J. Pharm. Pharmacol. 1998, 50: 789–794
Received December 2, 1997
Accepted March 5, 1998

Antioxidant and Hepatoprotective Activity of Punicalagin and Punicalin on Carbon Tetrachloride-induced Liver Damage in Rats

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Abstract

Punicalagin and punicalin, isolated from the leaves of *Terminalia catappa* L., are used to treat dermatitis and hepatitis. Both compounds have strong antioxidative activity. The anti-hepatotoxic activity of punicalagin and punicalin on carbon tetrachloride (CCl_4)-induced toxicity in the rat liver was evaluated.

Levels of serum glutamate-oxalate-transaminase and glutamate-pyruvate-trans-aminase were increased by administration of CCl_4 and reduced by drug treatment. Histological changes around the liver central vein and oxidation damage induced by CCl_4 also benefited from drug treatment.

The results show that both punicalagin and punicalin have anti-hepatotoxic activity but that the larger dose of punicalin induced liver damage. Thus even if tannins have strong antioxidant activity at very small doses, treatment with a larger dose will induce cell damage.

Terminalia catappa L. is a combretaceous plant widely distributed on tropical and subtropical beaches. The leaves of this plant have been used as a folk medicine for treating dermatitis and hepatitis in Taiwan, India, the Philippines, Malaysia and Indonesia (Perry 1980). Previous studies showed that *Terminalia catappa* L. had antioxidative, anti-inflammatory, and hepatoprotective activity, and also modulated mitomycin C-induced clastogenicity (Liu et al 1996; Lin et al 1997). The constituents of the leaves of this plant are chiefly hydrolysable tannins such as punicalagin, punicalin, chebulagic acid and geraniin, etc. (Tanaka et al 1986). Punicalagin and punicalin are the most abundant components and have the strongest antioxidative activity of this group of tannins. In related research they were shown to have anti-HIV replication effects (Nonaka et al 1990) and activity against carbonic anhydrase (Satomi et al 1993).

The hepatoprotective effect of punicalagin and punicalin has been investigated against the liver injury induced by carbon tetrachloride (CCl_4), a chemical often used in studies in the search for hepatoprotective agents (McCay et al 1984).

Active oxygen molecules such as the superoxide radical play an important role in the inflammation process after intoxication by CCl_4 (Slater & Sawyer 1971a, b). These radicals, which react with cell membranes and induce lipid peroxidation, have been implicated as important pathologic mediators in many clinical disorders (Slater 1984). A major defence mechanism is the antioxidant enzymes (especially superoxide dismutase, catalase and glutathione peroxidase) which convert active oxygen molecules into non-toxic compounds. In this study we were interested in evaluating the relationship between the hepatoprotective and antioxidant enzyme activity of punicalagin and punicalin.

Materials and Methods

Materials

Punicalagin and punicalin (Figure 1) were isolated from the dried leaves of *T. catappa* obtained commercially in Taiwan. The leaves were identified by comparative anatomical studies and repre-

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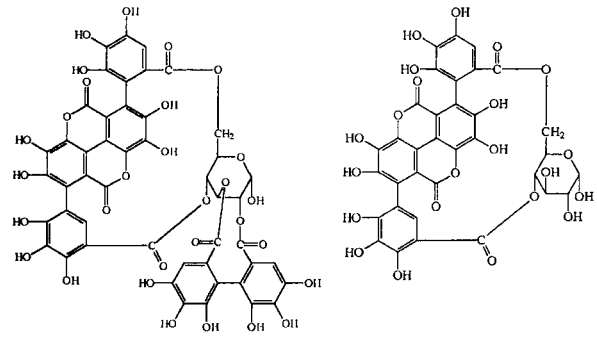

Punicalagin Punicalin

Figure 1. The chemical structures of punicalagin and punicalin.

sentative specimens were preserved in the Kaohsiung Medical College Herbarium, and authenticated by C. C. Lin, School of Pharmacy, Kaohsiung Medical College. The isolation methods were modifications of those reported by Tanaka et al (1986) and by Lin (1992).

Reagents and enzymes for antioxidant enzyme assays were purchased from Sigma (St Louis, MO). Kits B 8120 and B 8110 for measurement of serum glutamate-pyruvate-transaminase (GPT) activity were purchased from Menarini Industrie Farmaceutiche Riunite Divisione Diagnostici (Italy). Silymarin as reference drug was obtained from Aldrich (Milwaukee, WI). All other chemicals were of reagent grade and were used without further purification.

Animals, drug administration protocols and tissue collection

Male Wistar Albion rats, 4–6 weeks, 180–230 g, were housed in an air-conditioned room at 22±3°C, 55±5% humidity, 12-h light, and fed with a standard laboratory diet and tap water.

Rats were divided into seven groups of eight. Group 1 served as a control group, receiving normal saline only (10 mL kg^{-1}, i.p.). CCl_4 (3 mL kg^{-1} 50% in olive oil) was administered to animals of the other six groups by back subcutaneous injection. Punicalagin and punicalin (12·5 or 25·0 mg kg^{-1}, as 1·25 or 2·50 mg mL^{-1} solutions in normal saline, i.p.) and the reference drug silymarin (25·0 mg kg^{-1}, as a 2·50 mg mL^{-1} solution in 1% carmellose, i.p.) were administered to groups 3, 4, 5, 6 and 7, 2 h before and 24 and 48 h after CCl_4 administration. The remaining two groups, i.e. one given CCl_4 and one given vehicle only, were given no treatment. All animals were killed 72 h after CCl_4 administration, blood was drawn from the carotid artery and serum was separated for the different assays. Liver tissues were removed, liver sections were taken from each lobe of the liver and fixed in 10% neutral formalin; the remaining livers were cut into approximately 50–100 mg portions on ice and stored separately at − 70°C in plastic vials.

Assessment of liver function

After blood collection, the blood samples were left to coagulate at room temperature for 1 h. Serum was separated by centrifugation at 3000 rev min^{-1} and 4°C for 20 min. Serum glutamate-oxalate-transaminase (GOT) and GPT values were measured as described by Bergmeyer et al (1978).

Histopathological observation

After one week of fixing in 10% neutral formalin solution, the tissues were dehydrated with a sequence of aqueous solutions containing from 50–100% ethanol, embedded in paraffin, cut into 5-μm sections, stained with haematoxylin-eosin dye and observed under a photomicroscope. The morphological changes investigated were cell necrosis, fatty change and infiltration of lymphocytes and Kupffer cells.

Homogenate preparation

The frozen liver samples were homogenized in Tris–HCl or phosphate buffer solution to give a 20% homogenate. To measure lipid peroxidation levels homogenate was centrifuged at 1700 rev min^{-1} and 4°C for 10 min. For assay of catalase activity the homogenate was centrifuged at 3000 rev min^{-1} and 4°C for 15 min, then diluted to 0·5%. After centrifugation at 3000 rev min^{-1} for 15 min, the supernatant was again centrifuged either at 10 000 rev min^{-1} for 1 min and diluted to 2% for measurement of glutathione peroxidase activity or at 30 000 rev min^{-1} for 10 min before extraction of tissue superoxide dismutase activity with 20% ethanol.

The protein content of liver tissue was determined by the method of Lowry et al (1951); the result was correlated with the A280 curve, a standard calibration curve.

Measurement of tissue lipid peroxidation levels

Lipid peroxidation was quantified by measurement of thiobarbituric acid-reactive substances by spectrophotometric assay (Hitachi U-2000 spectrophotometer) as described by Ohkawa et al (1979). The level of lipid peroxides, expressed as nmol malondialdehyde (mg protein)$^{-1}$, was calculated from the absorbance at 532 nm using tetraethoxypropane as external standard. This method is an indirect measure of lipid peroxidation which is susceptible to interference by endogenous and exogenous substances; it should be regarded as an

indication rather than as an absolute measure of total tissue lipid peroxide levels (Draper & Hadley 1990).

Tissue enzyme activity assays

Superoxide dismutase activity was measured by the nitroblue tetrazolium reduction procedure (Beauchamp & Fridovich 1971) using 150 μL homogenate supernatant in a final assay volume of 3·0 mL. The units of enzyme activity were calculated from the absorbance at 560 nm using standard enzyme as a standard curve. The assays for catalase or glutathione peroxidase were performed by the methods of Aebi (1984) and Flohe & Gunzler (1984), respectively. Assays on a given sample were performed in triplicate, and enzyme-specific activities were expressed in units of activity $(\text{mg protein})^{-1}$.

Results

Effects of drug administration on serum GOT and GPT levels

The results from examination of the effects of punicalagin and punicalin on this liver-injury model are summarized in Table 1. The results indicate that 72 h after CCl_4 injection there were significant increases of serum GOT and GPT compared with the control group but that the GOT and GPT levels were significantly ($P < 0.005$ compared with the CCl_4-treated control) lower when the rats were given punicalagin (12·5 and 25 mg kg^{-1}). High serum GOT and GPT levels were, however, measured when rats were given 25 mg kg^{-1} punicalin.

Histopathological observation

This phenomenon was confirmed by histological examination (Figure 2). Massive fatty change, gross necrosis, broad infiltration of lymphocytes and Kupffer cells around the central vein, and loss of cellular boundary (Figure 2B) were observed in the livers of CCl_4-treated rats. Less damage was present in the livers of rats given punicalagin and punicalin (Figure 2D, E, and F), except for 25 mg kg^{-1} doses of punicalin, which caused the liver injury to deteriorate (Figure 2G).

Effects of drug administration on rat liver antioxidant enzyme and lipid peroxide levels

The results are shown in Table 2. Non-enzymatic lipid peroxidation was induced in the rat liver homogenate by $FeCl_2$-ascorbic acid and the effects of administration of the drugs were determined by assay of the malondialdehyde-thiobarbituric acid adduct at 532 nm (Wong et al 1987). There was a significant increase of malondialdehyde level compared with that measured for the normal control without $FeCl_2$-ascorbic acid. Punicalagin and punicalin reduced antioxidant enzyme activity, except for treatment with 25 mg kg^{-1} punicalin.

Discussion

Reactive oxygen species such as superoxide, hydroxyl radical, iron-oxygen complexes, hydrogen peroxide and lipid peroxides are generated by several reactions: metabolism of triplet oxygen molecules; one-electron reduction of oxygen; catalytic decomposition of hydrogen peroxide and lipid hydroperoxides by metal ions; attack of metal or of metal-oxygen complexes; light and X-ray irradiation; intake of exogenous radicals (Fridovich 1976).

These radicals react with biological molecules such as DNA, proteins and phospholipids and eventually damage membranes and other tissue (Vuillaume 1987). Aerobic organisms employ a battery of defence mechanisms such as antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) to prevent or mitigate oxidative tissue-damage (Halliwell & Gutteridge 1989). Superoxide dismutase removes the superoxide radical to prevent formation of the hydroxyl radical. Catalase deals especially effectively with the large amounts of hydrogen peroxide generated in the peroxisomes. Glutathione peroxidase is capable not only of utilizing hydroperoxides but also of

Table 1. The attenuating effects of drug treatment on serum glutamate-oxalate-transaminase and serum glutamate-pyruvate-transaminase levels in CCl_4-intoxicated rats.

Treatment	Serum glutamate-oxalate transaminase	Serum glutamate-pyruvate-transaminase
Control (NaCl)	125±5	28±4
CCl_4–olive oil (3 mL kg^{-1})	380±80	83±22
Silymarin (25 mg kg^{-1})	240±60	50±20
Punicalagin (12·5 mg kg^{-1})	190±30*	38±7*
Punicalagin (25 mg kg^{-1})	170±30*	35±5*
Punicalin (12·5 mg kg^{-1})	220±50	50±6
Punicalin (25 mg kg^{-1})	390±30	142±2

*$P < 0.005$, significantly different from CCl_4-treated rats.

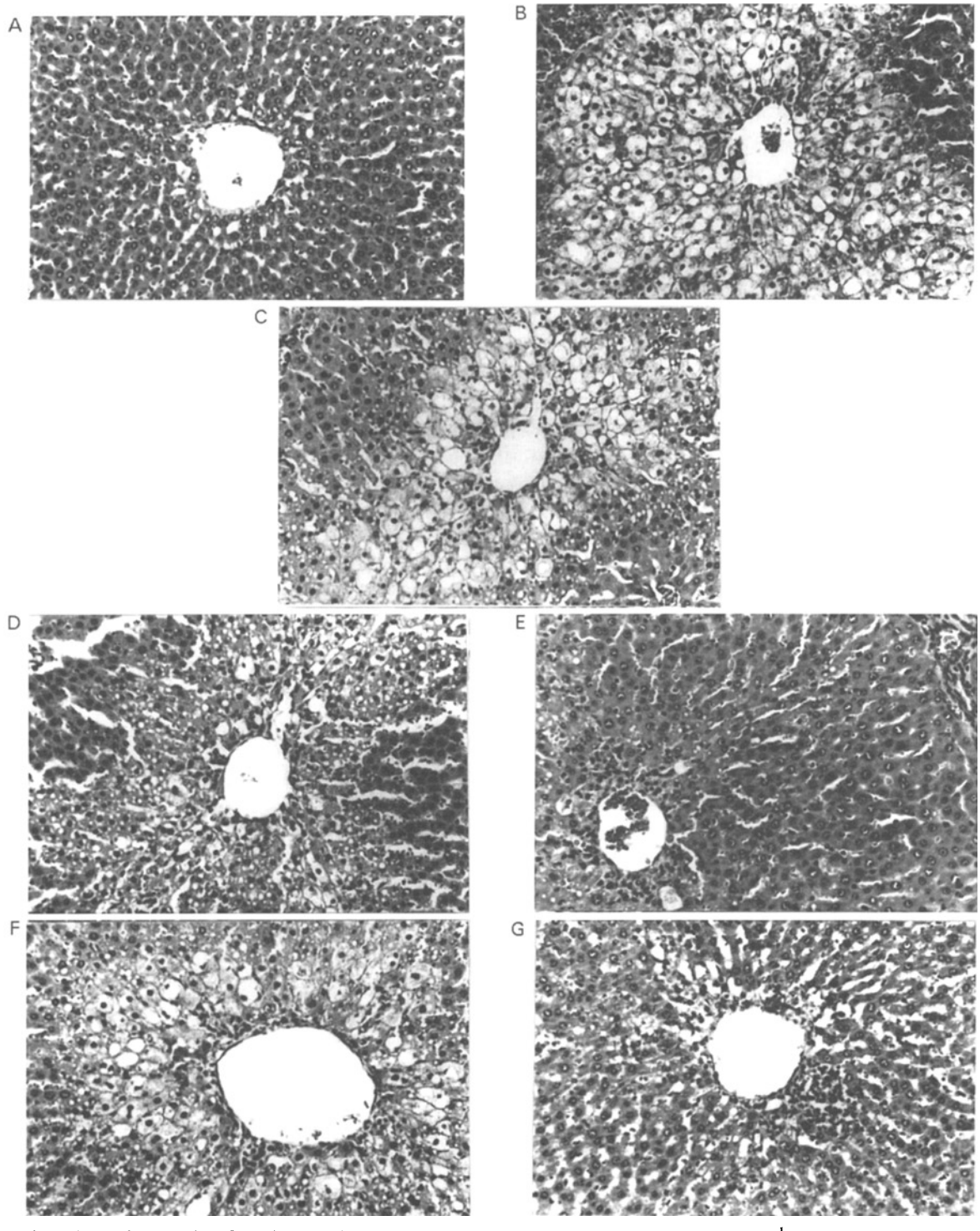

Figure 2. Photomicrographs of rat liver sections. A. Control (NaCl); B. CCl_4–olive oil (3 mL kg^{-1}); C. silymarin (25 mg kg^{-1}); D. punicalagin (12·5 mg kg^{-1}); E. punicalagin (25 mg kg^{-1}); F. punicalin (12·5 mg kg^{-1}); G. punicalin (25 mg kg^{-1}).

metabolizing hydrogen peroxide in both the cytosolic and mitochondrial compartments. At other sites intake of compounds which induce antioxidant enzyme activity or scavenge free radicals both prevent oxidative damage (Hochstein & Atallah 1988).

CCl_4 is metabolized by the mixed-function oxidase system in the endoplasmic reticulum of the liver. Cleavage of the carbon–chloride bond results in the formation of free trichloromethyl radicals (CCl_3), which are highly unstable and immediately react with membrane components (Recknagel &

Table 2. Effects of drug administration on the specific activities of liver antioxidant enzymes and on the levels of thiobarbituric acid-reactive substances in CCl_4-intoxicated rats.

Treatment	Enzyme activity (units (mg liver protein)$^{-1}$)			Thiobarbituric acid-reactive substances (nmol malondialdehyde (mg liver protein)$^{-1}$)
	Superoxide dismutase	Glutathione peroxidase	Catalase	
Control (NaCl)	75·81 ± 1·94	0·981 ± 0·030	296·83 ± 10·05	1·230 ± 0·389
CCl_4–olive oil (3 mL kg^{-1})	56·75 ± 0·50	0·704 ± 0·033	189·61 ± 5·56	1·790 ± 0·163
Silymarin (25 mg kg^{-1})	68·13 ± 2·54	0·848 ± 0·047	258·17 ± 6·31	1·262 ± 0·113*
Punicalagin (12·5 mg kg^{-1})	68·10 ± 0·54	0·934 ± 0·032*	240·74 ± 4·91*	1·747 ± 0·110
Punicalagin (25 mg kg^{-1})	92·55 ± 0·73*	0·973 ± 0·068*	254·28 ± 4·61	1·456 ± 0·083
Punicalin (12·5 mg kg^{-1})	67·89 ± 0·56	0·879 ± 0·062	264·92 ± 9·05*	1·668 ± 0·167
Punicalin (25 mg kg^{-1})	52·24 ± 0·53	0·736 ± 0·046	205·15 ± 4·17	1·834 ± 0·242

Specific activities of enzymes and levels of thiobarbituric acid-reactive substances in the liver are expressed as the means ± s.d., n = 8. *$P < 0·005$, significantly different from CCl_4-treated rats.

Glende 1973). They form covalent bonds with unsaturated fatty acids or abstract a hydrogen atom from the unsaturated fatty acids of membrane lipids, resulting in the production of chloroform and lipid radicals which react with molecular oxygen; this initiates peroxidative decomposition of phospholipids in the endoplasmic reticulum. The peroxidation process results in the release of soluble products that affect other membranes, such as cell membranes (Packer et al 1978). It has been found that microsomal oxidation of chloroform results in the formation of phosgene. It is thought that a secondary metabolite causes cell death (Shah et al 1979). Thus, protective agents against CCl_4-induced liver injury exert their action by impairing CCl_4-mediated lipid peroxidation by inhibiting the generation of free-radical derivatives (Castro et al 1974) or as a result of the antioxidant activity of the protective agent itself (Yasuda et al 1980).

Shigeki et al (1988) investigated superoxide (O_2^-) production in rat Kupffer cells in various pathological states. In CCl_4-induced liver cirrhosis, O_2^- production in Kupffer cells was remarkably reduced, and superoxide dismutase activity in the liver homogenate was also reduced.

In the current work, histological changes observed in the CCl_4 group were ballooning degeneration, fatty change, cell necrosis, lymphocyte infiltration, and increase in Kupffer cells. Treatment with increasing doses of punicalagin or punicalin reduced ballooning degeneration, fatty change and cell necrosis but not (in contrast with Shigeki's results) lymphocyte infiltration or Kupffer-cell proliferation. Also of interest was that treatment with punicalagin reversed the decrease in rat liver superoxide dismutase activity induced by CCl_4; treatment with 12·5 and 25 mg kg^{-1} punicalagin resulted in levels that were lower and higher, respectively, than that of the normal control group, but whereas treatment with 12·5 mg kg^{-1} punicalin had the same effect as the same level of punicalagin, treatment with 25 mg kg^{-1} punicalin resulted in a level similar to that in the untreated group (Table 2).

Oelrichs et al (1994) reported that punicalagin caused slight liver lesions, results in agreement with ours. We therefore suggest that although these condensed tannins have strong antioxidative activity in-vitro and can, in small doses, prevent CCl_4-induced liver injury, larger doses result in liver lesions. Measurement of CCl_4-induced serum GOT and serum GPT levels also support this suggestion.

In a previous study (unpublished), we found that punicalagin and punicalin had strong activity against lipid peroxidation and superoxide formation and strong superoxide-scavenging activity, but no hydroxyl-radical-scavenging activity in-vitro. On the basis of those results, we measured the activity of three antioxidant enzymes and levels of thiobarbituric acid-reactive substances in rat-liver homogenate in-vitro to determine the relationship between the drugs and the liver antioxidant defence system (Table 2). The activities of all the antioxidant enzymes were reduced by treatment with CCl_4 but treatment with the drugs reduced the decrease. Levels of thiobarbituric acid-reactive substances were higher in the CCl_4 group but the increase was reduced by drug treatment. These results demonstrated that although punicalagin and punicalin do not have hydroxyl-radical-scavenger activity, they restore the activity of hydroxyl-radical-scavenger enzymes (catalase and glutathione peroxidase).

According to the concept of traditional Chinese medicine, liver disease is thought to be caused by the stagnation of pathogenic damp-heat and liver stasis or invasion of the stomach and spleen by hepatic Qi. Patients with liver disease might manifest different syndromes in different phases (Tsai et al 1997). Therefore, one disease can be treated in

different ways, and the study rationalizes the traditional use of herbal prescriptions in liver diseases. Furthermore, a protective mechanism not specific to injury induced by CCl_4 might be responsible for the hepatoprotective activity of these prescriptions. Experiments are now in progress to study the mechanisms of activity and toxicity.

In conclusion, this study has demonstrated that although the leaves of this plant can be used as a crude hepatoprotective drug the medicine should be used carefully and should not be abused.

Acknowledgements

The authors wish to thank M. H. Yen and J. J. Yang for their technical assistance. We are grateful to The National Science Council of the Republic of China for financial support.

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