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Hypolipidemic effects of naringenin on phospholipase A and C during ethanol induced hepatotoxicity

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ABSTRACT

In this study the hypolipidemic effects of naringenin on liver fibrosis induced by exposure to ethanol are investigated in rats. Rats were divided into four groups, groups 1 and 2 received isocaloric glucose and 0.5% carboxymethyl cellulose (CMC); groups 3 and 4 received 20% ethanol equivalent to 6g/kg body weight every day for the total experimental period of 60 days. In addition, groups 2 and 4 were supplemented with naringenin (50mg/kg p.o) every day for the last 30 days of the experiment. The results showed significantly elevated levels/activities/expression of serum aspartate and alanine transaminases, plasma phospholipid, phospholipase A and C and decreased levels of tissue phospholipid in ethanol fed rats as compared to those of the controls. Supplementation with naringenin for the last 30 days of the experiment to ethanol-fed rats, significantly decreased the activities/expression of serum aspartate and alanine transaminases, plasma phospholipid, phospholipase A and C and decreased levels of tissue phospholipid in the liver as compared to the control rats. These findings suggest that naringenin has a protective effect on liver injury and can inhibit liver fibrosis induced by ethanol in rats. Naringenin improved the histological changes of fibrosis. The mechanism, possibly involves its hypolipidemic activity associated with its effect on inhibiting plasma phospholipid, phospholipase A and C and decreased levels of tissue phospholipid and suppressing the activation of hepatic stellate cells.

Keywords: Naringenin; Liver damage; Ethanol; Lipid Metabolizing Enzymes.

INTRODUCTION

Ethanol is a powerful inducer of hyperlipidemia. Oxidation of large amounts of alcohol results in the release of excess hydrogen ions, which alter the NAD/NADH ratio and changes the oxidation-reduction potential of liver cells. Ethanol-induced increase in the NAD/NADH ratio is a sign of major change in hepatic metabolism during ethanol oxidation [1]. The redox-related inhibition of fatty acid oxidation and the enhancement of triglyceride synthesis are the main pathogenic mechanisms in the development of alcoholic fatty liver [2]. Accumulation of lipids in the hepatocytes is the most striking manifestation of alcohol-induced liver injury. In chronic lipid accumulation, the liver cells become fibrotic, leading to impaired liver function. Ethanol also causes changes in the metabolism of lipoproteins [1].

Free radicals formed on alcohol consumption affects the permeability of hepatocytes, leading to leakage of enzymes such as serum transaminases (AST, ALT), alkaline phosphatase (ALP) [3]. An elevation in the activities of these serum enzymes is generally regarded as one of the most sensitive markers of liver damage.

Naringenin (4', 5, 7-trihydroxyflavanone) (Fig. 1) is a predominant flavonone abundant in fruits such as grapes, tangelo, blood orange, lemons, pummelo and tangerines [4]. Naringenin is the main metabolite of naringin which is the important flavonoid in *Exocarpium citri grandis*. Naringenin is used as a traditional medicine in China [5]. It has been reported to have several biological effects such as anticancer [6], antimutagenic [7], anti-inflammatory [8] antiatherogenic [9] and antifibrogenic [10] properties. Daily intake of citrus flavonoids has been estimated to be approximately 68g on an average in the USA, mainly ingested via fruit juices [11].

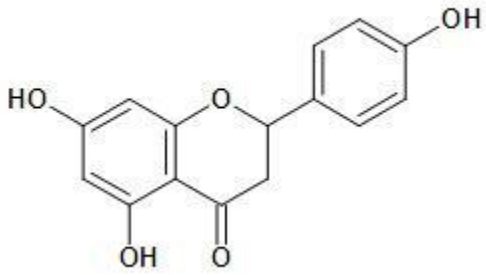


Figure:1 Struture of naringenin (4', 5, 7-trihydroxyflavonone)

Thus our present investigation was carried out to study the effect of naringenin on ethanol induced alterations in the hepatic fibrotic markers in male wistar rats.

MATERIALS AND METHODS

Chemicals and reagents

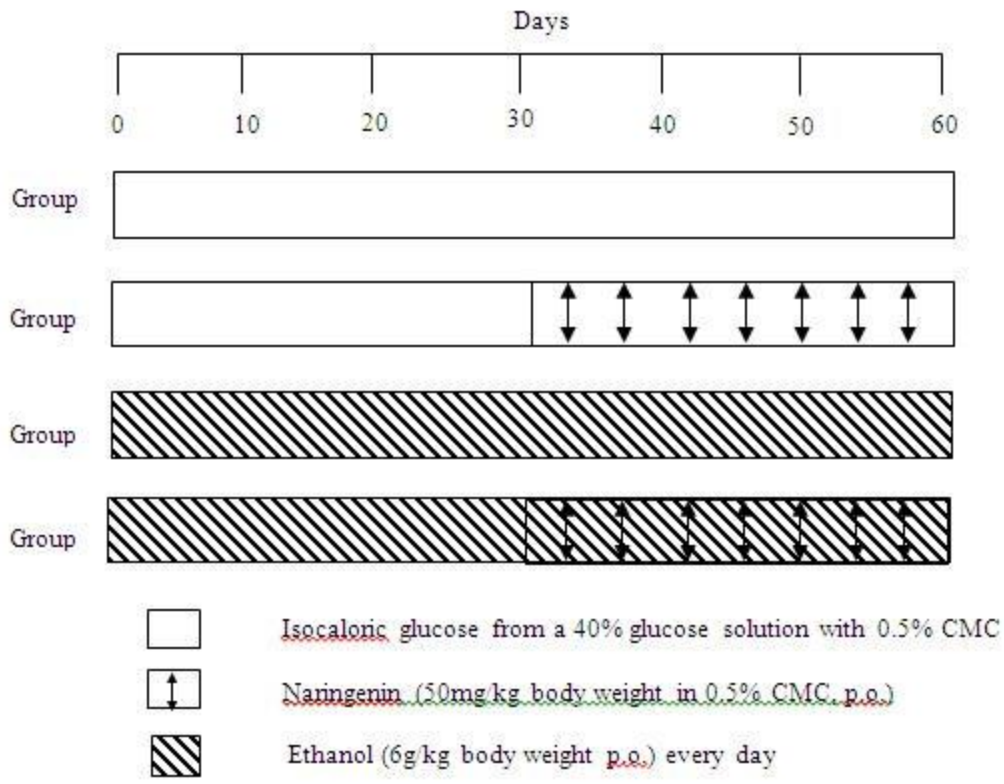
Naringenin was purchased from Sigma Chemical Co (St. Louis, MO, USA). Ethanol was obtained from E.I.D Parry India Ltd. (Nellikuppam, Cuddalore District, South India). All other chemicals used were of analytical grade and were obtained from Central Drug House Private, Ltd, Mumbai.

Animals

Adult male albino Wistar rats (150-170g) were assayed from the Central Animal House, Rajah Muthiah Medical College and Hospital, (RMMC&H), Annamalai University. The rats were housed in plastic cages under controlled conditions of 12-h light-dark cycle, 50% humidity and temperature of 28°C. They were all fed a standard pellet diet (Lipton Lever Mumbai, India) and water *ad libitum*. 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.

Study design

2 Diagrammatical representation of the experimental protocol



Animals were divided into four groups of 8 rats each and all were fed the standard pellet diet. Rats in groups 1 and 2 received isocaloric glucose from a 40% glucose solution and 0.5% CMC. Animals in groups 3 and 4 received 20% ethanol (equivalent to 6g/kg body weight) as an aqueous solution by intragastric intubation for 60 days as described previously [12]. At the end of this period, the dietary protocol of group1 and 3 animals was unaltered. However, group 2 animals received naringenin (50mg/kg body weight/day) suspended in 0.5% CMC for the next 30 days, and group 4 animals continued to receive ethanol every day along with naringenin as in group 2 for the next 30 days. The total experimental duration was 60 days. The study design is shown in Figure 2.

The animals were then fasted overnight, anesthetized with an intramuscular injection of ketamine hydrochloride (30mg/kg) and blood samples were collected by retro-orbital puncture. Blood samples were collected in heparinized tubes and centrifuged for the separation of plasma.

Extraction of lipids

Lipids were extracted from tissues by the method of Folch et al. [13] using chloroform - methanol mixture (2:1 v/v). A known weight of tissue was homogenized in 7.0 mL of chloroform-methanol using potter Elvehjam homogenizer. The contents were filtered into a previously weighed side arm flask; residue on the filter paper was scraped off and homogenized with 14 ml of chloroform- methanol mixture. This was again filtered into the side arm flask and the residue was successively homogenized in chloroform–methanol (2:1 v/v) and each time this extract was filtered.

The pooled filtrates in the flask were adjusted to a final volume using chloroform-methanol (2:1 v/v) and evaporated to dryness with a constant weight. The dried residue of lipid was dissolved in 5 ml of

chloroform - methanol mixture (2:1 v/v) and transferred into a centrifuge tube; 2 ml of 0.1M potassium chloride was added, shaken well and centrifuged. The upper aqueous layer containing gangliosides was discarded.

The chloroform layer was mixed with 1.0 ml of chloroform–methanol–potassium chloride mixture (1:10:10 v/v) and then centrifuged. This washing was repeated thrice and each time, the upper layer was discarded. The lower layer was made up to 5.0 ml and used for the analysis of total cholesterol, triglycerides, free fatty acids, phospholipids and α-tocopherol.

Biochemical estimations

Serum AST (EC 2.6.1.1) and ALT (EC 2.6.1.2) were assayed using a diagnostic kit based on the method of Reitman and Frankel. [14]. Phospholipids content was estimated by the method of Zilversmit and Davis [15].

Assay of phospholipase A

The enzyme activity was determined by the method of Rimon and Shapioro [16].

Assay of phospholipase C

The activity of the enzyme was determined according to the method of Kleiman and Land [17] (1969) by estimating the amount of phosphorylchloride liberated.

Statistical analysis

Data were analyzed 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 and the data are represented as mean ± SD for the absolute values or percent of controls as indicated in the vertical axis legends of figures. The statistical significance of differential findings between the experimental groups and control was determined.

RESULTS

Effect of naringenin and ethanol on liver marker enzymes

Table 1 shows the activities of serum AST and ALT. The activities of both the enzymes were significantly increased in ethanol fed rats as compared to the control rats. Supplementation with naringenin to ethanol-fed rats (group 4) significantly decreased the liver marker enzymes as compared to the unsupplemented ethanol fed rats (group 3; $P < 0.05$).

Table 1 Effect of naringenin and ethanol on hepatic marker enzymes of control and experimental rats

GROUPS	Aspartate transaminase (IU/L)	Alanine transaminase (IU/L)
Control	79.84±7.68[a]	28.86±2.77[a]
Control + Naringenin	82.13±7.90[a]	30.81±2.96[a]
Ethanol	112.40±10.81[b]	60.38±5.81[b]
Ethanol + Naringenin	87.27±8.40[a]	32.76±3.15[a]

Values are mean ± S.D. of eight rats in each group. Values not sharing a common superscript letter differ significantly at $p< 0.05$ (Duncan's multiple range test.

Effect of naringenin and ethanol on lipids and lipid metabolizing enzymes

Table 2 shows the concentrations of phospholipids (PL) in the plasma of control and experimental animals. Plasma concentrations of PL were significantly higher in ethanol fed rats (group 3; $p < 0.05$) as compared to the control rats. Supplementation with naringenin to ethanol fed rats markedly lowered (group 4; $p< 0.05$) the concentrations of PL as compared to the ethanol alone fed rats. Naringenin supplementation alone (group 2) did not produce any significant change in the activities of PL as compared to the control rats. In contrast, there was a significant decrease in the levels of PL in the liver, kidneys and heart of ethanol fed group (group 3; $p < 0.05$) which on naringenin supplementation showed increased levels (group 4; $p < 0.05$) as compared to the ethanol alone fed rats. Naringenin supplementation alone (group 2) did not produce any significant change in the levels of liver, kidney and heart FFA as compared to the control rats.

Table 2. Effect of naringenin and ethanol on serum and tissue phospholipids of control and experimental rats

Groups	Serum (mg/dL)	liver	kidney	Heart
		Phospholipids (mg/100g tissue)		
Control	88.8±8.55[a]	1765.1±169.9[b]	1449.0±139.4[b]	87.27±8.40[a]
Control+ Naringenin	87.8±8.45[a]	1825.3±175.7[b]	1516.8±146.0[b]	32.76±3.15[a]
Ethanol	127.6±12.28[b]	1265.4±121.8[a]	1213.8±116.8[a]	1528.3±147.1[b]
Ethanol+ Naringenin	1033.8±99.5[b]	1038.5±99.9[b]	731.54±70.41[a]	1021.22±98.30[b]

Values are mean ± S.D. of eight rats in each group. Values not sharing a common superscript letter differ significantly at p< 0.05 (Duncan's multiple range tests).

Table 3: Effect of naringenin and ethanol on liver phospholipase A and C of control and experimental rats.

GROUPS	Phospholipase A (mEq of ester hydrolyzed/min/mg protein)	Phospholipase C (mmoles of phosphorylcholine formed/min/mg protein
Control	0.03±0.003[a]	0.71±0.068[a]
Control + Naringenin	0.04±0.004[a]	0.77±0.074[a]
Ethanol	0.08±0.008[c]	1.08±0.103[b]
Ethanol + Naringenin	0.049±0.004[b]	0.81±0.078[a]

Values are mean ± S.D. of eight rats in each group. Values not sharing a common superscript letter differ significantly at p< 0.05 (Duncan's multiple range test

DISCUSSION

Chronic consumption of ethanol is known to cause injury to hepatocytes. The elevated activities of the serum enzymes such as AST and ALT observed in alcohol-fed rats may indicate increased permeability, damage or necrosis of hepatocytes [18]. In our study, chronic ethanol consumption caused a significant increase in the activities of AST and ALT, which could be due to severe damage to the liver cell membrane. The reduced activities of these serum enzymes on naringenin supplementation of ethanol-fed rats indicates the hepatoprotective potential of naringenin.

Phospholipids (PL) are basic components of cell membranes, mainly acting as regulators of membrane-bound enzymes and are also involved in membrane transport processes. This PL is important in determining the pathology of alcoholism [19]. Our results show significantly decreased levels of PL in the liver, kidney, heart and increased levels of PL in the plasma of ethanol fed rats as compared to the control group. The decreased levels indicate greater PL degradation [20] and can result in the modification of the composition, structure, and stability of cellular membranes and later membrane dysfunction [21]. Moreover PL are the primary targets of lipid peroxidation, which can be altered by ethanol [22]. Rukkumani et al., [21] have also reported a decrease in the PL content of the liver and kidney and an increase in the plasma levels in ethanol fed rats. The decrease in the levels of PL in the liver, kidney and heart of ethanol fed rats may be due to increased activity of phospholipases and increased degradation of muscle PL [23].

Our results show increased activity of phospholipases A and C in alcohol-fed rats as compared to control rats. In this context, Zhihong et al., [23] have found that chronic exposure to ethanol leads to a progressive increase in membrane phospholipase A2 activity. Ethanol ingestion also induces an increase in microsomal phospholipase C activity that correlates with an increase in the microsomal CYP2E1 and a decrease in microsomal arachidonic acid. Aruna et al., [2] have also shown increased activities of phospholipases A and C in the liver of ethanol administered rats.

Naringenin supplementation at the dose of 50 mg/kg body weight/day along with ethanol elevated the levels of PL to near normal levels and prevented the increase in the activities of phospholipases A and C. The results demonstrate the ability of naringenin to repair the cellular membrane damage caused by ethanol and its metabolic products, thereby preserving the membrane integrity. Huong et al., [24] have also reported a decrease in the serum PL content on naringenin supplementation to control male ICR mice.

CONCLUSION

The results of the study demonstrate the potential beneficial effects of naringenin on alcoholic liver damage. Naringenin exerts its hypolipidemic effect against ethanol induced toxicity by modulating the expression of transforming growth factor-β (TGF-β) and lipid changes.

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