain (0.1 mL, 0.4% w/v in 1% acetic acid) was added to each well and left in contact with the cells for 30 min, after which they were washed with 50 mL of 1% acetic acid and rinsed four times, leaving only the dye adhering to the cells. The plates were dried, and 0.1 mL of 10^{-2} mol dm^{-3} Tris buffer was subsequently added to the wells to solubilize the dye. The plates were shaken vigorously for 5 min, and the absorbance was measured using a microplate reader at 492 nm. IC_{50} was calculated as in the MTT assay. The CTC_{50} value is the average of the IC_{50} of the MTT and SRB assays.

Cell Viability Assay by Trypan Blue Dye Exclusion Assay. The trypan blue dye has the ability to penetrate into dead cells and gives them a blue color. This method gives the exact number of dead and viable cells. The treated and control cells were trypsinized and incubated for 15 min, and then 0.1 mL of growth medium was added to all of the wells. The cells were dispersed and cell suspension was transferred from the wells to Eppendorf tubes. A known aliquot of trypan blue dye was added, and the cell counts were determined using a hemocytometer

RESULTS AND DISCUSSION

Transition metal ions, namely, Mn, Fe, Ni, and Cu, combine with O, N, and S donor atoms via electron transfer. In flavanols

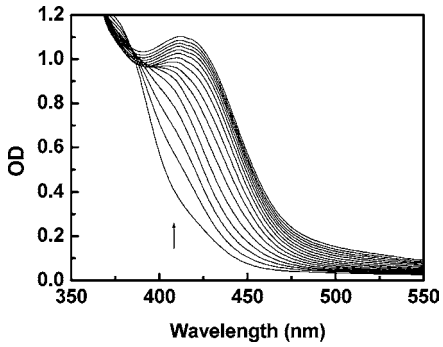

Figure 2. Change in UV–vis absorption spectra of aqueous solution containing 10^{-3} mol dm^{-3} phosphate buffer, 5×10^{-4} mol dm^{-3} punicalagin, and (0, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 400) $\times 10^{-6}$ mol dm^{-3} $CuCl_2$, pH 7.5, at 28 °C.

the phenolic groups especially of the catechol B-ring are responsible for metal chelating activity. Due to the polyphenolic structure of the flavonoids it is suggested that their antioxidant activity is due to two possible modes of action, free radical scavenging and metal chelation (*23*).

It is also well recognized that serum albumin is a major transport protein that has the ability to bind with drugs, antioxidants (*24*), metabolites, and unesterified fatty acids. Previous studies have shown that quercetin conjugates with albumin, even in the bound state, and retains its antioxidant properties (*25*). Recently, Labieniec and Gabryelak (*26*) have found that conformational changes occur in DNA as a result of intercalation of ellagic acid and gallic acid with calf thymus DNA. Keeping this in mind and in view of the fact that punicalagin also contains gallate moieties, the following studies were carried out.

Binding Properties of Punicalagin. Many polyphenols are known to show metal complexing ability, which is similar to what one observes in the case of proteins. It has been well-established that transition metal ions such as, iron, copper, and chromium in the presence of H_2O_2 generate highly oxidizing $^{\bullet}OH$ radicals (reaction 5) that are capable of oxidizing DNA

$$Fe^{2+}\ H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \qquad (5)$$

(*27*). Absorption of metal ions in humans is markedly reduced in the presence of polyphenols (*28*). Currently, desferrioxamine (*29*) is a drug useful for the treatment of iron overload. Because punicalagin is an active ingredient of pomegranate fruit, biologically compatible and having 16 −OH groups, it was thought to be worthwhile to assess its iron-binding ability at neutral pH. Selection of iron and copper is mainly based on the fact that iron is required for oxygen and carbon dioxide transport in blood and is bound to transferrin, whereas copper is a cofactor of many enzymes.

Chelation of iron and copper ions with punicalagin was evaluated at room temperature by the addition of 5 μL aliquots of 10^{-2} mol dm^{-3} Fe(II)/Cu(II) to 2 mL of 5×10^{-4} mol dm^{-3} punicalagin, containing 10^{-3} mol dm^{-3} phosphate buffer (pH 7.5). The M/L ratio was determined when the intensity of absorbance reached saturation, at the wavelength of maximum absorption.

Figure 2 shows the spectra of punicalagin complex at pH 7.5, in the presence of different concentrations [$(0.25-4) \times 10^{-4}$ mol dm^{-3}] of copper(II). Addition of copper(II) ions to punicalagin solution results in the oxidation of −OH groups of punicalagin to form a semiquinone type of radical and a consequential reduction of copper(II) to Cu(I) (*30*). The puni-

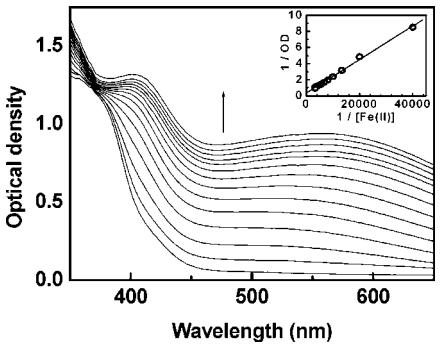

Figure 3. Change in UV–vis absorption spectra of aqueous solution containing 10^{-3} mol dm^{-3} phosphate buffer, 5×10^{-4} mol dm^{-3} punicalagin, and (0, 50, 100, 150, 200, 250, 300 350, 400, 450, 500, 550, 600) $\times 10^{-6}$ mol dm^{-3} ferrous ammonium sulfate, pH 7.5, at 28 °C. (Inset) Double-reciprocal plot of optical density versus iron(II) concentration at 550 nm.

calagin *o*-semiquinone can be further oxidized to *o*-quinone (*31*). Chelation of the metal ions occurs with the oxygen atoms of *o*-quinone and results in stabilization of the complex having an absorption maximum at 411 nm. Using Job's method, the metal/ligand ratio was found to be 1:1. A small red shift of ~7 nm was observed with increasing Cu(II)/punicalagin molar ratios.

By keeping the concentration of the ligand constant and by varying the concentration of metal ion, it was possible to determine the equilibrium constants. Equilibrium constants of a 1:1 M/L complex were determined from the following relationship (eq IV):

$$\mathrm{M} + \mathrm{L} = \mathrm{ML} \qquad \text{(I)}$$

$$K = \frac{[\mathrm{ML}]}{[\mathrm{M}][\mathrm{L}]} \qquad \text{(II)}$$

where $[\mathrm{M}]_0 = [\mathrm{M}] + [\mathrm{LM}]$ and $[\mathrm{L}]_0 = [\mathrm{L}] + [\mathrm{LM}]$, K is the equilibrium constant (expressed as logarithm), M is the amount of free metal ion, L is the amount of the free ligand such as phenol, $[\mathrm{M}]_0$ is the total concentration of the metal ion, and $[\mathrm{L}]_0$ is the total concentration of the ligand.

$$K = \frac{[\mathrm{ML}]}{([\mathrm{L}]_0 - [\mathrm{ML}])([\mathrm{M}]_0 - [\mathrm{ML}])} \qquad \text{(III)}$$

The equilibrium constant K for a 1:1 complex was determined by employing the Benesi−Hildebrand type of eq IV (*32*)

$$\frac{1}{[\Delta \mathrm{A}]} = \frac{1}{K\Delta\epsilon[\mathrm{M}]_0[\mathrm{L}]_0} + \frac{1}{\Delta\epsilon[\mathrm{M}]_0} \qquad \text{(IV)}$$

where $\Delta\epsilon$ is the change in the extinction coefficient at the wavelength of measurement. From the linear plot of $1/\Delta$A versus 1/[M], with slope $= 1/K\Delta\epsilon[\mathrm{L}]_0$, the value of K, the equilibrium constant, was determined to be 1.8×10^7 dm^3 mol^{-1}. This value is quite low but compares fairly with the binding constant of Cu(II)− with salicylaldehyde and β-phenylalanine (*33*).

The blue iron(II)−punicalagin complex (**Figure 3**) was formed in a similar manner to the Cu−punicalagin complex and showed two maxima, one at 400 nm and another one at 555 nm. The peak at 555 nm is attributed to a metal to ligand charge-transfer band. A similar blue complex ($\lambda = 555$ nm) was also observed in the case of metal complexes with green tea polyphenols (*34*). The metal/ligand ratio at this pH 7.5 was 1:1, and the equilibrium constant for the complex was $K = 1 \times 10^7$ dm^3 mol^{-1}, calculated from the double-reciprocal plot of optical density versus metal ion concentration (inset of **Figure 3**). This value is slightly higher than that reported by Reddan et al. (*35*) for a 1:1 Fe(II)/nPG complex but comparable to that for the 1:1 Cu(II)/punicalagin complex. It was previously shown (*13*) that compared to the reduction potential of the ligand the reduction potential of the complex (iron/copper) shows a greater shift to more cathodic potentials, when present in the same environment, thus indicating that the complexes can act as better reducing agents. Furthermore, pulse radiolysis studies had shown that the Fe(II)/*n*-propyl gallate complex formed in aqueous solutions at pH 9 showed good superoxide radical scavenging ability (*13*). It was thus expected that the Fe/Cu/punicalagin complexes when used under ideal metal/ligand ratios might show equivalent if not better $O_2^{\bullet -}$ radical scavenging properties because punicalagin has four moieties of *n*-propyl gallate. However, pulse radiolysis studies (pH 9) had shown that the rates were very low, possibly $<10^6$ dm^3 mol^{-1} s^{-1}. This shows that the structural conformation plays a significant role in superoxide activity.

Binding of Punicalagin to BSA. Albumin is an important protein present in the blood plasma of humans, the concentration being 3.1−4.3 g/dL. This constitutes about 50% of the total proteins in the blood. Albumin can bind with bilirubin, drugs, and diverse organic compounds. Fluorescence quenching of protein could be used to retrieve much drug protein binding information. Hence, the interaction of punicalagin with BSA was measured by the fluorescence quenching of the BSA by punicalagin. To a freshly prepared aqueous 10^{-3} mol dm^{-3} Tris-HCl buffer solution, pH 7.4, was added 1.3×10^{-6} mol dm^{-3} BSA. A 2.5 mL aliquot of the above solution was taken in a quartz cuvette, maintained at 27 °C, and titration was carried out by a stepwise addition of aqueous Tris-HCl buffered punicalagin solution of 1×10^{-3} mol dm^{-3} in the concentration range of $(0-25) \times 10^{-5}$ mol dm^{-3}. A constant lag time was maintained between two additions. Keeping the excitation wavelength at 295 nm, the fluorescence emission spectrum was recorded from 300 to 400 nm. BSA used in the present study contains two tryptophanyl residues (Trp-135 and Trp-214) that act as fluorophors in BSA. At the excitation wavelength of 280 nm, both tryptophan and tyrosine amino acid residues in protein show fluorescence emission, whereas excitation at 295 nm results in fluorescence by tryptophan only. The presence of a large number of hydroxyl groups makes punicalagin hydrophilic in nature; hence, it will tend to seek hydrophilic sites present in protein. Addition of punicalagin to a BSA solution resulted in quenching of BSA fluorescence ($\lambda_{max} = 343$ nm). As mentioned above, the time interval between each addition and measurement was short, indicating that the equilibrium was attained quickly. Although the fluorescence quenching spectra did not show any marked shift (~4 nm) in the fluorescence maximum on the addition of punicalagin, it does suggest that as a result of antioxidant−BSA binding there is a change in the microenvironment of tryptophan residues present in BSA. These changes were used to determine the binding constant of punicalagin with BSA.

Figure 4 shows the percentage quenching of BSA fluorescence ($\lambda_{ex} = 295$ nm) on the addition of punicalagin. The quenching percentage reflects the energy transfer from the excited state of tryptophan residue of BSA to punicalagin, present in the ground state, and this was found to be concentration dependent. It was observed that saturation in the quenching was achieved with a 10-fold increase in punicalagin concentration over BSA concentration.

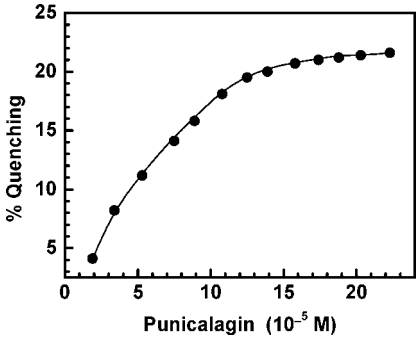

Figure 4. Plot of percentage fluorescence quenching of BSA in aqueous solution on the stepwise addition of punicalagin at pH 7, 27 °C, and λ_{ex} = 295 nm. (Matrix) To 2.5 mL of 1.3×10^{-6} mol dm^{-3} BSA in 10^{-3} mol dm^{-3} Tris buffer was added aqueous punicalagin solution in the concentration range of (0–25) $\times$ 10^{-5} mol dm^{-3}.

Quenching of the relative fluorescence intensity of BSA by punicalagin was analyzed in terms of the binding of punicalagin to protein using an established method (*36*, *37*) with the assumption that the binding of each molecule of punicalagin causes the same degree of fluorescence quenching. The percentage quenching of the fluorescence intensity of proteins by punicalagin was corrected empirically for internal absorption and filtration by subtracting the percentage quenching of the fluorescence of *N*-acetyltryptophan amide by the same concentration of punicalagin, equivalent in absorption to protein at 295 nm. Quenching studies show that the quencher molecules are present in close proximity to the tryptophan molecules present in BSA and contribute to the fluorescence of BSA.

The equilibrium constant, *K*, is given by eq V

$$K = \frac{\beta}{1-\beta} \times \frac{1}{Cf} \qquad \text{(V)}$$

where $\beta = Q/Q_{max}$. Q_{max} is maximum quenching, which was determined by extrapolation of a double-reciprocal plot of Q against C_T to intercept. C_T is the total concentration of punicalagin added.

$$Cf = C_T - n\beta T \qquad \text{(VI)}$$

The value of K is given by the slope of a plot of $\beta/(1-\beta)$ against Cf; the free ligand concentration was found to be 2.7×10^5 dm^3 mol^{-1}. This value seems to be quite plausible when compared with the binding constant values of ferulic acid and chlorogenic acid with HSA (*38*), which are in the range of (2−4) $\times$ 10^4 dm^3 mol^{-1}, and the binding constant of sulfasalazine (*39*) with native BSA is 3×10^6 dm^3 mol^{-1}. The binding of punicalagin to albumin could be due to hydrogen-bonding interactions at hydrophilic sites.

From a physiological point of view binding of punicalagin to BSA is an important phenomenon, because it could enhance the body's total antioxidant status when the complex acts as a free radical trap in circulating body fluid.

Binding of Punicalagin with DNA. The double-helical DNA is the most important biomolecule containing stacked base pairs that can be considered as a conjugated π-electron system (*40*) and helps in charge transfer. Current interest is focused on finding efficient DNA-binding agents with a view to develop new chemotherapeutic and antiviral drugs.

For determining the binding of punicalagin to DNA all measurements were made in 1 cm quartz cells at 27 °C. Titrations were performed using 1 mL aliquots, containing 50 μL of 1 mg /mL of DNA and 950 μL of Tris buffer-HCl at pH 7.4. To 1 mL of this solution was added 10 μL aliquots of 5×10^{-4} mol dm^{-3} punicalagin, and the spectral changes were recorded after each addition, keeping the time interval constant. The spectral changes were recorded on stepwise addition of punicalagin to DNA solution. We did not observe any significant shift in the absorption maximum as a consequence of binding between punicalagin and DNA. The equilibrium constant value (K = 45 dm^3 mol^{-1}) calculated form these data was quite low and is suggestive of nonspecific interactions.

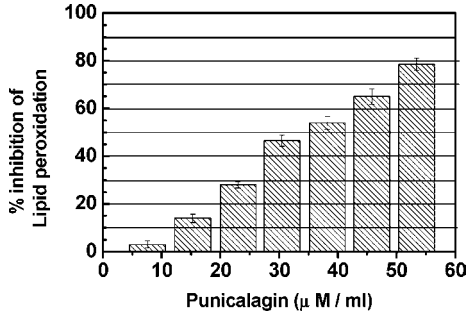

Figure 5. Antioxidant activity of punicalagin in inhibiting lipid peroxidation in a dose-dependent manner. Values are expressed as means ± SD from at least three determinations.

Inhibition of Lipid Peroxidation. Lipid peroxidation is a free radical mediated propagation of oxidative insult to polyunsaturated fatty acids, involving several types of free radicals, and termination occurs through enzymatic means or by scavenging of free radicals in the presence of antioxidant (*41*). Oxidation of low-density lipids (LDL) has been implicated in inflammatory and vascular diseases. Addition of antioxidants to LDL is known to inhibit their oxidation (*42*). To evaluate the antioxidant activity of punicalagin (to inhibit the lipid peroxidation in biological systems), a rat liver microsomal system was used. The concentration of punicalagin was varied from 10 to 100 μM/mL. **Figure 5** shows that inhibition of lipid peroxidation was dependent on the concentration of punicalagin. The IC_{50} value (amount of punicalagin required to inhibit peroxidation by 50%) was determined to be 36.8 ± 0.6 μg/mL in comparison to an IC_{50} value of ~86.32 ± 1.5 μg/mL for BHA, a reference antioxidant used in lipid peroxidation experiments. These results indicate that punicalagin is about twice as effective as BHA in inhibiting lipid peroxidation; hence, it acts as a good antioxidant even at very low concentrations. It is pertinent to mention here that control experiments showed that punicalagin itself did not interfere with the TBARS measurements.

The aforementioned results showed that punicalagin forms a chelate with metal ions. Thus, the protective effect of punicalagin against lipid peroxidation might be due to its chelation properties as well as due to radical scavenging. To substantiate this, the scavenging activity of punicalagin against the stable ABTS radical anion was also measured, as the assay results are known to show correlation with the degree of protection of lipid peroxidation activities of these test compounds. The ABTS method is useful for determining the total radical scavenging activity of antioxidants, especially from plant extracts.

The diammonium salt of ABTS exists in the anionic form as $ABTS^{2-}$ at neutral pH. It undergoes oxidation to form the $ABTS^{\bullet-}$ radical. Scavenging of this radical by punicalagin resulted in its discoloration, which was determined according to the method of Re et al. (*16*). Briefly, to a 50 mL aqueous solution of 2×10^{-3} mol dm^{-3} $ABTS^{2-}$ containing 10^{-3} mol dm^{-3} phosphate was added 0.2 mL of 7×10^{-2} mol dm^{-3} potassium persulfate, and the solution was kept in the dark for 24 h (27 °C), which resulted in the formation of $ABTS^{\bullet-}$ radical

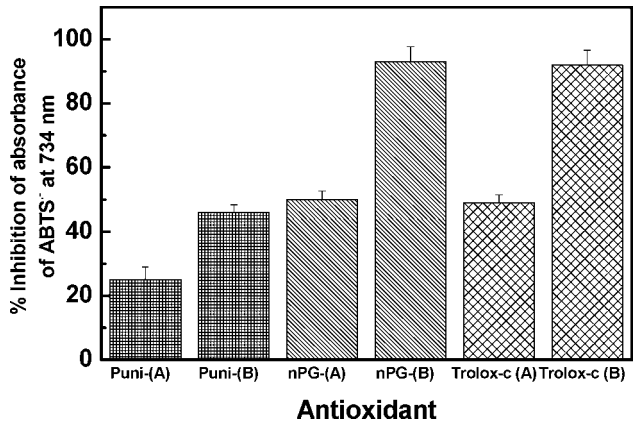

Figure 6. Total ABTS•− radical scavenging capacity of punicalagin, *n*-propyl gallate, and Trolox-c: (A) 50 μL and (B) 100 μL of 5×10^{-4} mol dm^{-3} punicalagin, *n*-propyl gallate, or Trolox-c. Values are expressed as means ± SD from at least three determinations.

having an absorption maximum at 734 nm. This solution was appropriately diluted to yield an initial absorbance of ~0.8. The radical scavenging activity of punicalagin was assessed by adding 0.05 or 0.1 mL of 5×10^{-4} mol dm^{-3} punicalagin to 2 mL of appropriately diluted buffered ABTS•− solution. The resulting change in the absorbance of ABTS•− solution due to discoloration after 6 min of addition of antioxidant was monitored, and the results were compared with that of *n*-propyl gallate (nPG) and Trolox-c, using equimolar concentrations of both antioxidants and under similar experimental conditions. All determinations were run in triplicate. An appropriate solvent blank was run with each assay. The antioxidant capacity was expressed as percent inhibition of absorbance of ABTS•− radical at 734 nm. **Figure 6** shows the percent decrease in the absorbance of ABTS•− radical upon addition of 50 and 100 μL of either punicalagin, nPG, or Trolox-c. It can be seen that both nPG and Trolox-c have almost identical radical scavenging abilities, whereas that of punicalagin is nearly half that of nPG and Trolox-c. Thus, it appears that punicalagin inhibits lipid peroxidation more significantly by binding with metal ions. However, the protection effect due to a free radical scavenging mechanism cannot be ruled out completely. This was more evident from the subsequent studies carried out using pulse radiolysis technique.

Pulse Radiolysis Studies. This is an important in vitro technique to determine the reactivity of compounds with free radicals, namely, •OH, $N_3^•$, $NO_2^•$, and NO•, and also to study the repair of DNA and proteins.

(a) Free Radical Reactions with Punicalagin. Pulse radiolysis of N_2O-saturated aqueous solution containing 5×10^{-2} mol dm^{-3} sodium azide, 10^{-3} mol dm^{-3} phosphate buffer, and 5×10^{-4} mol dm^{-3} punicalagin at pH 6.0 resulted initially in the formation of azide radical ($N_3^•$) (reactions 1−3). The $N_3^•$ is a specific one-electron oxidant, having a very narrow absorption band with $\lambda_{max} = 274$ nm. This radical subsequently reacts with the substrate to generate the punicalagin phenoxyl radical (reaction 6), having an absorption maximum at 425 nm (**Figure 7**), which is similar to that of nPG, reported by Deeble et al. (*43*).

$$N_3^• + \text{Pun-(OH)}_{16} \rightarrow N_3H + \text{Pun-(OH)}_{15}O^• \qquad (6)$$

As reported in the case of tea polyphenols (*44*), the azide radical can oxidize more than one phenolic site (i.e., moiety of

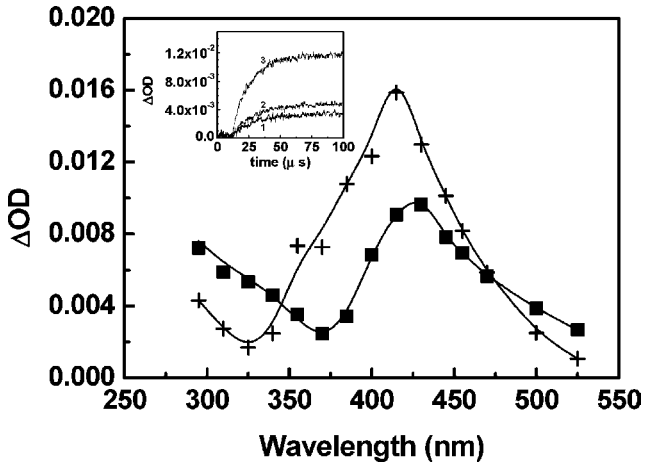

Figure 7. Transient time-resolved spectra of the semioxidized species formed by the reaction of azide radicals with punicalagin at (■) 5 and (+) 15 μs after the pulse. (Matrix) N_2O-saturated aqueous solution containing 5×10^{-2} mol dm^{-3} sodium azide, 10^{-3} mol dm^{-3} phosphate buffer, and 5×10^{-4} mol dm^{-3} punicalagin at pH 6.0. (Dose = 12 Gy/pulse.) (Inset) Formation of absorbances at 425 nm at (1) 10, (2) 20, and (3) 50×10^{-6} mol dm^{-3} punicalagin concentration.

n-propyl gallate) of punicalagin, resulting in the formation of a mixture of phenolic radicals, which on further transformation leads to the formation of a phenoxyl radical with lowest reduction potential. The rate constant for the formation of transient species was obtained by plotting the pseudo-first-order rates obtained from the build-up traces at 425 nm for different substrate concentrations versus the substrate concentration and was found to be 4.7×10^8 dm^3 mol^{-1} s^{-1}. This value is quite comparable with some of the reported values for the azide radical reaction with some phenolic derivatives at pH 5.8 (*45*). Because punicalagin phenoxyl radical is stable over a long time scale, it was not feasible to determine its decay rate.

Among the various ROS, the •OH radical is the most reactive oxidant and is also one of the most predominant ROS. Hence, the effective scavenging of this radical is of utmost importance from the point of view of various health perspective reasons. The hydroxyl radical is generated by the radiolysis of water (reactions 1 and 2) and can also be generated endogenously during aerobic metabolism. It is a strong electrophile and can react with aromatic substrates by addition to the phenolic ring, by electron transfer from the −OH group, or by abstraction of an H atom. Pulse radiolysis of N_2O-saturated 10^{-3} mol dm^{-3} phosphate buffer and 5×10^{-4} mol dm^{-3} punicalagin at pH 6.0 showed an absorption spectrum similar to that observed in azide radical reaction and attributed to the formation of punicalagin phenoxyl radical (reaction 7).

$$\text{Pun-(OH)}_{16} + {}^•OH \rightarrow \text{Pun-(OH}_{15})O^• + H_2O \qquad (7)$$

Formation of the above transient was exponential with time, which gave the pseudo-first-order rate constants. When the pseudo-first-order rate constants were plotted versus the solute concentration [punicalagin], the bimolecular rate constant for the reaction was found to be 2.5×10^9 dm^3 mol^{-1} s^{-1}, showing the high reactivity of punicalagin with •OH radicals.

The $^•NO_2$ radical is a moderate one-electron oxidant ($E^1 = 1$ V vs NHE) that can damage lipids (*46*) and can undergo addition reaction, resulting in nitration of tyrosine (*47*), a constituent of proteins. It is found in cigarette smoke and exhausts from vehicles. Therefore, its reactivity with punicalagin was also studied. The transient spectrum was recorded by pulsing N_2O-

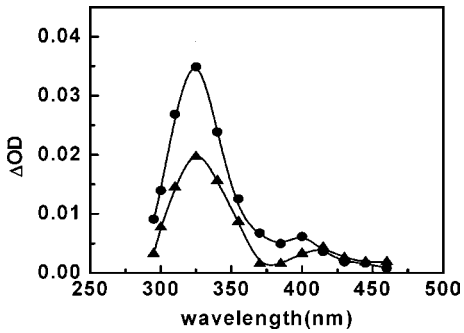

Figure 8. Transient time-resolved spectra of the semioxidized species formed by the reaction of $NO_2^{\bullet}$ radicals with punicalagin at (▲) 5 and (●) 15 μs after the pulse. (Matrix) N_2O-saturated aqueous solution containing 5×10^{-2} mol dm^{-3} sodium nitrite, 10^{-3} mol dm^{-3} phosphate buffer, and 5×10^{-4} mol dm^{-3} punicalagin at pH 7.0. (Dose = 12 Gy/pulse.)

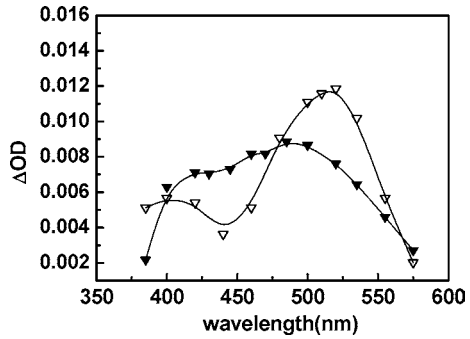

Figure 9. Transient absorption spectrum attributed to the formation of (▽) (Trp$^{\bullet}$) radical on pulsing N_2O-saturated aqueous solution containing 5×10^{-2} mol dm^{-3} sodium azide and 10^{-3} mol dm^{-3} phosphate buffer and of tryptophan, in the absence of punicalagin, pH 7, and at 80 μs after pulse, (▼) in the presence of 8×10^{-5} mol dm^{-3} punicalagin, pH 7, and at 80 μs after pulse. (Dose = 12 Gy/pulse.)

saturated aqueous solution containing 5×10^{-2} mol dm^{-3} sodium nitrite, 10^{-3} mol dm^{-3} phosphate buffer, and 5×10^{-4} mol dm^{-3} punicalagin at pH 7.0 obtained by the reaction of $^{\bullet}NO_2$ radical with punicalagin as shown in **Figure 8**. The absorption maximum at ~325 nm is probably due to the addition of $^{\bullet}NO_2$ radical with the substrate, which at longer time results in a growth at ~400 nm, that is, formation of punicalagin phenoxyl radical. The bimolecular rate constant for the above reaction was calculated to be 1×10^9 dm^3 mol^{-1} s^{-1} by plotting the pseudo-first-order rate constants for at least four different concentrations of punicalagin in the range of $(1-6) \times 10^{-5}$ mol dm^{-3}.

(b) Repair Reactions of Punicalagin. (1) Repair of ABTS Radicals. When N_2O-saturated aqueous solution containing 5×10^{-2} mol dm^{-3} sodium azide, 10^{-3} mol dm^{-3} phosphate buffer, and 2×10^{-3} mol dm^{-3} $ABTS^{2-}$ at pH 7.0 was pulse irradiated, a characteristic absorption was observed with λ_{max} = 417, 645, and 728 nm, which is well characterized and attributed to the formation of one-electron oxidized radical $(ABTS)^{\bullet-}$ (*48*). In the additional presence of $(0.5-5) \times 10^{-5}$ mol dm^{-3} punicalagin, the absorbance at 728 nm showed a decay that increased with increasing punicalagin concentration (reactions 8 and 9). It might be noted that at this wavelength, there is no interference from the punicalagin radicals. Hence, by plotting the pseudo-first-order rate constants versus punicalagin concentration the bimolecular rate constant for the repair of $ABTS^{\bullet-}$ radical by punicalagin was found to be 8.8×10^6 dm^3 mol^{-1} s^{-1}. This value compares well with the repair of $ABTS^{\bullet-}$ radical by ascorbate (*49*) ($k = 8 \times 10^6$ dm^3 mol^{-1} s^{-1}).

$$N_3^{\bullet} + ABTS^{2-} \rightarrow ABTS^{\bullet-} + N_3^- \quad (8)$$

$$ABTS^{\bullet-} + \text{Pun-(OH)}_{16} \rightarrow ABTS^{2-} + \text{Pun-(OH)}_{15}O^{\bullet} + H^+ \quad (9)$$

(2) Repair of Tryptophan Radicals. We have shown earlier that punicalagin is able to bind with BSA. To show that the bound punicalagin can bring about repair of any damage to proteins, we have used tryptophan as a model amino acid. Pulse radiolysis is a convenient method to generate tryptophan radicals. For this, initially the azide radical was produced via reactions 1−3 by irradiating a N_2O-saturated aqueous solution containing 5×10^{-2} mol dm^{-3} sodium azide, 10^{-3} mol dm^{-3} phosphate buffer, and 3×10^{-3} mol dm^{-3} tryptophan, pH 7. The $N_3^{\bullet}$ radical generated subsequently reacts with tryptophan (reaction 9) to form the tryptophan radical (Trp$^{\bullet}$), which has a well-characterized $\lambda_{max} \sim 510$ nm (*50*). In the additional presence of $(0.5-4) \times 10^{-5}$ mol dm^{-3} punicalagin the tryptophan radical was scavenged as evidenced by the decrease in the absorption at 510 nm, and a consequential increase in the absorbance at 440 nm (**Figure 9**) is due to the formation of punicalagin phenoxyl radical.

$$N_3^{\bullet} + TrpH \rightarrow Trp^{\bullet} + N_3^- \quad (10)$$

$$Trp^{\bullet} + \text{Pun-(OH)}_{16} \rightarrow TrpH + \text{Pun-(OH)}_{15}O^{\bullet} \quad (11)$$

By a proper choice of experimental conditions, such that the reactivity of azide radical with tryptophan was ≥25, the rate constants for the repair of tryptophan radical (reaction 11) was determined from the decay rate of the tryptophan radical (λ = 520 nm) with increasing punicalagin concentration, as well as from the formation of punicalagin radical, as a consequence of repair of tryptophanyl radical at 440 nm were 1.5 and 1.7×10^8 dm^3 mol^{-1} s^{-1}, respectively. Both the rates showed good agreement.

(3) Repair of Guanosine Base Radicals. Although our study has shown that binding of punicalagin to DNA is rather poor, this does not preclude the possibility of its repairing DNA base radicals. The guanosine radical (*51*) is a moderately strong oxidant ($E^1 = 1.29$ V). Also, earlier studies have shown that various phytochemicals have the potential to reduce the oxidized forms of DNA base (*52*). Hence, an attempt has been made to study the repair of guanosine radicals.

Pulse radiolysis of an N_2O-saturated aqueous solution of 5×10^{-3} mol dm^{-3} Tl(I) and 3×10^{-4} mol dm^{-3} guanosine (R-G) at pH ~8.0 results in the initial generation of Tl(II) ion (reaction 12). The Tl(II) ion is a strong oxidant ($E^1 = 2$ V) and oxidizes guanosine to initially form a guanosine radical cation, $k = 1.3 \times 10^9$ dm^3 mol^{-1} s^{-1}. In neutral solutions, the cation radical is rapidly deprotonated (*50*) to yield a neutral radical (reactions 13 and 14). In the additional presence of $(0.8-2) \times 10^{-5}$ mol dm^{-3} punicalagin, a decay of guanosine radical (reaction 15) was observed at 520 nm. The rate constant for the electron-transfer reaction was 2.5×10^7 dm^3 mol^{-1} s^{-1}. This value seems to be a bit slower than the rate constant for

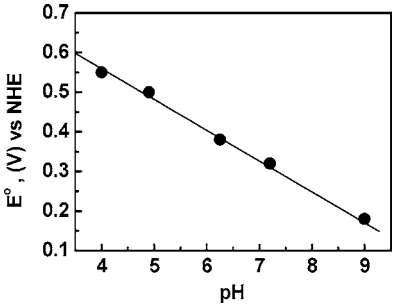

Figure 10. Effect of pH on the dependence of potential (E^0) of punicalagin. (Matrix) Punicalagin (5×10^{-4} mol dm^{-3}), 10^{-3} mol dm^{-3} phosphate buffer, and 10^{-1} mol dm^{-3} KCl (pH 7.5) at 28 °C.

the repair of oxidized guanosine radical by uric acid (*53*).

$$\mathrm{Tl(I)} + {}^{\bullet}\mathrm{OH} \rightarrow \mathrm{Tl(II)} + \mathrm{OH}^- \quad (12)$$

$$\mathrm{Tl(II)} + \mathrm{R\text{-}G} \rightarrow \mathrm{R\text{-}G}^{\bullet+} + \mathrm{Tl(I)} \quad (13)$$

$$\mathrm{R\text{-}G}^{\bullet+} \rightleftarrows \mathrm{R\text{-}G(-H)} + \mathrm{H}^+ \quad (14)$$

$$\mathrm{R\text{-}G(-H)} + \mathrm{Pu\text{-}(OH)_{15}\,OH} \rightarrow \mathrm{R\text{-}G} + \mathrm{Pu\text{-}(OH)_{15}O}^{\bullet} \quad (15)$$

The above repair studies using ABTS, tryptophan, and guanosine demonstrate the antioxidant action of punicalagin in bringing about the restitution process at near physiological pH values is by electron transfer.

Cyclic Voltametric Experiments. The antioxidant action of punicalagin can be assessed from the ease with which they can donate an electron to an oxidizing radical in aqueous solution. To investigate this, a differential pulse voltammetric technique was employed to transfer electrons from punicalagin to a glassy carbon electrode in aqueous media at different pH values.

The differential pulse voltammograms (DPV) obtained for punicalagin solutions at different pH values were well resolved and were carried out with pulse amplitudes of 25 mV. E^0 was calculated from $E^0 = (E_p + E_h)/2$, where E_p is the voltammetric peak potential and E_h is the applied pulse amplitude. **Figure 10** shows a linear plot of peak potential E^0 versus solution pH over the range of 4−9. Furthermore, because there was no change in the slope of the line over the pH range of 4−9, one can conclude that the protons are still participating in the oxidation process over this range. From the above figure one concludes that the compound can be more easily oxidized at near-neutral pH than at a lower pH of 4. The reduction potential of punicalagin radical at pH 7 was $E_7 = 0.32 \pm 0.20$ V versus NHE and was found to be lower than that of gallocatechin radical at pH 7, namely, ∼0.43 V versus NHE, and comparable to that of quercetin radical, $E_7 = 0.32 \pm 0.20$ V versus NHE (*54*). A lower reduction potential of punicalagin radical in comparison to that of gallocatechin radical suggests that the former is a better electron donor than gallocatechin.

In Vitro Assay for Cytotoxicity of Punicalagin. A critical module in the characterization of a functional molecule is to assess the safety of the candidate in a series of acute and subacute toxicity assays. An ideal antioxidant molecule should exert antioxidant properties in a concentration range that has no adverse or toxic effect on human health. As a model, the simplest method to determine the toxicity of a molecule is in vitro cytotoxicity studies against the animal cell lines cultured under laboratory conditions. It is well-known that different cell lines exhibit different sensitivities to a chemical compound. Therefore, the use of more than one cell line was considered to be necessary in assessing the cytotoxicity of punicalagin. Therefore, punicalagin was tested against three different cell lines, namely, Vero, Hep-2, and A-549, which are from different origins and possess different morphologies and tumorigenic properties. A linear increase in cytotoxic activity was observed in relation with concentration gradient of punicalagin against all three cell lines tested. The CTC_{50} values were about 460.8 ± 21, 740 ± 12, and 830 ± 18 μM against Vero, Hep-2, and A-549 cell lines, respectively (**Table 1**). Results reveal that punicalagin exhibits very low cytotoxicity. It is interesting to note that the constituent molecules of punicalagin, namely, ellagic acid and gallic acid, are both reported to be toxic at much lower concentration, that is, in the range of 1−185 μM, against digestive cells of the freshwater mussel *Unio tumidu* (*55*). The wide variation in the cytotoxicity may be attributed to both the structural variation in a molecule as well as cell lines used in the present study. For example, in a study on the cytotoxicity of phenolics, namely, quercetin and rutin, the two pharmacologically well-studied antioxidant phenolics revealed that quercetin at higher doses (50−100 μM) was cytotoxic; on the contrary, rutin had no cytotoxic effect at any dose on human hepatoma cell line (HepG2) (*56*). Gupta et al. (*57*) reported that tea polyphenols produce cytotoxicity only at high concentrations. Although many reports are available on the cytotoxicity of phenolics, their modes of action are not yet well established. Damianaki et al. (*58*) have proposed that protein binding ability, in particular, membrane protein of cell lines, may be the cause that affects the cell growth and its viability. The binding of punicalagin to BSA may to some extent be viewed as a process occurring on similar lines as mentioned above. However, variation in the cytotoxic activity of punicalagin against different cell lines suggests its nonspecific type of toxicity, and its mode of action is, hence, as yet inconclusive. However, this kind of investigation allows us to apply these functional phytochemicals in treatments using their beneficial properties at concentrations that have no toxic effect.

Table 1. Cytotoxicity Activity of Punicalagin against Three Cell Lines

cell line	cytotoxicity of punicalagin (CTC_{50}, μM)
Vero	460 ± 21
Hep-2	740 ± 12
A-549	830 ± 18

Conclusion. It is believed that the redox active iron present endogenously exists in the bound state to proteins and is hence unavailable for initiating $^{\bullet}$OH radical mediated oxidative reactions in biological systems, especially in the presence of hydrogen peroxide. However, under certain pathological conditions, its presence in the free state may prove to be deleterious. The chelating ability of punicalagin toward transition metal ions such as iron and copper, although not sufficiently large enough in comparison to that of desferrioxamine, shows that it may still offer protection against H_2O_2-induced damage to DNA/proteins. The results obtained by in vitro studies demonstrated the ability of punicalagin to scavenge a variety of ROS and RNS known to produce disease status through cellular damage and point out its protective function. The reduction potential of punicalagin at pH 7 is quite low. The present in vitro studies have shown not only that ROS such as $^{\bullet}$OH and $NO_2^{\bullet}$ radicals are scavenged at pH 7 but also that the punicalagin antioxidant shows good ability to transfer electrons to model compounds such as $ABTS^{\bullet-}$, guanosine, and tryptophan radicals and also to oxidized lipids, thereby bringing about their restitution. The binding constant value of punicalagin with BSA is relatively low when compared to that of Cu-EDTA ($K \cong 10^{18}$ dm^3 mol^{-1}),

but it nevertheless does suggest that it is biologically available to scavenge ROS in addition to endogenously present antioxidant defense in humans. The cytotoxicity studies revealed that this molecule is not toxic at low concentrations. Thus, scavenging of ROS as well as binding to metal ions are the two pathways by which punicalagin acts as an antioxidant. The above properties along with its water-soluble nature suggest that it can act as a good antioxidant in living systems to fight oxidative stress. These factors make it a probable candidate as a nutraceutical molecule possessing promising health-beneficial properties.

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