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Effects of naringenin on fibrotic markers (MMPs and TIMP1) during ethanol induced hepatotoxicity

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ABSTRACT

To investigate the antifibrotic effects of naringenin on liver fibrosis induced on exposure to ethanol in rats. Rats were divided into four groups, groups 1 and 2 received isocaloric glucose and 0.5% carboxy methyl 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, matrix metallo proteinases (MMPs) and tissue inhibitor of metallo proteinases (TIMPs) in ethanol fed rats as compared to those of the control. Ethanol administration caused liver damage and fibrosis as evidenced by liver histology and various fibrogenic factors. 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, MMP2, MMP9 and TIMP1 in the liver as compared to the control rats. These findings suggest that naringenin has 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 anti-inflammatory activity associated with its effect on inhibiting MMP2, MMP9 and TIMP1and suppressing the activation of hepatic stellate cells.

Keywords: Naringenin, Liver damage, Ethanol, Fibrogenic factors, Histo chemistry.

INTRODUCTION

The World Health Organization reports about two billion alcohol consummers worldwide and 76.3 million people with diagnosable alcohol use disorders. Globally, alcohol causes 3.2% deaths for all causes (1.8 million deaths annually) and accounts for 4.0% of disease burden [1]. Excessive ethanol consumption is an important preventable cause of morbidity. The association between ethanol intake and alcoholic liver disease has been well documented, though liver cirrhosis develops only in a small proportion of heavy drinkers. Twenty percent of alcoholics and heavy drinkers develop fatty liver and only 10 to 15 percent of these will develop cirrhosis. The point prevalence of cirrhosis is 1% in people who drink 30 to 60 g of alcohol a day, and up to 5.7% in those consuming 120 g daily. In this matter, mortality studies have

demonstrated that heavy drinkers and alcoholics die from cirrhosis at a much higher rate than the general population [2].

Alcoholic liver diseases progress from fatty infiltration, inflammation, and fibrosis, and ultimately lead to irreversible damage-cirrhosis. Liver fibrosis is a wound-healing process characterized by accumulation of extracellular matrix (ECM) proteins, especially collagen types I and III, as well as an increase in other ECM constituents, which reflects alteration in the synthesis of matrix proteins, their degradation, or both [3]. Elevated levels of collagen in the liver have been documented in bile duct obliteration-induced liver injury [4] and alcoholic liver disease [5]. Numerous proteases have been implicated in the proteolytic degradation of collagen and other ECM components; the most prominent among them are matrix metalloproteinases (MMPs).

MMPs or matrixins are zinc- and calcium-dependent multidomain endopeptidases that, with few exceptions, share a basic structural organization comprising propeptide, catalytic hinge, and C-terminal (hemopexin-like domain). Over 60 MMPs have been sequenced to date, of which at least 17 are human MMPs [6]. MMPs collectively can degrade ECM proteins, such as collagens, proteoglycans, elastin, laminin, fibronectin, and other glycoprotein [7]. MMPs are also involved in tissue remodeling during fibrotic and/or inflammatory processes, embryonic development, morphogenesis, angiogenesis, and tissue repair [8]. The activities of most matrixins are very low or negligible in the normal steady-state tissues, but expression is transcriptionally controlled by inflammatory cytokines, growth factors, hormones, and cell–cell and cell–matrix interactions. Matrixins are also regulated by activation of the precursor zymogens and inhibition by endogenous inhibitors: tissue inhibitor of matrix metalloproteinases (TIMPs).

Currently, four TIMPs (TIMP 1 to 4) have been identified in vertebrates. The increasing evidence also supports that there is an over expression of TIMP-1 and TIMP-2 in hepatic fibrosis, while the role of TIMP-3 and TIMP-4 in liver diseases is so far unknown [9]. Besides their MMP inhibitor activity, TIMPs exhibit cell growth-promoting, antiapoptotic, steroidogenic, and embryogenic activities [10]. TIMPs are also controlled during tissue remodeling and physiological condition to maintain a balance in the metabolism of the ECM. Disruption of this balance may result in diseases associated with uncontrolled turnover of matrix components. Matrixins and their inhibitors have received considerable attention as targets for drug development because of their potential role in connective tissue degradation associated with fibrosis [11].

Naringenin (4', 5, 7-trihydroxyflavanone) (Fig. 1) is a predominant flavonone abundant in fruits such as grapes, tangelo, blood orange, lemons, pummelo and tangerines [12]. 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 [13]. It has been reported to have several biological effects such as anticancer [14], antimutagenic [15], anti-inflammatory [16] antiatherogenic [17] and antifibrogenic [18] properties. Daily intake of citrus flavonoids has been estimated to be approximately 68g on an average in the USA, mainly ingested via fruit juices [19].

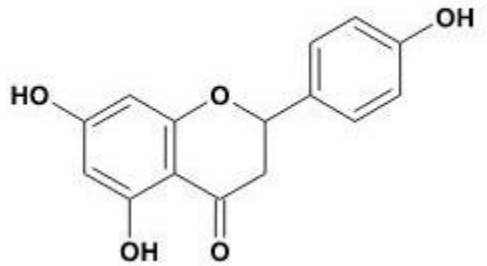


Figure: 1 Struture of naringenin (4', 5,7-trihydroxyflavonone)

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

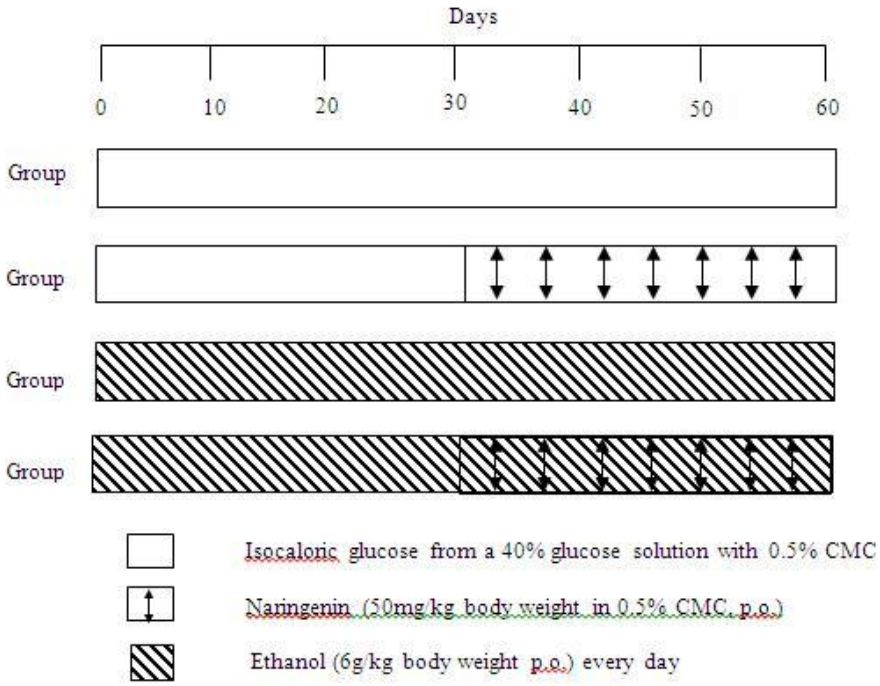
Animals

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 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

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 bodyweight) as an aqueous solution by intragastric intubation for 60 days as described previously [20]. At the end of this period, the dietary protocol of group1 and 3 animals were unaltered. However, group 2 animals received naringenin (50mg/kg bodyweight/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.

Figure: 2 **Diagrammatical representation of the experimental protocol**



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

Western Blot Analysis

Liver proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane. Briefly, the presoaked membranes were blocked in 5% non-fat dry milk in Tris –buffered saline (TBS) for 1 h at room temperature, membranes were incubated with primary antibody MMP-2 (Mouse monoclonal antibody, Catalogue no. SC-13594, (2C1), Santa cruz Biotechnology, INC). MMP-9 (Goat polyclonal antibody, Catalogue no.SC-6840, (C20), Santa cruz Biotechnology, INC). TIMP-1 (Rabbit polyclonal antibody, Catalogue no.SC-5538), (H150) Santa cruz Biotechnology, INC)] (1:1000 dilution) in PBS containing 0.2% Tween-20 overnight at 4°C. After 3–5 washes, the membranes were incubated with secondary antibodies (1:5000 dilutions) [MMP-2 (Goat–anti rabbit IgG HRP; Catalogue no. SC-2001, Santa cruz Biotechnology, INC). MMP-9 (Donkey-antigoat IgG HRP; Catalogue no.SC-2020, Santa cruz Biotechnology, INC). TIMP-1 (Goat antimouse IgG HRP; Catalogue no.SC-2005), Santa cruz Biotechnology, INC)] for 1 hr. Protein-antibody complexes were detected by the addition of diaminobenzidine (DAB) as substrate and the intensities of bands were captured with a Gel Documentation system (Bio-Rad Lab, Hercules, CA), and quantified using Quantity One software (Bio-Rad Lab, Hercules, CA). β-actin was used as a loading control. Blot was performed in triplicate.

Biochemical estimations

Serum AST (EC 2.6.1.1) and ALT (EC 2.6.1.2) were assayed using a diagnostic kits based on the method of Reitman and Frankel. (1957).

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 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 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.

Table 1 shows the activities of serum AST and ALT. 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; $P < 0.05$).

Effect of naringenin and ethanol on MMP-2, MMP-9 and TIMP1

Immunoblot analysis was performed for MMP-2 and MMP-9 in different experimental animal groups (Fig.4. A and B). The results showed induction of MMP-2 and MMP-9 expression in ethanol fed rats (group 3) as compared to control rats (group 1). Naringenin challenge along with

ethanol exhibited notable down regulation of both MMP-2 and MMP-9 expression. Western blot analysis was performed for TIMP-1 in different experimental animal groups. The results showed induction of TIMP-1 expression in ethanol fed rats (group 3) as compared to control rats (group 1). Naringenin challenge along with ethanol exhibited marginal down regulation of TIMP-1 expression.

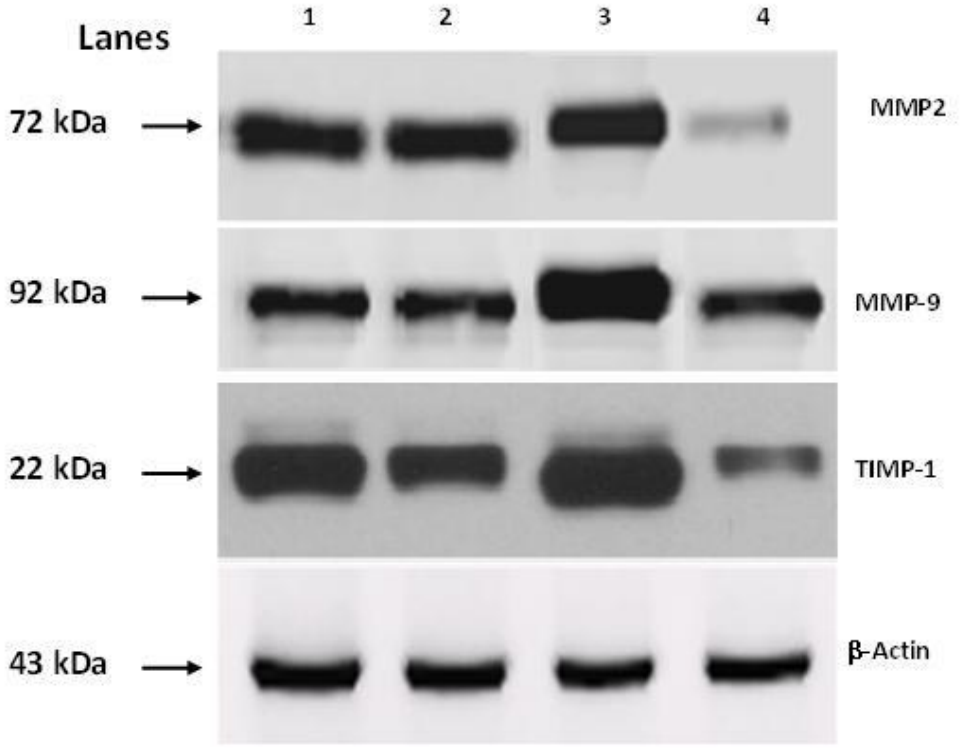
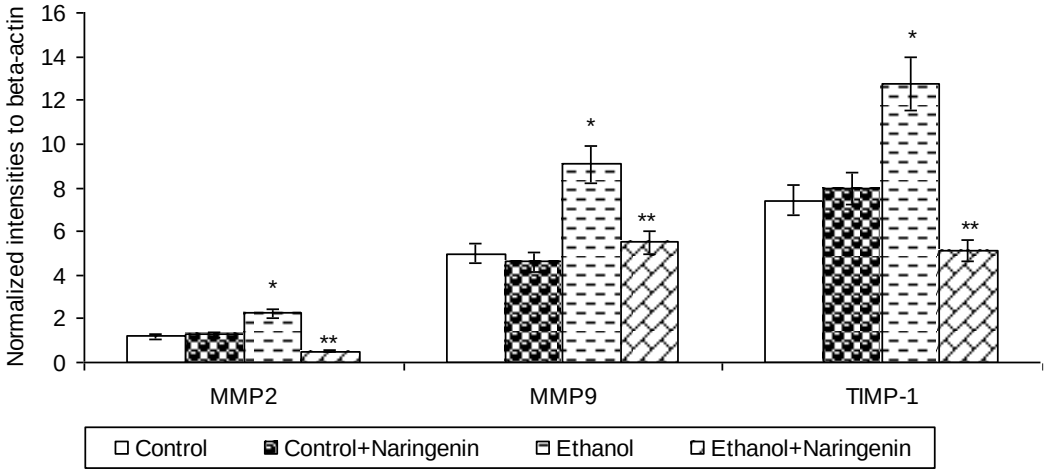


Figure: 3 Effect of naringenin and ethanol on MMP-2, MMP-9 and TIMP-1 in the liver of control and experimental rats.



Expression of MMP-2, MMP-9 and TIMP-1 in the liver of control and experimental rats. (A). western blot analysis liver MMP-2, MMP-9 and TIMP-1 expression of four experimental groups (Lane 1: Control; Lane 2: Control+naringenin; Lane: 3 Ethanol; Lane 4: Ethanol+naringenin) (B). Each lane was analyzed by densitometry and the expression in control was considered as normalized intensities to β-actin. β-actin was used as loading control. The column heights are the means ± SDs of

six determinants. SDs are shown as error bars. * significantly ($p<0.05$) compared with ethanol and control groups, significantly ** ($p<0.01$) compared with control groups.

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 [21]. 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 to ethanol-fed rats indicates the hepatoprotective potential of naringenin.

Hepatic fibrosis is a prepathologic state of cirrhosis that occurs as a consequence of severe liver damage in diverse chronic liver diseases. Cirrhosis is known to play a role in the several types of liver cancer. Fibrosis reflects increased deposition of the physiological components of ECM. The liver ECM is a passive structural support since it plays a key roles in providing a structural framework and maintaining the differentiated phenotype and normal function of hepatocytes, sinusoidal endothelial and stellate cells [22]. A major group of enzymes responsible for ECM degradation is the MMP family, including collagenase, gelatinase, and stromelysin. MMPs and TIMPs play an important role in various fibrotic diseases [23] expression of TIMP-1 is increased in hepatic fibrosis and reflects the changes of liver fibrosis [24]. Gelatinase is comprised of gelatinase A (MMP-2) and gelatinase B (MMP-9) and can degrade main components of the ECM (John and Tuszynski, 2001). TIMP-1 is a natural tissue inhibitor of gelatinases and can inhibit the gelatinolytic activities of gelatinases [25].

In our study, we observed increased MMP expression in the liver of ethanol fed rats. Two possible mechanisms can be postulated for the increased activities of MMPs during alcohol ingestion. First, ethanol oxidation via CYP2E1 plays a key role in the production of reactive oxygen species (ROS) in the liver, which has been reported to activate latent resident MMP enzymes [26]. The hepatic mRNA expression for the MMPs increases with the amount of connective tissues, suggesting that in advancing fibrosis, the production of enzymes for matrix degradation also increases. In this context, Herbst et al., [27] have reported an up-regulation of MMP-2 and MMP-9 expression in fibrotic versus normal liver. Moreover, Saravanan et al., [28] demonstrated elevated MMP-2 and MMP-9 expression in rat cirrhosis.

The second possible mechanism for the increased induction of MMPs might be due to production of the cytokines and other inflammatory mediators during liver injury. Reports have shown that the promoter region of MMP genes is induced by growth factors, cytokines, and other environmental factors in contact with ECM [29]. Among cytokines, TGF-β plays a central role in fibrosis, contributing to both influx and activation of inflammatory cells as well as activation of stellate cells. Activated stellate cells in turn become an important source of TGF-β, which are the major producers of MMPs during liver injury [30]. Possibly, from the above mechanism, the increased expression of MMPs observed in the alcohol administered group might be due to the increased production of ECM and/or ROS.

The results of the present study indicate that treatment with naringenin significantly decreased the activities of MMPs as compared to the ethanol fed group. Naringenin is an effective antioxidant and has been previously reported to scavenge free radicals [31]. Reactive oxygen intermediates are thought to contribute to hepatic injury via generation of lipid peroxides, which may have a direct stimulatory effect on matrix production by activated HSC. Naringenin might have attenuated the collagen deposition and MMP expression though, at least in part, by its antioxidant effects. It has been reported that naringenin inhibits cytochrome P450, in particular CYP2E1, in the hepatocytes [32] and consequently decreases ROS-mediated MMP expression in the liver. Furthermore, a report has shown that naringenin effectively decreased collagen levels in dimethyl nitrosamine (DMN)-induced toxicity. In addition, naringenin by its antiinflammatory property inhibits cytokines, which plays a crucial role in fibrosis. In our study naringenin might have attenuated the inflammatory response associated with alcohol-mediated hepatic injury and subsequently decreased MMPs. From the above findings, it could be speculated that naringenin, via

regulating HSC activation, decreased hydroxyproline formation, and declined the secretion of MMPs, thereby bringing down the MMP expression to near normal, and thus helping in the maintenance of the structural integrity of the tissue.

The extent of ECM remodeling depends upon the ratio of active MMPs to TIMPs. A ratio in favour of MMP activity results in ECM degradation, whereas a ratio favouring TIMPs inhibits ECM degradation and is permissive for ECM deposition. An increase in the TIMP level is one of the pathogenic mechanisms of liver fibrosis. Cytokines, which are increased during liver fibrosis, which are known to induce TIMPs. Herbst et al., [27] by performing an in vitro study has shown that TGF-β and IL-6, increased during fibrosis, which are known to induce TIMP expression in HSCs. In our study, we have observed increased levels of TIMPs in alcohol fed rats. Normally, TIMPs are reported to inhibit the activities of MMPs. Inspite of an increase in the MMP expression pattern, there was a concomittant increase in the TIMP levels in the ethanol fed rats. The underlying mechanism, for this could the activation of proMMPs by TIMP. Thus the increased expression of MMPs during the progressive stage of fibrosis (i.e., in the ethanol group) overwhelms the TIMPs. Hence, we could have observed increased activities of MMPs and TIMPs in the ethanol fed rats. The results of the present study indicate that supplementation with naringenin significantly decreased the levels of TIMP-1 as compared to the ethanol fed rats. Naringenin has been shown to decrease liver injury through its antioxidant, anti-inflammatory properties, and regulation of HSC and modulation of the MMP expression pattern. Consequently, from the above-mentioned properties, it is conceivable that naringenin might have maintained the MMP and TIMP ratio by decreasing the levels of TIMPs and thus protecting the structural integrity of tissues. Moreover, naringenin might react with some other ligands in the system and thus might not be completely available for quenching free radicals.

CONCLUSION

The results of the study demonstrate the potential beneficial effects of naringenin on alcoholic liver fibrosis. Naringenin exerts its antifibrotic effect against ethanol induced toxicity by modulating the expression of matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinases (TIMPs).

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