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Pomegranate peel extract prevents liver fibrosis in biliary-obstructed rats

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Abstract

Punica granatum L. (pomegranate) is a widely used plant that has high nutritional value. The aim of this study was to assess the effect of chronic administration of pomegranate peel extract (PPE) on liver fibrosis induced by bile duct ligation (BDL) in rats. PPE (50 mg kg^{-1}) or saline was administered orally for 28 days. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) levels were determined to assess liver function and tissue damage. Pro-inflammatory cytokines (tumor necrosis factor-alpha and interleukin 1 beta) in the serum and antioxidant capacity (AOC) were measured in plasma samples. Samples of liver tissue were taken for measurement of hepatic malondialdehyde (MDA) and glutathione (GSH) levels, myeloperoxidase (MPO) activity and collagen content. Production of reactive oxidants was monitored by chemiluminescence assay. Serum AST, ALT, LDH and cytokines were elevated in the BDL group compared with the control group; this increase was significantly decreased by PPE treatment. Plasma AOC and hepatic GSH levels were significantly depressed by BDL but were increased back to control levels in the PPE-treated BDL group. Increases in tissue MDA levels and MPO activity due to BDL were reduced back to control levels by PPE treatment. Similarly, increased hepatic collagen content in the BDL rats was reduced to the level of the control group with PPE treatment. Thus, chronic PPE administration alleviated the BDL-induced oxidative injury of the liver and improved the hepatic structure and function. It therefore seems likely that PPE, with its antioxidant and antifibrotic properties, may be of potential therapeutic value in protecting the liver from fibrosis and oxidative injury due to biliary obstruction.

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Introduction

Flavanoids are natural products widely distributed in the vegetable kingdom and consumed regularly in moderate amounts by man through his daily diet. There is considerable evidence that flavanoids interact with various biological systems (Di Carlo et al 1999). Epidemiological studies have demonstrated that consumption of phenol-rich food retards the progression of arteriosclerosis and reduces the incidence of heart disease by preventing oxidative stress (Gil et al 2000). Considerable research has been done on the antioxidant effect of flavanoids, but many of the therapeutic benefits of flavanoids and related compounds from innumerable natural sources remain to be explored.

Pomegranate juice is an important source of phenolic compounds, particularly anthocyanins and especially the 3-glucosides and 3,5-diglucosides of delphinidin, cyanidin and pelargonidin. These components, along with gallagyl-type tannins, ellagic acid derivatives and other hydrolysable tannins could contribute in some way to the antioxidant activity of pomegranate juice. On the other hand, methanolic pomengrate peel extract (PPE) has been shown to have high antioxidant activity (Chidambara Murthy et al 2002).

Hepatic fibrosis, the main complication of chronic liver disease, is usually initiated by hepatocyte damage, leading to recruitment of inflammatory cells and platelets, activation of Kupffer cells and subsequent release of cytokines and growth factors (Kullak-Ublick & Meier 2000). It is known that increased concentration of bile acids induces lipid peroxides, probably relating to stimulation of phagocytic activity in polymorphonuclear leucocytes and inflammatory cells that are present after biliary tract obstruction, and enhance the tissue injury (Sener et al 2006, 2007). Several clinical and experimental studies have shown that

oxygen free radicals have a role in the pathogenesis of tissue injury in obstructive jaundice (Peres et al 2000; Padillo et al 2002; Sener et al 2006, 2007). Free radical ablation for the treatment of cholestatic liver injury has been shown to have beneficial effects in the prevention of fibrosis and oxidative damage following biliary obstruction.

In the current study we used biochemical and histopathological approaches to examine the putative protective effect of PPE against oxidative damage and fibrosis induced by biliary obstruction.

Materials and Methods

The plant was collected from Istanbul in October 2005 and was identified by Dr Melek U. Dumlu of the Department of Pharmacognosy, Marmara University. The voucher specimens are maintained in our laboratory.

The peel and pulp were separated manually. The separated fresh peel was cut into small pieces and extracted with methanol. The extracts were filtered and residue was evaporated to dryness under vacuum. Although we have previously studied peel, juice and seed extracts, in this study we used only peel extract as it had the highest antioxidant activity.

In vitro experiments

Free radical scavenging ability

Free radical scavenging ability was measured using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method. Briefly, 50, 100, 250 and 500 mg L^{-1} solutions of PPE were mixed with 1 mL 1 mM methanolic solution of DPPH radical. The mixtures were shaken and left to stand for 30 min in the dark at room temperature. The absorbance of the final solutions was measured at 517 nm against a blank sample. Ascorbic acid was used as the control (Dumlu & Gurkan 2006). The % inhibition was calculated from $[(A_C - A_E)/A_C] \times 100$, where A_C is the absorbance of the control and A_E is the absorbance of the extract.

Reducing power

PPE (2.5 mL of each concentration: 50, 100, 250 and 500 mg L^{-1}) was mixed with 2.5 mL phosphate buffer (pH 6.6) and 2.5 mL 1 % potassium ferricyanide, and the mixtures incubated at 50°C for 30 min; 2.5 mL 10% trichloroacetic acid (TCA) (w/v) was then added and the mixtures centrifuged at 3000 rpm for 10 min. The upper layer (5 mL) was mixed with 5 mL deionized water and 1 mL 0.1% ferric chloride, and the absorbance of the mixtures measured at 700 nm against a blank sample. Butylated hydroxyanisol (BHA) and α-tocopherol were used as controls (Dumlu and Gurkan, 2006).

Determination of phenolic content

The analytical procedure described by Poyrazoglu et al (2002) was used with some modifications. PPE was diluted with distilled water and centrifuged at 7500 rpm for 10 min using 0.45 μm micro-spin centrifuge filter tubes (Alltech Associates, Deerfield, IL, USA). Filtered extract (20 μL) was injected onto an Agilent 1100 model HPLC system (Waldbronn, Germany) consisting of a quaternary pump with vacuum degasser, a temperature-controlled column oven and a photodiode array detector. Chromatographic separations were performed on an Atlantis dC18 column (4.6 × 150 mm, 3.0 μm) supplied by Waters (Milford, MA, USA). A gradient elution profile was carried out at 40°C using a mixture of formic acid and water (5:95 v/v) as solvent A and methanol as solvent B at a flow rate of 1.0 mL min^{-1}. The elution profile was 5% solvent B isocratic for 3 min followed by a 5–50% linear gradient with solvent B for 7 min, holding with 50% solvent B for 5 min, followed by a 50–5% linear gradient with solvent B for 5 min. The chromatogram was monitored simultaneously at 280 and 320 nm with 2 nm bandwidth, with spectra taken continuously throughout the elution.

Animal model of liver fibrosis and treatment procedure

Male Wistar albino rats (250–300 g) were housed at a mean constant temperature of 22 ± 2°C with a 12 h light–dark cycle, and free access to standard pellet chow and water. The study was conducted according to the ethical guidelines of Marmara University Animal Care and Use Committee.

Liver fibrosis was induced by biliary obstruction achieved by bile duct ligation (BDL). Rats were anaesthetized with ketamine (100 mg kg^{-1} i.p.) and chlorpromazine (0.75 mg kg^{-1} i.p.) and the common bile duct was exposed and ligated by double ligatures with silk suture. The first ligature was tied below the junction of the hepatic ducts and the second above the entrance of the pancreatic ducts. The common bile duct was resected between the double ligatures (Shimizu et al 1999). In sham-operated rats, an incision was made in the abdomen, which was then closed without any treatment.

PPE (50 mg kg^{-1} p.o.) or saline treatment was initiated on the day of surgery and continued for 28 days. At the end of treatment, rats were killed by decapitation and trunk blood was collected for biochemical analysis of the serum. Samples of liver tissue were stored at −70°C for measurement of malondialdehyde (MDA) and glutathione (GSH) levels, myeloperoxidase (MPO) activity, collagen content and luminol and lucigenin chemiluminescence (CL).

Biochemical analysis of serum

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) (Moss et al 1987) and lactate dehydrogenase (LDH) levels (Martinek 1972) were determined spectrophotometrically using an automated analyser (Olympus AU 2700, Hamburg, Germany). Total bilirubin level was assayed using a Bayer Opera Autoanalyser (Bayer, Mannheim, Germany). The total antioxidant capacity (AOC) in plasma was measured using a colorimetric test system (ImAnOx, Immunodiagnostic, Bensheim, Germany), according to the manufacturer's instructions.

Measurement of cytokines

Serum tumour necrosis factor (TNF)-α and interleukin (IL)-1β were quantified using a commercial ELISA assay specific for these rat cytokines (Biosource International, Nivelles, Belgium). These particular assay kits were selected because

of their high degree of sensitivity, specificity, inter- and intra-assay precision and small amount of serum sample required for the assay.

Biochemical analysis of liver tissue

Chemiluminescence assay

To assess the role of reactive oxygen species (ROS) in BDL-induced hepatic damage, luminol and lucigenin CL were measured as indicators of radical formation. Lucigenin (bis-N-methylacridiniumnitrate) and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) were obtained from Sigma (St Louis, MO, USA). Measurements were made at room temperature using a Junior LB 9509 luminometer (EG&G Berthold, Bad Wildbad, Germany). Samples were put into vials containing phosphate-buffered saline (PBS)–HEPES buffer (0.5 M PBS containing 20 mM HEPES, pH 7.2). ROS were quantified after the addition of the enhancers, lucigenin or luminal, at a final concentration of 0.2 mM. Luminol detects a group of reactive species (i.e. $^{\bullet}OH$, H_2O_2, HOCl radicals) while lucigenin is selective for $O_2^{-\bullet}$ (Davies et al 1994). Counts were obtained at 1 min intervals and the results were given as the area under the curve for a counting period of 5 min. Counts were corrected for wet tissue weight and are given in relative light unit (rlu) per mg tissue) (Davies et al 1994).

Malondialdehyde and glutathione assays

Tissue samples were homogenized with ice-cold TCA (1 g tissue plus 10 mL 10% TCA) in an Ultra Turrax tissue homogenizer (Ika, Heidelberg, Germany). MDA levels were measured as products of lipid peroxidation by monitoring formation of thiobarbituric acid reactive substance, as described previously (Beuge & Aust 1978). Lipid peroxidation is expressed in terms of MDA equivalents using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{cm}^{-1}$ and the results are expressed as nmol MDA per g tissue. Glutathione measurements were performed using a modification of the Ellman procedure (Beutler 1975). Briefly, after centrifugation at 2000 *g* for 10 min, 0.5 mL supernatant was added to 2 mL 0.3 M $Na_2HPO_4 \cdot 2H_2O$ solution. Dithiobisnitrobenzoate (0.2 mL 0.4 mg mL^{-1} solution containing 1% sodium citrate) was then added and the absorbance at 412 nm measured immediately after mixing. GSH levels were calculated using an extinction coefficient of $1.36 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. The results are expressed in μmol GSH per g tissue.

Measurement of liver myeloperoxidase activity

Tissue samples (0.2–0.3 g) were homogenized in 10 vol ice-cold potassium phosphate buffer (50 mM K_2HPO_4, pH 6.0) containing hexadecyltrimethylammonium bromide (HETAB; 0.5%, w/v). The homogenate was centrifuged at 30000 *g* for 10 min at 4°C and the supernatant discarded. The pellet was then rehomogenized with an equivalent volume of 50 mM K_2HPO_4 containing 0.5% (w/v) HETAB and 10 mM EDTA (Sigma). MPO activity was assessed by measuring the hydrogen-peroxide-dependent oxidation of O-dianizidine·2 HCl. One unit of enzyme activity was defined as the amount of the MPO present per gram of tissue weight that caused a change in absorbance of 1.0 per min at 460 nm and 37°C (Hillegass et al 1990).

Measurement of liver collagen

Tissue collagen was measured as a marker of free-radical-induced fibrosis. Tissue samples were cut with a razor blade and immediately fixed in 10% formalin. Samples were embedded in paraffin and sections (approximately 15 μm thick) obtained. Collagen content was evaluated using a published method based on selective binding of the dyes Sirius Red and Fast Green to collagen and non-collagenous components, respectively (Lopez De Leon & Rojkind 1985). Both dyes were eluted readily and simultaneously by using 0.1 M NaOH–methanol (1:1, v/v). Finally, the absorbance at 540 and 605 nm was used to determine the amount of collagen and protein, respectively.

Preparation of samples for light microscopy

Liver samples for light microscopy were fixed with 10% formaldehyde, dehydrated in graded alcohol series, cleared with toluene and embedded in paraffin. Tissue sections (5 μm) were stained with haematoxylin and eosin (H&E) for general morphology, and with Masson's trichrome for the determination of fibrosis, and examined under an Olympus BX51 photomicroscope (Tokyo, Japan).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). All data are expressed as mean ± s.e.m. Groups of data were compared using analysis of variance followed by Tukey's multiple comparison test. Values of P below 0.05 were regarded as significant.

Results

In-vitro experiments

Free radical scavenging ability

PPE had free radical scavenging activity in the concentration range 50–500 mg L^{-1}. The assay showed that the methanolic PPE has significant free radical scavenging ability. The results were compared with data using ascorbic acid: the 50, 100, 250, 500 and mg L^{-1} concentrations of extract had activities of 50, 62, 68 and 83% when compared with that of ascorbic acid (98%).

Reducing power

PPE had reducing power activity in the concentration range 50–500 mg L^{-1}. BHA and α-tocopherol were used as positive controls. The 50, 100, 250, 500 mg L^{-1} extracts had activities of 0.43, 0.58, 0.76 and 0.87, respectively when compared with that of BHA (1.0) and α-tocopherol (0.98).

Phenolic content

Water extract of the pomegranate peel was characterized by the presence of three main compounds, labelled 1, 2 and 3 in the chromatogram shown in Figure 1. Compounds 1 and 2 were tentatively identified as punicagalin 1 and punicagalin 2 from their UV spectra (Figure 2), which had maxima at 378 and 258 nm, characteristic of gallagic acid derivatives. These

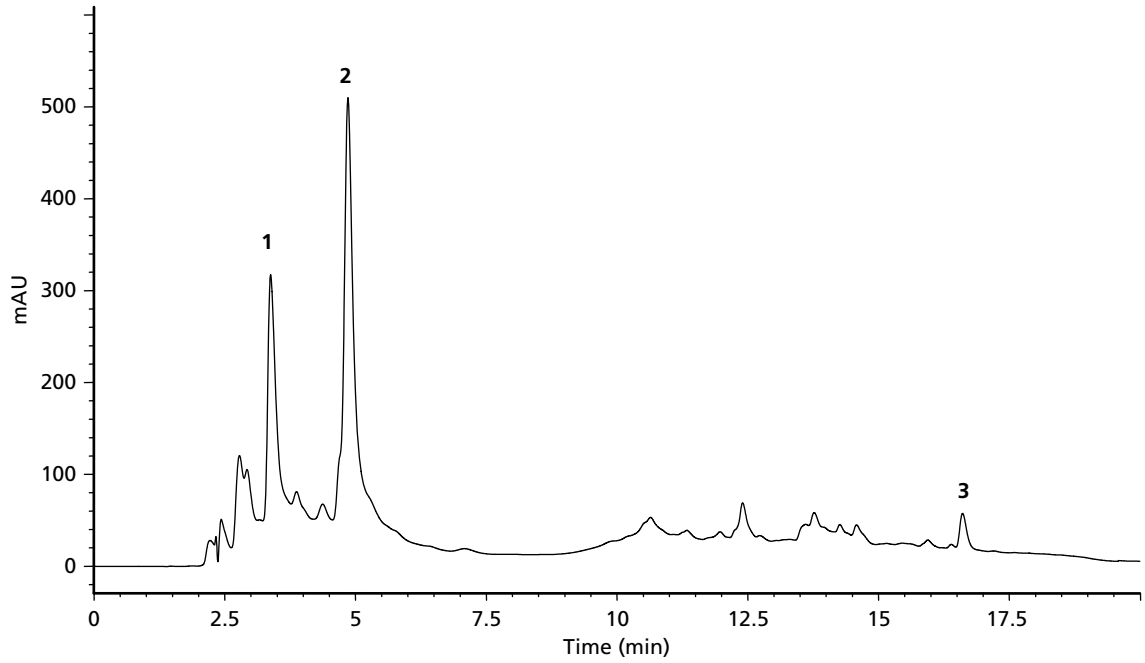

Figure 1 HPLC chromatogram of pomegranate peel extract recorded at 280 nm. Peak 1 is punicagalin 1; peak 2 is punicagalin 2; peak 3 is ellagic acid.

compounds were the main constituents of a water extract of pomegranate peel, and had a characteristic yellow colour. Presence of these two compounds has been previously reported by others, as confirmed by both UV and mass spectra (Gil et al 2000). In addition, compound 3 was identified as ellagic acid, with characteristic UV spectra showing 254, 300, 360 and 378 nm in the water extract of pomegranate peel. Presence of ellagic acid was further confirmed by comparing the retention time of pure ellagic acid standard to that of compound 3.

Biochemical analysis of serum

Cholestasis was evident 5 days after surgery by a significant increase in the level of total bilirubin, which was still high 28 days after surgery (Table 1). As shown in Table 1, serum AST, ALT and LDH were significantly higher ($P < 0.05$–0.01) in saline-treated BDL rats compared with controls, while administration of PPE to BDL rats reduced these values ($P < 0.05$–0.01). On the other hand, AOC, which was significantly reduced in the saline-treated BDL group compared with the sham-operated control group ($P < 0.001$), was reversed significantly by PPE treatment ($P < 0.05$).

In the saline-treated BDL group, TNF-α and IL-1β levels were significantly increased compared with the sham-operated control group ($P < 0.001$) but this BDL-induced rise in serum cytokine levels was significantly reduced by treatment with PPE ($P < 0.001$) (Table 1).

Biochemical analysis of liver tissue

BDL caused significant increases in luminol and lucigenin CL but levels decreased significantly in PPE-treated BDL rats ($P < 0.05$) (Table 2).

Liver MDA was significantly higher in the saline-treated BDL group than in the sham-operated control group. Treatment with PPE decreased the elevated MDA level back to control levels (Table 2). The level of GSH in the hepatic tissue was decreased significantly in the BDL group compared with the control group; PPE treatment significantly reversed the GSH reduction (Table 2). PPE treatment alone slightly increased the GSH level but this increase was not statistically different from control.

When compared with the sham-operated control groups, MPO activity was 2.5-fold higher than in the BDL group. This elevation in the MPO activity induced by BDL was reversed almost back to the control level with PPE treatment (Table 2). Hepatic collagen content of the saline-treated BDL rats was two-fold higher than that of sham-operated control rats; PPE treatment significantly reduced this increase in the hepatic collagen content, to levels that were close to control values (Table 2).

Histological evaluation

Light microscopy of liver samples from control (Figure 3A and B) and PPE-treated groups (Figure 3C and D) revealed a regular morphology of liver parenchyma, with intact hepatocytes and sinusoids. However, in the BDL group, hepatic cords were disorganized and hepatocytes showed degeneration and increased eosinophilia. Fibrosis was evident in the form of delicate bands around the acini. Other findings in this group were bile duct proliferation and mononuclear inflammatory infiltration in the portal tract (Figures 3E and F). PPE treatment of BDL rats resulted in a decrease in the number of degenerated and eosinophilic hepatocytes and there was only mild disorganisation of hepatic cords. Moreover, there was mild fibrosis around the acini and inflammatory cell infiltration within the portal tract (Figures 3G and H).

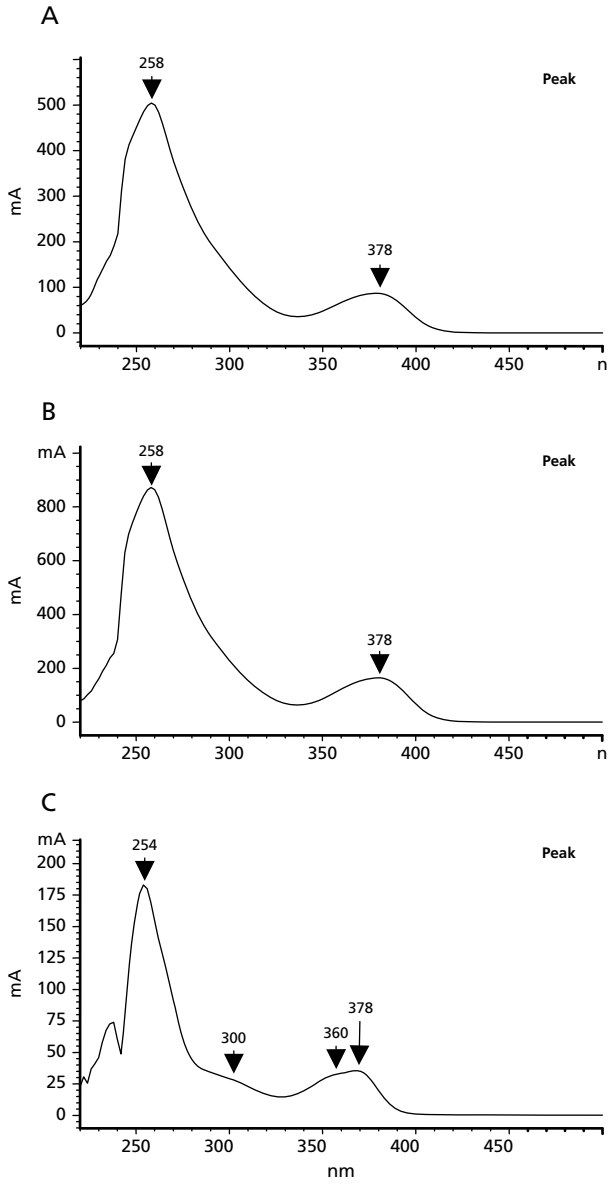

Figure 2 UV spectra of peaks 1 (punicagalin 1) (top trace), 2 (punicagalin 2) (middle) and 3 (ellagic acid) (bottom) shown in Figure 1.

Discussion

Punica granatum L. (Punicaceae) is a native tree of occidental Asia and Mediterranean Europe. For centuries the bark, leaves, flowers and fruits of the plant have been used to ameliorate diseases ranging from conjunctivitis to haematuria (Vidal et al 2003). The pomengrate fruits are one of the oldest known edible fruit. The arils are the edible part of the fruit, constituting about 52% of the total fruit (w/w) and comprising 78% juice and 22% seeds. The fresh juice contains 85.4% moisture and considerable amounts of sugars, reducing sugars, anthocyanins, phenolic compounds, ascorbic acid and proteins. Recently, the antioxidant activity of ethylacetate, methanolic and water extracts of pomegranate peel and seeds in various in-vitro models has been reported (Singh et al 2002) and suggested that, among all the extracts, methanolic PPE had the highest antioxidant activity in various models (Chidambara Murthy et al 2002; Singh et al 2002). We have shown for the first time that the hepatic fibrosis induced by biliary obstruction is prevented by methanolic PPE. PPE treatment improved BDL-induced impairment in liver function and decreased BDL-induced elevations in serum LDH activity and plasma TNF-α and IL-1β levels. Furthermore, increased hepatic lipid peroxidation, MPO activity and collagen content, and decreased GSH levels following BDL were reversed by PPE treatment. Histological findings also support the protective effect of PPE against BDL-induced hepatic damage.

Hepatic fibrosis, characterized by increased production and deposition of extracellular matrix components, accompanies most chronic liver disorders and is a major factor contributing to hepatic failure (Friedman 2003). Although the mechanisms of liver fibrosis are not fully understood, activated hepatic stellate cells play an important role in synthesis and deposition of connective tissue during fibrogenesis (Nan et al 2000). Oxidative stress caused by accumulation of hydrophobic bile acids also has a role in fibrogenesis (Zhong et al 2003; Muriel and Moreno 2004).

BDL induces a type of liver fibrosis that aetiologically and pathogenetically resembles biliary fibrosis in humans. Injury to hepatocytes results in the generation of lipid peroxides, which may have a direct stimulatory effect on matrix production by activated stellate cells (Jeong et al 2005). Liu et al (2001) demonstrated excessive production of superoxide radicals and hydroxyl radicals in blood and liver in rats with obstructive jaundice induced by ligation of the common bile duct. In our study, BDL caused significant increases in the level of hepatic MDA, an end product of lipid peroxidation, and PPE treatment prevented this increase, probably in part by scavenging the highly reactive hydroxyl and peroxyl radicals. Attenuation of the increase in hepatic luminol- and lucigenin-enhanced CL levels by PPE treatment in BDL animals also supports the scavenging ability of the agent.

The liver is an important organ in the metabolism of cytokines, with the capacity to both produce and remove them (Simpson et al 1997). Serum levels of IL-1β, IL-6, TNF-α and interferon gamma are elevated in patients with chronic liver disease (Tilg et al 1992). In rat studies, chronic BDL significantly increases most plasma and hepatic cytokines (Liu et al 2001; Fernandez-Martinez et al 2006). Various investigators have demonstrated that circulating pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, which trigger hepatic injury, were increased, at least in part, by a free-radical-mediated apoptotic mechanism. Therefore, it seems reasonable to propose that antioxidants or free radical scavengers counteract the oxidant stress produced by cholestasis. In our study, increased TNF-α and IL-1β levels in BDL rats tended to decrease with PPE treatment, supporting the notion that PPE ameliorates oxidative liver injury caused by BDL through its antioxidant effect. Although not many studies have looked at the effects of pomegranate peel on cytokines,

Table 1 Serum total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH), plasma antioxidant capacity (AOC) and TNF-α and IL-1 levels in control (saline) or pomegranate peel extract (PPE)-treated sham-operated control and BDL groups. Each group consisted of 8 animals

	Sham		**BDL**	
	Saline-treated	PPE-treated	Saline-treated	PPE-treated
Bilirubin (mg dL^{-1})	0.26 ± 0.05	0.24 ± 0.02	7.61 ± 0.52***	6.08 ± 0.59***
AST (mg dL^{-1})	157.8 ± 6.8	143.8 ± 6.1	249.4 ± 25.5**	$152.5 \pm 9.3^{++}$
ALT (mg dL^{-1})	42.8 ± 3.56	44.8 ± 2.5	95.4 ± 41.9**	$50.2 \pm 4.8^{+}$
LDH (U L^{-1})	1904 ± 46.1	1730 ± 85.8	2353 ± 160.3*	$1951 \pm 24.7^{+}$
AOC (pg mL^{-1})	476.7 ± 37.8	483.5 ± 44.0	240.8 ± 25.7***	$421.8 \pm 12.4^{+}$
TNF-α (pg mL^{-1})	2.9 ± 0.6	2.9 ± 0.8	28.7 ± 8.6***	$7.0 \pm 1.8^{+++}$
IL-1β (pg mL^{-1})	12.0 ± 2.0	10.2 ± 1.5	42.3 ± 4.8***	$15.5 \pm 2.1^{+++}$

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs saline-treated sham-operated control group.
$^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ vs saline-treated BDL group.

Table 2 Tissue luminol and lucigenin chemiluminescence and malondialdehyde (MDA), glutathione (GSH), myeloperoxidase (MPO) and collagen levels in control (saline) and pomegranate peel extract (PPE)-treated sham-operated control and BDL groups. Each group consisted of 8 animals

	Sham		**BDL**	
	Saline-treated	PPE-treated	Saline-treated	PPE-treated
Luminol (rlu mg^{-1})	8.9 ± 0.9	9.8 ± 1.1	16.5 ± 3.4***	$12.6 \pm 1.7^{+}$
Lucigenin (rlu mg^{-1})	12.5 ± 1.8	12.1 ± 2.4	16.9 ± 3.4*	$12.8 \pm 1.5^{+}$
MDA (nmol g^{-1})	30.0 ± 2.8	29.3 ± 2.9	54.9 ± 3.7***	$31.9 \pm 4.0^{+++}$
GSH (pg mL^{-1})	2.0 ± 0.2	2.2 ± 0.2	1.3 ± 0.1***	$1.9 \pm 0.3^{+}$
MPO (U g^{-1})	8.0 ± 1.0	8.2 ± 0.9	19.8 ± 3.5***	$11.6 \pm 2.7^{+}$
Collagen (μg mg^{-1})	13.5 ± 0.7	12.9 ± 1.8	25.4 ± 7.0***	$15.5 \pm 2.5^{+++}$

*$P < 0.05$, ***$P < 0.001$ vs saline-treated sham-operated control group.
$^{+}P < 0.05$, $^{+++}P < 0.001$ vs saline-treated BDL group.

the polyphenolic phytochemicals in the pomegranate juice have been shown to play an important role in the modulation of inflammatory cell signalling in colon cancer cells via its antioxidant effects (Adams et al 2006). In a recent study, Guo et al (2007) demonstrated the antioxidant properties of peel, juice and seed extracts of pomegranate. The peel extract of white pomegranate had the best scavenging ability on hydrogen peroxide. A study by Ahmed et al (2005) showed that pomegranate fruit extract inhibited the IL-1β-induced expression of matrix metalloproteinases via inhibition of mitogen activated protein (MAP) kinases and the DNA binding activity of the transcription factor NF-kappaB in osteoarthritic chondrocytes. Taken together, these results indicate that pomegranate juice, or compounds derived from it, may inhibit inflammatory responses that cause oxidative damage.

It is well known that oxidant injury is initiated by free radicals and ROS, which are generated by activated neutrophils, monocytes and mesangial cells during inflammatory processes (Kullak-Ublick & Meier 2000). These inflammatory cells, by their very phagocytic nature, contain many lytic enzymes that are expelled into the tissue on death of the inflammatory cells in-situ. Upon phagocytosis, polymorphonucleocytes undergo a respiratory burst, resulting in the production of ROS via elevation of the mitochondrial transmembrane potential.

Ohta et al (2003) reported a strong correlation between MPO activity and subsequent lipid peroxidation and tissue damage in BDL rats. In accordance with this, in our study BDL caused an increase in MPO activity – an index of tissue neutrophil infiltration – while PPE administration to BDL rats decreased these values, showing that PPE can also inhibit neutrophil infiltration.

The key antioxidant GSH is an important constituent of intracellular protective mechanisms against various noxious stimuli, including oxidative stress. By contrast, reduced GSH, which is a major component of the endogenous non-protein sulfhydryl pool, is a major low-molecular-weight scavenger of free radicals in the cytoplasm (Ross 1988). Because of their exposed sulfhydryl groups, non-protein sulfhydryls bind

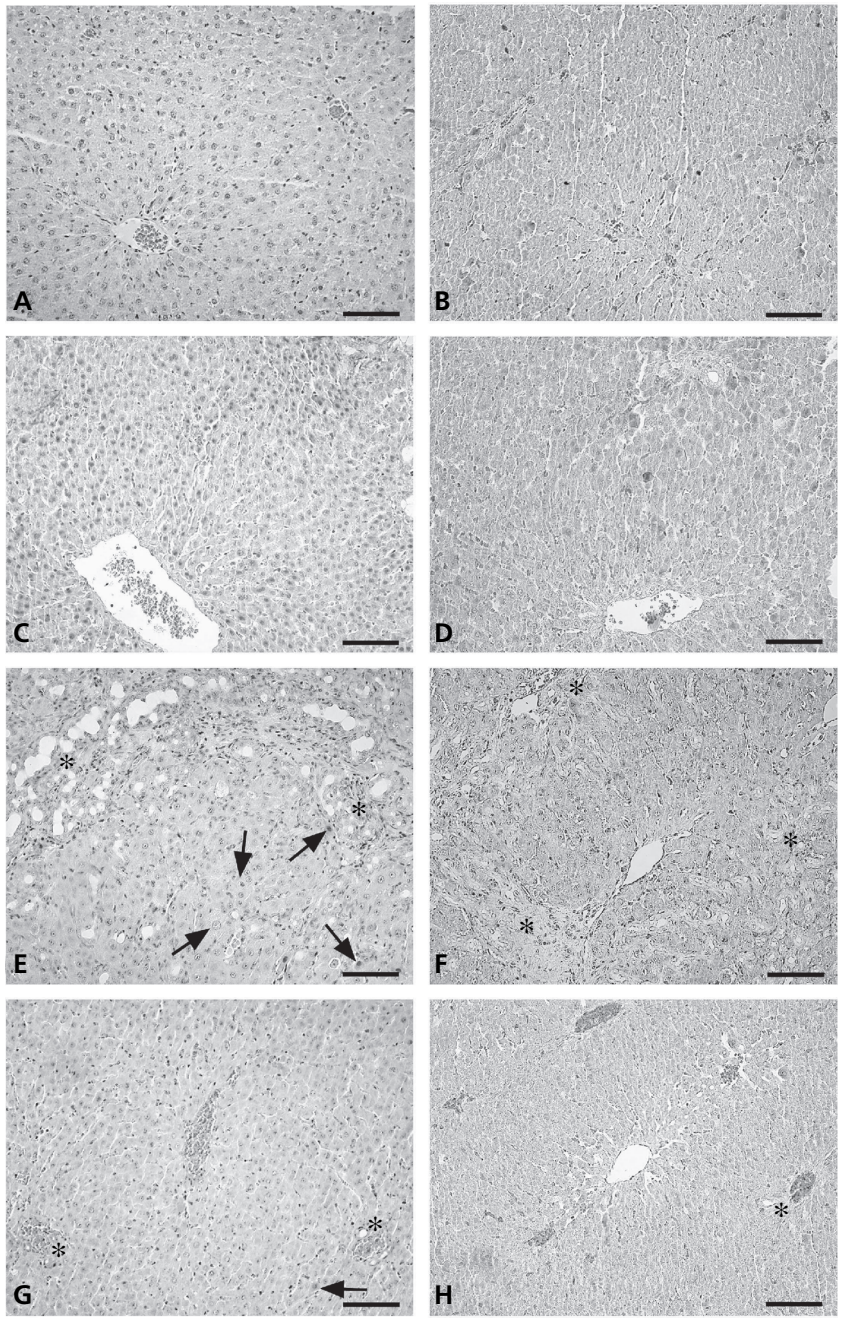

Figure 3 Light microscopy of liver samples. Left-hand side (A, C, E, G): H&E staining; right-hand side (B, D, F, H): Masson's trichrome staining. Scale bars: 25 μm. Control (A, B) and pomegranate-peel-extract (PPE)-treated (C, D) groups showed regular liver parenchyma with hepatocytes and sinusoids. Liver from bile-duct ligated (BDL) rats (E, F) showed disorganisation of hepatic cords, degeneration and increased eosinophilia in hepatocytes (arrows), fibrosis in the form of delicate bands around the acini and bile duct proliferation within the portal tract (asterisks). Liver from the BDL + PPE group (G, H) showed mild disorganization of hepatic cords, a decrease in degenerated and eosinophilic hepatocytes (arrow), mild fibrosis around the acini and decreased bile duct proliferation (asterisks).

a variety of electrophilic radicals and metabolites that may be damaging to cells. Huang et al (2003) studied mitochondrial function in BDL rats and suggested that biochemical and molecular changes are related to oxidative stress in the liver. In accordance with previous reports, our results also support the notion that depletion of tissue GSH, as observed in BDL-induced hepatic injury, is one of the major factors that permits lipid peroxidation and subsequent tissue damage. Since administration of PPE prevented the depletion of hepatic GSH, it appears that the protective effect of the extract

involves maintenance of antioxidant capacity, protecting hepatic tissue against oxidative stress. In parallel to tissue GSH, reduction of plasma antioxidant capacity was prevented by PPE treatment.

Conclusion

Targeting oxidative stress with antioxidants or boosting endogenous levels of antioxidants is likely to be beneficial in the treatment of chronic inflammatory diseases such as hepatic fibrosis due to biliary obstruction. The results of this study indicate for the first time that methanolic PPE provides protection against hepatic fibrosis induced by biliary obstruction, as shown by biochemical and histopathological studies conducted on the liver and blood. Although pomegranate peel is rich in phenolic compounds, its composition can vary. Further studies with phenolic compounds of pomegranate peel are needed to elucidate the mechanisms of action.

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