JPP Journal of Pharmacy and Pharmacology

JPP 2009, 61: 1383–1390

Received May 17, 2009
Accepted July 16, 2009
DOI 10.1211/jpp/61.10.0016
ISSN 0022-3573

Research Paper

Potential beneficial effect of naringenin on lipid peroxidation and antioxidant status in rats with ethanol-induced hepatotoxicity

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Abstract

Objectives The aim was to study the effect of naringenin, a biologically active compound, on tissue antioxidant status and lipid peroxidation in ethanol-induced hepatotoxicity in rats.
Methods Rats were divided into four groups: Groups 1 and 2 received isocaloric glucose and 0.5% carboxymethyl cellulose; groups 3 and 4 received 20% ethanol equivalent to 6 g/kg daily for 60 days. In addition, groups 2 and 4 were given naringenin (50 mg/kg) daily for the last 30 days of the experiment.
Key findings The results showed significantly elevated levels of serum aspartate and alanine transaminases, γ-glutamyl transpeptidase, tissue thiobarbituric acid reactive substances, conjugated dienes, lipid hydroperoxides and protein carbonyl content, and significantly lowered activities/levels of antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase, reduced glutathione and vitamins C and E in ethanol-treated rats compared with control rats. Administration of naringenin to rats with ethanol-induced liver injury significantly decreased the levels of serum aspartate and alanine transaminases, γ-glutamyl transpeptidase, tissue thiobarbituric acid reactive substances, conjugated dienes, lipid hydroperoxides and protein carbonyl content and significantly elevated the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase, and the levels of reduced glutathione and vitamins C and E in the tissues compared with unsupplemented ethanol-treated rats. Histological changes observed in the liver correlated with the biochemical findings.
Conclusions Taken together these findings suggest that naringenin has a therapeutic potential in the abatement of ethanol-induced hepatotoxicity.
Keywords alcoholic liver disease; reactive oxygen species; protein carbonyl content; flavanone; naringenin

Introduction

Alcoholism is associated with numerous degenerative and inflammatory disorders that affect many organs, including liver, brain, kidney, heart, skeletal muscle and pancreas.[1] The development and manifestations of alcoholic liver disease are influenced by genetic, psychosocial and environmental factors.[2] Ethanol is a fat-soluble non-electrolyte, which is readily absorbed from the gastrointestinal tract, diffuses readily into the circulation and is distributed uniformly throughout the body.[3] Ethanol is an addictive drug with a complex mechanism of action and a variety of pharmacological and toxic effects.[4]

Reactive oxygen species (ROS) have been implicated in a growing number of diseases. In general, the toxicity caused by superoxide ions and H_2O_2 in biological systems is believed to be due to the conversion of these ROS to more potent oxidants, such as hydroxyl species.[5] Free radicals have been implicated in the pathogenesis of alcohol-induced liver injury in humans and experimental animals.[6] A number of reports have suggested that acute ingestion of ethanol results in the formation of free radicals and ROS.[7,8]

Enzymatic antioxidants such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione reductase (GR), and non-enzymatic antioxidants such as reduced glutathione (GSH), vitamin C and

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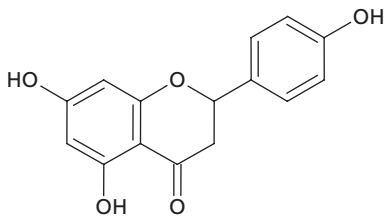

Figure 1 Struture of naringenin (4′,5,7-trihydroxyflavanone)

vitamin E[9,10] are essential in the prevention of cellular damage caused by free radicals and free-radical-induced lipid peroxidation.[11] There is evidence that these antioxidants are altered in alcoholics. Free radicals formed on alcohol consumption affect the permeability of hepatocytes, leading to leakage of enzymes such as serum aspartate and alanine transaminases (AST and ALT) and γ-glutamyltransferase (GGT).[12]

Flavonoids are diverse in chemical structure and characteristics. They occur naturally in fruits, vegetables, nuts, seeds, flowers and bark and are an integral part of the human diet.[13] Flavonoids are described as 'high level' natural antioxidants on the basis of their abilities to scavenge free radicals.[14]

Naringenin (Figure 1) is a predominant flavanone abundant in fruits such as grapes, tangelo, blood orange, lemons, pummelo and tangerines.[15] Naringenin has been reported to have several biological effects such as anticancer,[16] antimutagenic,[17] anti-inflammatory,[18] antioxidant,[19] antifibrogenic[20] and antiatherogenic[21] properties. Daily ingestion of citrus flavonoids has been estimated to be approximately 68 g on an average in the USA, mainly via fruit juices.[22] The concentration of naringenin in grapefruit juice has been estimated at 1283 μmol/l (349 mg/l).[23]

Diet is one of the vital factors essential for sustaining life and promoting health. Hence, developing drugs from dietary substances and evaluating natural compounds such as naringenin would be potentially beneficial in reducing the risk of various pathological conditions. There are no reports in the literature on the protective effect of naringenin against alcohol-induced hepatotoxicity. This study was designed to test the hypothesis that naringenin would reduce ethanol-induced toxicity by modulating lipid peroxidation and antioxidant levels.

Materials and Methods

Chemicals and reagents

Naringenin was purchased from Sigma Chemical Co. (St Louis, MO, USA). Ethanol was obtained from E.I.D. Parry India Ltd (Nellikuppam, Cuddalore District, South India). All other chemicals were of analytical grade and were obtained from Central Drug House Pvt. Ltd (New Delhi, India).

Animals

Adult male albino Wistar rats (150–170 g) were obtained from the Central Animal House, Rajah Muthiah Medical College and Hospital of Annamalai University. The rats were housed in plastic cages under controlled conditions of 12 h light–dark cycle, 50% humidity and 28°C and were fed a standard pellet diet (Lipton Lever Mumbai, India) and water *ad libitum*. All animal handling and experimental procedures were approved by the Institutional Animal Ethics Committee, Annamalai University (registration no. 160/1999/CPCSEA/557) and animals were cared for in accordance with the Indian National Law on animal care and use.[24]

Study design

The rats were divided into four groups of eight. Groups 1 and 2 received isocaloric glucose (40% glucose in drinking water) and 0.5% carboxymethyl cellulose (CMC, p.o. daily). Groups 3 and 4 received 20% ethanol (equivalent to 6 g/kg) as an aqueous solution by intragastric intubation for 30 days, as described previously.[25] At the end of this period, the dietary protocol of groups 1 and 3 was unaltered. Group 2 animals received naringenin (50 mg/kg) suspended in 0.5% CMC for the next 30 days, while group 4 continued to receive ethanol daily along with naringenin, as in group 2. The study design is shown in Figure 2.

Preparation of tissue homogenates

Rats were anaesthetised with ketamine (30 mg/kg i.m.) and then decapitated. Immediately after death, blood samples were collected in heparinised test tubes/plain tubes and centrifuged to separate plasma and serum. The erythrocytes were washed with 0.9% saline three times, and then distilled water was added to induce haemolysis. The haemolysate was then used to estimate the total haemoglobin content. The liver, kidneys and heart were quickly excised, rinsed with saline, blotted dry on filter paper and weighed. Homogenates (10% w/v) were prepared with appropriate buffer using a tissue homogeniser and the supernatants used for various biochemical assays.

Biochemical estimations

Serum AST (EC 2.6.1.1) and ALT (EC 2.6.1.2) were measured using a diagnostic kit based on the method of Reitman and Frankel.[26] Serum GGT (EC 2.3.2.2) activity was measured according to the method of Rosalki and Rau.[27]

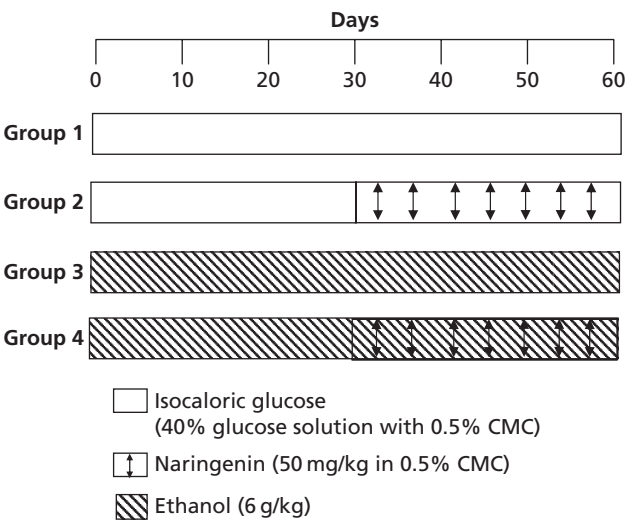

Figure 2 The experimental protocol. CMC, 0.5% carboxymethyl cellulose. All treatments were given orally by gavage.

Thiobarbituric acid reactive substances (TBARS) in the tissues were measured by the method of Ohkawa *et al*.[28] The pink chromogen produced by the reaction of secondary products of lipid peroxidation such as malondialdehyde with thiobarbituric acid was estimated at 532 nm. The concentration of tissue lipid hydroperoxides (LOOH) was estimated by the method of Jiang *et al*.[29] Conjugated dienes were estimated by the method of Rao and Recknagel.[30] This method is based on the arrangement of double bonds in polyunsaturated fatty acids to form conjugated dienes with an absorbance maximum at 233 nm. The protein carbonyl content in tissues was determined using the 2,4-dinitro phenyl hydrazine method as described by Levine *et al*.[31] and was calculated from the maximum absorbance (360–370 nm) using a molar absorption coefficient of 22 000 mol/l per cm. The results are expressed as nmol carbonyls/mg protein.

SOD (EC 1.15.1.1) activity was determined using the method of Kakkar *et al*.[32] One unit of enzyme is defined as the quantity of SOD required to produce 50% inhibition of nitro blue tetrazolium (NBT) reduction/min per mg protein. Catalase (EC 1.11.1.6) activity was measured by monitoring the decomposition of H_2O_2, as described by Sinha.[33] The specific activity of catalase was expressed as μmol H_2O_2 utilised/min per mg protein. GPx (EC 1.11.1.9) activity was determined using the method of Rotruck *et al*.[34] A known amount of the enzyme preparation was incubated with H_2O_2 in the presence of GSH for a specific time period. The amount of H_2O_2 utilised was determined using the Ellman method.[35] GST (EC 2.5.1.18) activity was measured using the method of Habig *et al*.[36] The change in absorbance at 340 nm was measured and enzyme activity calculated as μmol 1-chloro-2,4-dinitrobenzene (CDNB) conjugate formed per min per mg protein, using a molar extinction coefficient of 9.6 mmol/l per cm. GR (EC 1.6.4.2) activity was determined by the method of Carlberg and Mannervik.[37] One unit of enzyme is defined as nmol NADPH consumed per min per mg protein.

GSH in the tissues was measured using the method of Ellman,[35] based on the development of yellow colour when 5,5-dithiobis (2-nitro benzoic acid) is added to compounds containing sulfhydryl groups. Ascorbic acid (vitamin C) was estimated by the method of Roe and Kuether,[38] and α-tocopherol (vitamin E) by the method of Baker *et al*.[39] Proteins were estimated by the method of Lowry *et al*.[40] using bovine serum albumin as the standard.

Statistical analysis

Data were analysed by one-way analysis of variance followed by Duncan's multiple range test using SPSS for Windows (v. 11.0; SPSS Inc., Chicago, IL, USA). Results are presented as means ± SD of eight rats in each group. Values of $P < 0.05$ were regarded as statistically significant.

Results

Table 1 shows the average weight gain, food intake and liver-to-body-weight ratios of control and experimental rats. Food intake and weight gain were significantly reduced in ethanol-treated rats compared with control rats. The liver-to-body-weight ratio was significantly increased in the naringenin-supplemented rats (group 4) compared with the ethanol-treated rats (group 3) from Day 31, showing a significant weight gain, increased food intake and decreased liver-to-body-weight ratio ($P < 0.05$).

Table 2 shows the activities of serum AST, ALT and GGT. Activities of all three enzymes were significantly increased in ethanol-fed rats compared with control rats. Naringenin supplementation to ethanol-fed rats (group 4) significantly decreased the liver marker enzymes compared with the unsupplemented ethanol-fed rats (group 3; $P < 0.05$).

Table 3 shows the levels of TBARS and LOOH, which were significantly higher in the liver, kidneys and hearts of ethanol-fed rats (group 3) compared with control rats (group 1). Tissue TBARS and LOOH levels were significantly lowered ($P < 0.05$) by naringenin supplementation to ethanol-fed rats (group 3). Naringenin supplementation alone (group 2) did not produce any significant change in the levels of TBARS and LOOH compared with control rats.

Table 1 Effect of naringenin on body weight and liver-to-body-weight ratios

	Control	Control + naringenin	Ethanol	Ethanol + naringenin
Bodyweight, day 1	153.1 ± 14.7^{a}	150.05 ± 14.4^{a}	146.02 ± 14.05^{a}	152.3 ± 14.6^{a}
Bodyweight, day 60	235.1 ± 22.6^{c}	233.6 ± 22.4^{c}	156.5 ± 15.07^{a}	202.1 ± 19.4^{b}
Net gain (g)	81.99 ± 7.89^{c}	83.62 ± 8.05^{c}	10.55 ± 1.01^{a}	49.79 ± 4.79^{b}
Average food intake (g)	10.40 ± 1.00^{b,c}	10.60 ± 1.02^{a,c}	7.35 ± 0.70	9.38 ± 0.90^{b}
Liver-to-body weight ratio (×100)	2.88 ± 0.27^{a}	2.98 ± 0.28^{a}	4.73 ± 0.45^{b}	3.19 ± 0.30^{a}

Values are means ± SD ($n = 8$). Values that do not share a common superscript letter differ significantly ($P < 0.05$; Duncan's multiple range test).

Table 2 Effect of naringenin on plasma markers of hepatic activity

	Control	Control + naringenin	Ethanol	Ethanol + naringenin
Aspartate transaminase (IU/l)	79.84 ± 7.68^{a}	82.13 ± 7.90^{a}	112.40 ± 10.81^{b}	87.27 ± 8.40^{a}
Alanine transaminase (IU/l)	28.86 ± 2.77^{a}	30.81 ± 2.96^{a}	60.38 ± 5.81^{b}	32.76 ± 3.15^{a}
γ-Glutamyl transpeptidase (IU/l)	2.32 ± 0.22^{a}	3.18 ± 0.30^{b}	8.03 ± 0.77^{d}	4.18 ± 0.40^{c}

Values are means ± SD ($n = 8$). Values that do not share a common superscript letter differ significantly ($P < 0.05$; Duncan's multiple range test).

Table 3 Effect of naringenin on levels of thiobarbituric acid reactive substances and lipid hydroperoxides

	Control	Control + naringenin	Ethanol	Ethanol + naringenin
TBARS (nmoles/g tissue)				
Liver	29.89 ± 2.88[a]	28.77 ± 2.77[a]	59.67 ± 5.74[b]	32.44 ± 3.12[a]
Kidney	0.52 ± 0.05[a]	0.49 ± 0.05[a]	2.61 ± 0.26[b]	0.69 ± 0.07[c]
Heart	0.67 ± 0.06[a]	0.71 ± 0.07[a]	1.63 ± 0.16[b]	0.87 ± 0.08[c]
LOOH (mmoles/g tissue)				
Liver	67.19 ± 6.47[a]	66.10 ± 6.36[a]	86.74 ± 8.35[b]	61.36 ± 5.91[a]
Kidney	0.79 ± 0.08[a]	0.72 ± 0.07[a]	3.16 ± 0.30[b]	0.99 ± 0.10[c]
Heart	88.64 ± 8.53[a]	81.73 ± 7.87[a]	117.13 ± 11.27[b]	81.73 ± 7.87[a]

Values are means ± SD ($n = 8$). LOOH, lipid hydroperoxides; TBARS, thiobarbituric acid reactive substances. Values that do not share a common superscript letter differ significantly ($P < 0.05$; Duncan's multiple range test).

Levels of conjugated dienes and protein carbonyl content in the livers, kidneys and hearts (Table 4) were significantly higher in ethanol-fed rats (group 3) than control rats (group 1) and were significantly lowered ($P < 0.05$) on naringenin supplementation to ethanol-fed rats (group 4). Naringenin supplementation alone (group 2) did not produce any significant effects on levels of conjugated dienes or protein carbonyl content compared with control rats.

SOD and catalase in the livers, kidneys and hearts (Table 5) were significantly lower in the ethanol-fed rats (group 3) than control rats (group 1). Tissue SOD and catalase levels were increased significantly on naringenin supplementation to ethanol-fed rats (group 4) ($P < 0.05$). Naringenin supplementation alone did not produce any significant difference in the levels of SOD and catalase compared with control rats.

Levels of GPx, GST and GR in the livers, kidneys and hearts (Table 6) were significantly lower in the ethanol-fed rats (group 3) than the control rats (group 1). Tissue GPx, GST and GR levels were increased significantly on naringenin supplementation to ethanol-fed rats (group 4; $P < 0.05$). Naringenin supplementation alone did not produce any significant difference in the levels of GPx, GST and GR compared with control rats.

Levels of GSH, vitamin C and vitamin E in the livers, kidneys and hearts (Table 7) of ethanol-fed rats (group 3) were significantly lower than in control rats (group 1) ($P < 0.05$). Tissue GSH, vitamin C and vitamin E levels were increased significantly on naringenin supplementation to ethanol-fed rats ($P < 0.05$). Naringenin supplementation alone did not produce any significant difference in the levels of GSH, vitamin C and vitamin E compared with control rats.

Table 4 Effect of naringenin on levels of conjugated dienes and protein carbonyl content

	Control	Control + naringenin	Ethanol	Ethanol + naringenin
Conjugated dienes (mmoles/g tissue)				
Liver	105.94 ± 10.20[a]	99.96 ± 9.62[a]	142.80 ± 13.76[b]	112.24 ± 10.80[a]
Kidney	100.99 ± 9.72[a]	101.49 ± 9.77[a]	140.76 ± 13.55[b]	105.16 ± 10.12[a]
Heart	41.79 ± 4.02[a]	40.09 ± 3.86[a]	84.64 ± 8.15[b]	49.98 ± 4.81[c]
Protein carbonyl content (nmoles/mg protein)				
Liver	0.83 ± 0.08[a]	0.82 ± 0.08[a]	3.06 ± 0.29[b]	0.88 ± 0.08[a]
Kidney	1.89 ± 0.18[a]	1.73 ± 0.17[a]	4.28 ± 0.41[b]	1.94 ± 0.19[a]
Heart	4.99 ± 0.48[a]	4.59 ± 0.44[a]	6.12 ± 0.59[b]	5.10 ± 0.49[a]

Values are means ± SD ($n = 8$). Values that do not share a common superscript letter differ significantly ($P < 0.05$; Duncan's multiple range test).

Table 5 Effect of naringenin on the activities of superoxide dismutase and catalase

Groups	Control	Control + naringenin	Ethanol	Ethanol + naringenin
Superoxide dismutase (50% inhibition of NBT reduction/min per mg protein)				
Liver	7.7 ± 0.7[a]	7.9 ± 0.7[a]	3.5 ± 0.3[b]	7.4 ± 0.7[a]
Kidney	5.0 ± 0.4[a]	5.4 ± 0.4[a]	3.2 ± 0.3[b]	4.2 ± 0.4[c]
Heart	8.7 ± 0.8[a]	8.3 ± 0.8[a]	6.3 ± 0.6[b]	7.2 ± 0.7[c]
Catalase (μmol H_2O_2 utilised/min per mg protein)				
Liver	79.1 ± 7.6[a]	81.8 ± 7.8[a]	51.2 ± 4.9[b]	75.2 ± 7.2[a]
Kidney	46.7 ± 4.4[a]	46.9 ± 4.5[a]	35.9 ± 3.4[b]	45.1 ± 4.3[a]
Heart	50.0 ± 4.8[a]	49.3 ± 4.7[a]	38.5 ± 3.7[b]	48.0 ± 4.6[a]

Values are means ± SD ($n = 8$). NBT, nitro blue tetrazolium. Values that do not share a common superscript letter differ significantly ($P < 0.05$; Duncan's multiple range test).

Table 6 Effect of naringenin on the activities of glutathione-dependent enzymes

	Control	Control + naringenin	Ethanol	Ethanol + naringenin
Glutathione peroxidase (μg GSH utilised/min per mg protein)				
Liver	16.3 ± 1.5^{a}	16.5 ± 1.5^{a}	7.9 ± 0.7^{b}	14.4 ± 1.3^{c}
Kidney	14.4 ± 1.3^{a}	14.9 ± 1.4^{a}	7.0 ± 0.7^{b}	13.4 ± 1.2^{a}
Heart	8.4 ± 0.8^{a}	8.3 ± 0.8^{a}	5.3 ± 0.5^{b}	7.2 ± 0.6^{c}
Glutathione reductase (nmol NADPH utilised/min per mg protein)				
Liver	25.3 ± 2.4^{a}	25.1 ± 2.4^{a}	12.2 ± 1.18^{b}	22.4 ± 2.1^{a}
Kidney	26.6 ± 2.5^{a}	$24.5 \pm 2.3^{a,b}$	18.5 ± 1.7^{c}	22.4 ± 2.1^{b}
Heart	7.5 ± 0.7^{a}	6.3 ± 0.6^{b}	3.12 ± 0.3^{c}	6.00 ± 0.5^{b}
Glutathione S-transferase (μmol CDNB–GSH conjugate formed/min per mg protein)				
Liver	9.5 ± 0.9^{a}	$9.3 \pm 0.9^{a,b}$	5.6 ± 0.5^{c}	8.4 ± 0.8^{b}
Kidney	7.9 ± 0.7^{a}	7.6 ± 0.7^{a}	4.1 ± 0.3^{b}	6.1 ± 0.5^{c}
Heart	0.9 ± 0.09^{a}	0.8 ± 0.08^{a}	0.5 ± 0.05^{b}	0.7 ± 0.08^{a}

Values are means ± SD ($n = 8$). CDNB–GSH, 1-chloro-2,4-dinitrobenzene–glutathione conjugate. Values not sharing a common superscript letter differ significantly ($P < 0.05$; Duncan's multiple range test).

Table 7 Effect of naringenin on the levels of glutathione, vitamin E and vitamin C

	Control	Control + naringenin	Ethanol	Ethanol + naringenin
Glutathione (mmol/mg tissue)				
Liver	19.69 ± 1.89^{a}	20.61 ± 1.98^{a}	10.93 ± 1.05^{b}	19.07 ± 1.83^{a}
Kidney	17.65 ± 1.70^{a}	18.57 ± 1.79^{a}	11.54 ± 1.11^{b}	18.57 ± 1.79^{a}
Heart	99.26 ± 9.55^{a}	101.18 ± 9.74^{a}	78.75 ± 7.58^{b}	90.88 ± 8.74^{a}
Vitamin E (mg/100 g tissue)				
Liver	7.75 ± 0.75^{a}	7.47 ± 0.72^{a}	4.41 ± 0.42^{b}	7.61 ± 0.73^{a}
Kidney	5.30 ± 0.51^{a}	5.52 ± 0.53^{a}	3.27 ± 0.31^{b}	4.65 ± 0.45^{c}
Heart	4.07 ± 0.39^{a}	4.07 ± 0.39^{a}	2.51 ± 0.24^{b}	3.53 ± 0.34^{c}
Vitamin C (mg/100 g tissue)				
Liver	0.95 ± 0.09^{a}	0.97 ± 0.09^{a}	0.84 ± 0.08^{b}	1.01 ± 0.10^{a}
Kidney	0.80 ± 0.08^{a}	0.81 ± 0.08^{a}	0.53 ± 0.05^{b}	0.77 ± 0.07^{a}
Heart	0.67 ± 0.06^{a}	0.68 ± 0.07^{a}	0.53 ± 0.05^{b}	0.63 ± 0.06^{a}

Values are means ± SD ($n = 8$). Values that do not share a common superscript letter differ significantly ($P < 0.05$; Duncan's multiple range test).

Histological changes in the liver are shown in Figure 3. Portal inflammation, micro- and macrovesicular changes observed in ethanol-fed rats were significantly reduced on naringenin supplementation.

Discussion

Alcoholic liver disease is a common consequence of chronic alcohol misuse.[41] Studies in our laboratory are in agreement with this hypothesis, in that the ethanol-fed rats showed decreased food intake and increased oxidative stress.[42] Ethanol supplementation resulted in less weight gain than in the control rats, which may be due to the ability of ethanol to reduce the absorption of foodstuff and nutrients from the intestine.[43] Since body weight is considered to be a putative indicator of health, the weight gain on naringenin supplementation suggests beneficial protective effect against ethanol injury. The liver-to-body-weight ratio showed significant decrease in ethanol-fed rats supplemented with naringenin compared with unsupplemented ethanol-fed rats. This may be because the naringenin increases the elimination of ethanol directly from the intestines without absorption or prevents fat accumulation in the liver.

Chronic consumption of ethanol is known to cause injury to hepatocytes. The elevated activities of the serum enzymes such as AST, ALT and GGT observed in alcohol-fed rats may indicate increased permeability, damage or necrosis of hepatocytes.[44] In our study, chronic ethanol consumption caused a significant increase in the activities of AST, ALT and GGT, which could be due to severe damage to the liver cell membrane. The reduced activities of these serum enzymes on naringenin supplementation to ethanol-fed rats indicates the hepatoprotective potential of naringenin.

Lipid peroxidation is known to damage cell membranes and other critical cellular macromolecules. Lipid peroxidation is measured by the formation of TBARS, LOOH and conjugated dienes.[9,45,46] In addition to cellular lipids, studies have shown that cellular proteins may also be affected by accumulation of free radicals.

Protein carbonyl content is the most general indicator and by far the most commonly used marker of protein oxidation.[47] The carbonyl derivatives of proteins may result from oxidative modification of amino acid chains and reactive oxygen-mediated peptide cleavage. Further, Remmer *et al.*[48] have reported that the primary target of the oxygen-radical attack promoted by ethanol is on cellular

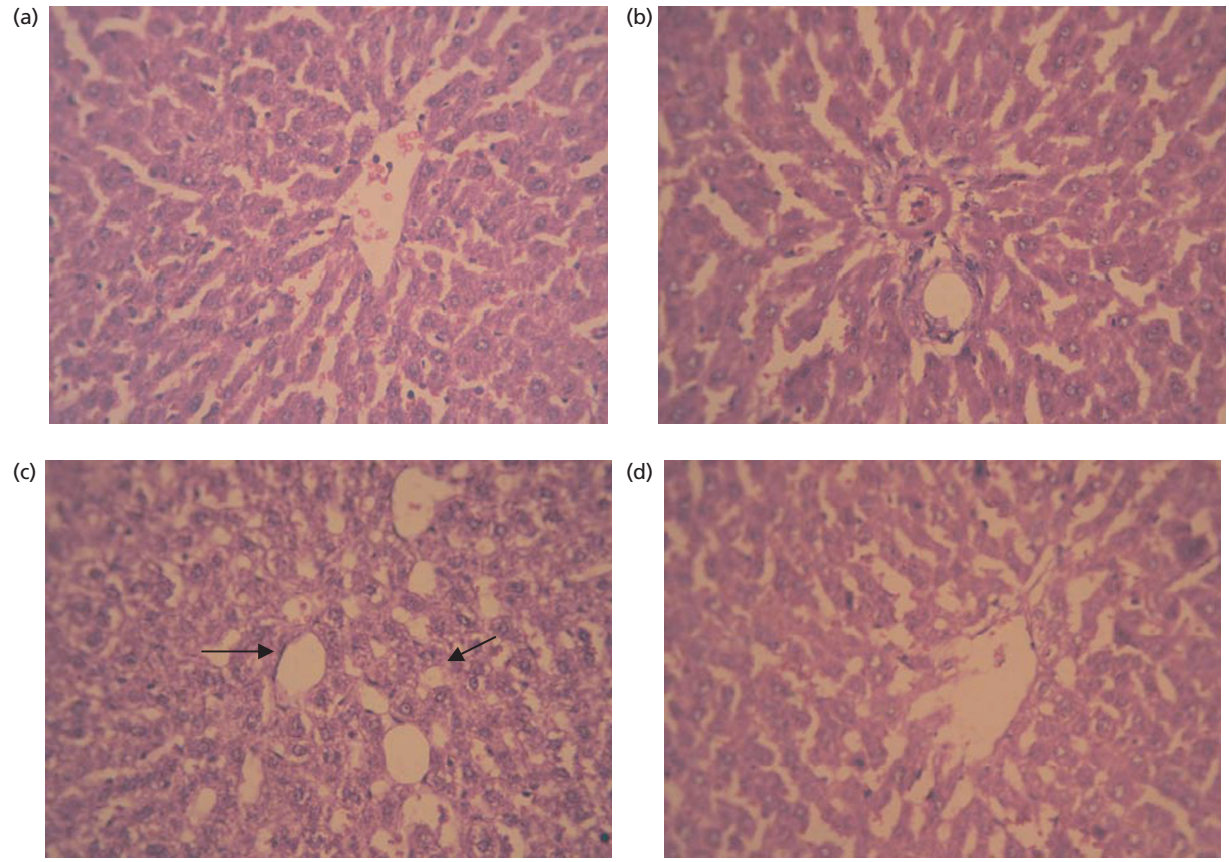

Figure 3 Liver sections (×20) stained with haematoxylin and eosin to show histopathological changes in the liver. Normal hepatocytes in (a) control rats and (b) naringenin-treated rats. (c) Livers of ethanol-treated rat show marked sinusoidal dilatation, portal inflammation, steatosis and both microvesicular and macrovesicular fatty changes, indicated by arrows. (d) Ethanol-treated rats supplemented with naringenin show normal histology with fewer and smaller fatty cysts.

proteins. Thus, increased protein carbonyl content associated with chronic ethanol consumption has been reported in both animal model and human clinical trials. In agreement with these findings, in our study, the levels of TBARS, LOOH, conjugated dienes and the protein carbonyl content were increased significantly in the tissues of ethanol-fed rats, due to either their increased production or decreased destruction.

Naringenin supplementation to ethanol-fed rats significantly decreased the levels of TBARS, LOOH, conjugated dienes and the protein carbonyl content, which may be due to its antioxidant property. Naringenin, by its free-radical-scavenging action, may prevent free-radical attack on amino acids and thus diminish the production of carbonyl groups. The polar nature of naringenin may facilitate its adherence to the lipid bilayer, which may reduce the formation of free radicals and protect the cell membrane.[49]

SOD is considered to be the first line of defence against the deleterious effects of oxygen radicals in the cells; it scavenges ROS by catalysing the dismutation of superoxide to H_2O_2.[50] Inhibition of SOD may result in an increased flux of superoxide ions into cellular compartments, which may be the reason for the increased lipid peroxidation indices observed in the rats fed ethanol in our study. Catalase acts as a preventive antioxidant and plays an important role in protection against the deleterious effects of lipid peroxidation. The activities of SOD and catalase were significantly decreased in the tissues of rats exposed to ethanol. These results confirm our previous studies.[9] Administration of naringenin elevated the activities of SOD and catalase in the tissues of ethanol-treated rats, which may reflect the ability of naringenin to reduce the accumulation of free radicals generated during ethanol-induced lipid peroxidation. Naringenin is known to inhibit malondialdehyde production from ethyl arachidonate[51] and to scavenge hydroxyl groups,[52] emphasising its antioxidant role.

Chronic ingestion of ethanol resulted in a significant decrease in GPx activity, which may be due to either free-radical-dependent inactivation of the enzyme or depletion of its co-substrates (i.e. GSH and NADPH).[53] GST plays an essential role in the liver by eliminating toxic compounds by conjugating them with glutathione. GR is concerned with the maintenance of cellular levels of GSH by catalysing the reduction of oxidised glutathione to the reduced form,[53] thus altering the GSH/GSSG ratio. GR activity was also lowered upon ethanol administration compared with control rats, which in turn may inactivate many enzymes containing-SH groups and inhibit protein synthesis.[54]

Naringenin supplementation increased the activities of GR, GPx and GST in the tissues of ethanol-treated rats, which may reflect an ability to reduce accumulation of free radicals generated during ethanol-induced lipid peroxidation. Naringenin effectively quenches the free radicals and ROS, which can be attributed to the electron-donating properties of the hydroxyl group in its B ring.[55]

Non-enzymatic antioxidants such as GSH, vitamin C and vitamin E are closely interlinked with each other and play a vital role in protecting cells from lipid peroxidation. The depleted levels of GSH in alcohol-induced toxicity may reflect increased utilisation for scavenging of toxic radicals, inhibition of its synthesis and increased turnover rate.[56] Exposure to ethanol, which triggers oxidative stress, may deplete GSH levels, and GSH biosynthesis may also be inhibited at a gene level.[57] Shaw *et al*.[58] have also shown that chronic ethanol feeding causes depletion of hepatic GSH and initiation of lipid peroxide, which correlates with the results of this study, as the hepatic GSH content was significantly decreased in ethanol-treated rats.

Vitamin C is a potent scavenger of ROS in plasma and extracellular compartments, whereas vitamin E, a major lipid-soluble antioxidant, is the most effective chain-breaking antioxidant within the cell membrane and protects membrane fatty acids from lipid peroxidation.[59] Vitamin C scavenges and destroys free radicals in combination with vitamin E and GSH.[60] Decreased plasma vitamin E has been proposed as a biological marker of ethanol-evoked oxidative stress in alcohol-fed rats,[61] and the maintenance of normal concentrations of vitamin C is essential to prevent lipid peroxidation provoked by ethanol.[62] Thus it is well known that the depletion of non-enzymatic antioxidants occurs as the severity of alcoholism increases.[63] In agreement with these reports, we observed decreased levels of GSH and vitamins E and C on ethanol feeding, which may be due to their increased utilisation for scavenging of ethanol-induced free radicals. Naringenin supplementation to alcohol-fed rats resulted in near-normal plasma levels of GSH, vitamin C and vitamin E. In this context, naringenin is known to restore GSH-dependent protection against lipid peroxidation in vitamin-E-deficient liver microsomes[55] and inhibits malondialdehyde production.[13] Moreover, Galvez *et al*.[64] have demonstrated the anti-peroxidative activities of naringenin against chemically induced lipid peroxidation in rat liver. Naringenin is also known to protect against hepatocyte autophagy and endocytosis induced by okadaic acid.[65]

Conclusions

Our data suggest that naringenin has beneficial effects against alcohol-induced tissue damage, as evidenced by decreased tissue lipid peroxidation and enhanced non-enzymatic and enzymatic antioxidants. Thus, naringenin could be exploited as a therapeutic agent and offers opportunities to develop new formulations of functional foods. Further experiments, including preclinical efficacy and study on the mechanisms of action, are warranted to fully evaluate the hepatoprotective effects of naringenin and to understand its mode of action.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

The research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

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