Rzeszów University Press 2025
ISSN 2544-1361 (online)
doi: 10.15584/ejcem.2025.3.14

http://www.ejcem.ur.edu.pl
European Journal of Clinical and Experimental Medicine
Eur J Clin Exp Med 2025; 23 (3): 626–632

ORIGINAL PAPER

Therapeutic effect of naringenin on ethanol-induced liver fibrosis in rats

Jayachitra Jayaraman[1], Nalini Namsivayam[2], Kavitha Manivannan [3], Uma Sundaresan [4]

[1] P.G and Research Department of Biochemistry, Rabiammal Ahamed Maideen College for Women, Tiruvarur, Tamilnadu, India
[2] Faculty of Science, Department of Biochemistry and Biotechnology, Annamalai University, Tamilnadu, India
[3] Department of Microbiology, Tharb Camel Hospital, Qatar
[4] School of Bioengineeing, SRM University, Tamilnadu, India

ABSTRACT

Introduction and aim. Liver fibrosis, a progressive disorder marked by the surplus buildup of extracellular matrix proteins, frequently results from long-term ethanol intake. Our aim of study is to investigate how naringenin's antifibrotic properties impact ethanol induced liver fibrosis in rats.

Material and methods. Rats were divided into four groups: groups 1 and 2 received carboxymethylcellulose (CMC) containing 0.5% glucose, while groups 3 and 4 received 20% ethanol (6 g/kg of body weight) over a 60-day period. In the last 30 days, naringenin (50 mg/kg) was administered each day to groups 2 and 4.

Results. Rats treated with ethanol exhibited liver damage and fibrosis, leading to elevated serum concentrations of aspartate and alanine transaminases. Expression levels of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), alpha-smooth muscle actin (α-SMA) and related proteins were compared with the control group.

Conclusion. Ethanol-fed rats showed an increase in serum matrix metalloproteinases, TIMPs, α-SMA, transaminases, and other proteins compared to the control group. The administration of ethanol led to liver damage and fibrosis. During the final 30 days of the trial, the inclusion of naringenin in the diets of rats notably reduced the levels of α-SMA, MMP2, MMP9, TIMP1, along with serum levels of aspartate and alanine transaminase levels and significant differences were observed compared to control group.

Keywords. ethanol, fibrogenic factors, histopathology, liver damage, naringenin

Introduction

The WHO estimates two billion alcohol consumers worldwide, with 76.3 million having alcohol use disorders, leading to significant disease burden and mortality.[1,2] A percentage of heavy drinkers develop liver cirrhosis, making excessive use of ethanol an avoidable cause of illness. Alcoholics and heavy drinkers are more likely to develop cirrhosis.[3] Worldwide, excessive alcohol use causes cirrhosis, liver fibrosis, liver injury, fat accumulation, cell death, and decreased liver cell renewal, among other sociomedical and public health problems. The importance of prevention and treatment in public health is growing.[4-6] In this cycle, cells change into myofibroblasts, the matrix expands, fat, inflammation, fibrosis to permanent harm, and collagen types I and III in ECM(extracellular matrix), build up due to changes in the assembly or breakdown, resulting in 4,000 deaths annually in the UK, with two-thirds occurring before

Corresponding author: Jayachitra Jayaraman, e-mail: jayachitra.manikandan@gmail.com

Received: 7.06.2024 / Revised: 3.08.2024 / Accepted: 11.04.2025 / Published: 30.09.2025

Jayaraman J, Namsivayam N, Manivannan K, Sundaresan U. Therapeutic effect of naringenin on ethanol-induced liver fibrosis in rats. *Eur J Clin Exp Med.* 2025;23(3):626–632. doi: 10.15584/ejcem.2025.3.14.

the age of 65.[7-10] Matrix metalloproteinases (MMPs) are proteases influencing various processes. in tissue remodeling and the breakdown of excess collagen. 17 of the more than 60 MMPs that have been sequenced are human MMPs. Growth factors, hormones, interactions between cells and the cell matrix, and inflammatory cytokines all alter their activity through transcription.[11,12]

Endopeptidases known as matrixins degrade extracellular matrix (ECM) proteins such as collagen and fibronectin, which are crucial for tissue remodeling, embryonic growth, and repair.[13] Their expression is regulated by growth factors, hormones, inflammatory cytokines, and interactions among cells within a matrix. In vertebrates, four TIMPs (TIMP 1–4) have been discovered; their functions in liver diseases are still not well understood and they are overproduced in liver fibrosis. TGF-β1 is a key factor in the progression and development.[14-21] Naringenin, a flavanone in citrus fruits, is used in traditional Chinese medicine and shows various health benefits, including anti-inflammatory and anticancer effects, with adults consuming approximately 68 g daily.[22]

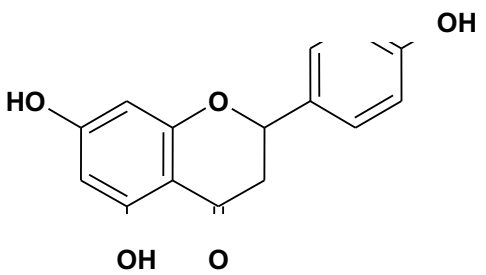

Fig. 1. Naringenin structure

Aim

The study investigates the effects of naringenin's impact on liver fibrosis from ethanol in rats.

Material and methods

Chemicals and reagents

Naringenin was sourced from Sigma, ethanol from E.I.D Parry, and antibodies for MMP and TIMPs from Santa Cruz.

Animals

Adult male albino Wistar rats (150–170 g) were obtained from Annamalai University and kept in regulated conditions featuring a 12-hour light/dark cycle, 50% humidity, and a temperature of 28°C, with the experimental protocols sanctioned by the Institutional Animal Ethics Committee (Reg. No. 160/1999/CPCSEA/557).

Study design

The study categorized the animals into four groups, each with eight rats on a standard pellet diet. Groups 1 and 2 received isocaloric glucose, while groups 3 and 4 received 20% ethanol for 60 days. Subsequently, group II received naringenin with CMC, and group IV received ethanol alongside naringenin (50 mg/kg/day), based on a previously published study, ensuring similar dietary conditions for groups 1 and 3. Since ethanol-induced liver fibrosis is prevalent in India, ethanol induction is used instead of CCl4 and bile duct ligation. The entire experiment lasted for sixty days. Fig. 2 shows the study design. Retroorbital punctures were used to obtain blood samples.

Animals received 30 mg / kg of ketamine intramuscularly, starved overnight, and blood samples were centrifuged in heparinized tubes to separate plasma.

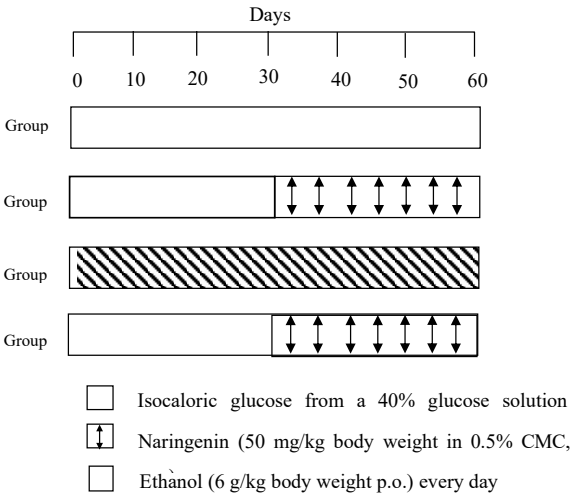

Fig. 2. Study design

Quantitative real-time PCR

Total RNA was obtained from tissues using the QIAGEN Total RNA Kit, and subsequently, DNase treatment was performed to eliminate genomic DNA. Reverse transcription utilized 200 units of Superscript reverse transcriptase, 2 μg RNA, and 0.25 μg random hexamer, incubated at 25°C and 50°C. Real-time PCR employed 3 μL cDNA with SYBR Green Master Mix and specific primase.

PCR primer design

S.No.	Symbol	Forward primer	Reverse primer
1	Alpha-SMA	CAGTTCTTTGGCCACACTCA	CCAGGATTAAGGCCGATGTA

Western blot analysis

Liver proteins were isolated using SDS-PAGE, followed by coating a PVDF membrane with the proteins. The membranes were immersed in 5% non-fat dry milk in TBS for one hour at room temperature prior to the addition of primary antibodies: MMP-9, TIMP-1, and MMP-2. Incubation overnight with Santa Cruz Biotechnology antibodies (1:1000 dilution) was carried out at 4°C, and secondary antibodies (1:5000 dilution) were added after three to five washes.

Liver histopathology and sirus red staining

After sacrifice, the liver is extracted and preserved in 10% formal saline for 48 hours for histological analysis. Subsequently, it was dehydrated with ethanol and water, cleaned utilizing xylene, and encased in paraffin. Sections, cut to a thickness of 5–6 µm, are stained with hematoxylin and eosin, mounted in neutral DPX, and analyzed microscopically.

Masson trichrome staining method for collagen

Liver slices were stained for collagen using Masson's trichrome, a method from 1929. Acid fuchsin, phosphomolybdic acid, glacial acetic acid, potassium dichromate, concentrated HCl, and quick green were among the reagents. The materials were dehydrated with ethanol after being absorbed in trays with distilled water, ethanol, and xylene. The slides were mounted in a resinous substance after cleaning with xylene.

Statistical analysis

Results presented as mean±SD for eight rats; significance $p<0.05$ (SPSS software, IBM, Armonk, NY, USA).

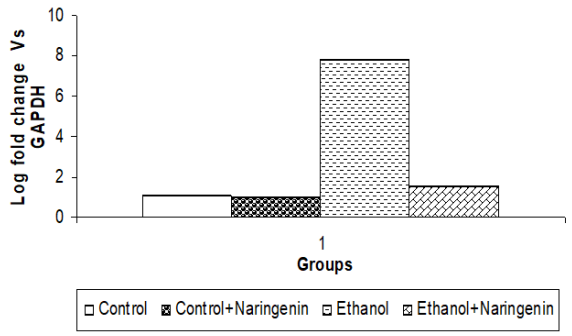

Fig. 3. Effect of narigenin and ethanol on alpha-SMA in rat livers

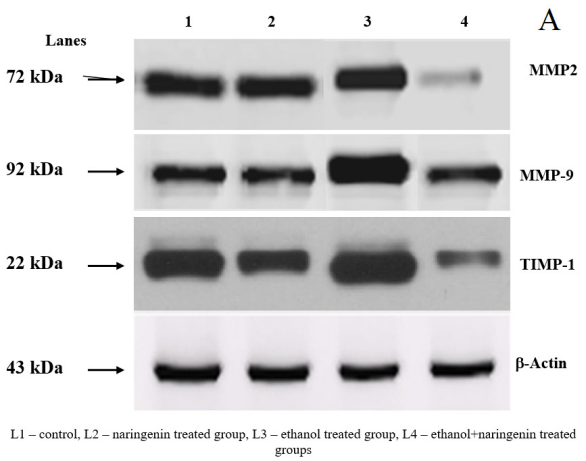

Fig. 4A. Effect of narigenin and ethanol on MMP-2, MMP-9, and TIMP-1 in the liver of control and experimental rats

Results

Hepatic fibrosis, an early pathological condition of cirrhosis, is caused by significant liver damage in several chronic liver illnesses. Cirrhosis is recognized to contribute to several types of liver cancer. Numerous experimental animal groups participated in immunoblot investigations of MMP-2 and MMP-9 (Figs. 3, 4A and 4B).

The results showed that rats given ethanol (group 3) had higher blood levels than rats in the control group (group 1). The combined administration of ethanol and naringenin resulted in a considerable reduction in the expression of MMP-2 and MMP-9. Western blot analysis was used to look at TIMP-1 in a number of experimental animal groups. TIMP-1 levels improved in rats compared to control rats.

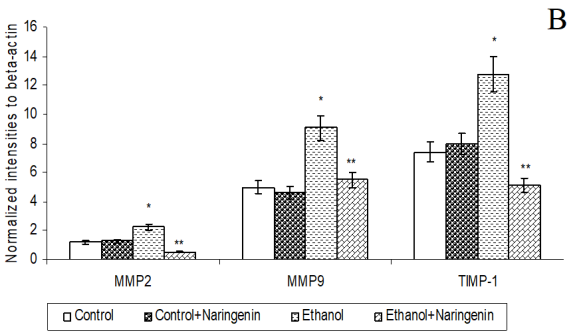

Fig. 4B. Naringenin and ethanol influence liver enzymes MMP-2, MMP-9

When ethanol and naringenin were combined, TIMP-1 expression somewhat decreased. The accumulation of normal chemicals in the extracellular matrix causes fibrosis. In order to provide a framework and maintain the regular operation of different liver cells, the liver extracellular matrix (ECM) provides structural support. Numerous biological functions depend on the regulation of collagen and ECM stability, which is strictly regulated by endogenous inhibitors (TIMPs). Several disorders arise as a result of dysregulation of MMP activity.[26] Additionally, it interacts with other molecules to carry out unrelated MMP processes, which are well documented in the invasion, migration, and development of cancer. Recent data clearly indicate that CD147 is important in PF, induces MMP, and transforms fibroblasts into myofibroblasts. In a number of fibrotic diseases, TIMPs and MMPs are essential.[27,28] The primary constituents of ECM29 can be broken down by gelatinase, which is made up of gelatinase A (MMP-2) and B (MMP-9). TIMP-1 is a naturally occurring tissue inhibitor of gelatinases that can prevent them from performing their gelatinolytic work.[30] We discovered that the livers of rats given ethanol expressed more MMP.

Alcohol consumption increases MMP activity by two mechanisms: ethanol oxidation through CYP2E1

produces reactive oxygen species that activate MMPs, and liver injury leads to cytokine release, further enhancing MMP activation and correlating with increased liver fibrosis.MMP-13 levels are elevated in various cancers, contributing to tumor progression and metastasis by cleaving collagens I, II, III and other ECM components. In addition to causing ECM remodeling, its action frequently results in the release of several angiogenetic and sequestered growth factors that promote the development, invasion, and angiogenesis of tumor cells.[32] Corresponding to activated stellate cells there after serving as a significant source of TGF-β, the primary mediator of MMPs during liver damage.[33] According to the above process, increased ROS generation of ROS and/or ECM in the group that received alcohol may be what caused the increased expression of MMPs. Naringenin may reduce MMPs and fibrosis by lowering the inflammatory response linked to alcohol-mediated liver injury due to its anti-inflammatory properties.

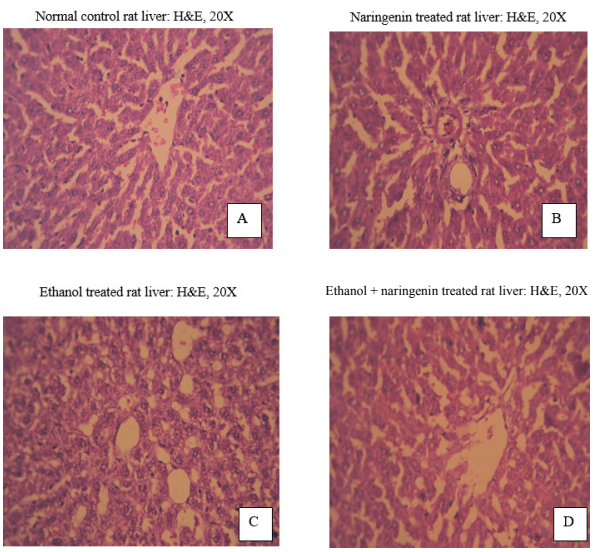

Fig. 5. Histopathological changes in the liver after ethanol administration

The data reported above suggest that naringenin inhibited the formation of hydroxyproline and the secretion of MMP by regulating HSC activity. This would help preserve the tissue's structural integrity by bringing MMP expression down to nearly normal. Figure 5 displays the liver's histological alterations. Hepatocytes in the liver of control rats were normal (Fig 5A).

Liver samples from rats given ethanol showed sinusoidal enlargement, ortal inflammation, and steatosis with micro- and macrovesicular alterations (Fig. 5C). The liver treated with ethanol and naringenin (Fig. 5D) exhibited normal histological characteristics with reduced and smaller fatty cysts. Rats treated with naringenin exhibited normal hepatocytes (Fig. 5B). These results aligned with our biochemical and cellular discovery that naringenin supplementation protects against ethanol-induced liver damage (Fig. 6). Serial sections were stained with Sirius red to visualize collagen. The liver of control rats did not show histological alteration (Fig. 6A). The livers of rats fed ethanol showed high collagen levels and clusters of collagen fibers surrounding the lobes. The central vein and the portal triad show a notable accumulation of collagen (Fig. 6C). Liver treated with ethanol and naringenin (shown in Fig. 6D) exhibited lower levels of collagen, reduced collagen deposition around the portal triad and central vein, a reduction in collagen fiber bundles, and decreased liver fibrosis scores. No alterations were observed in the histology of rats administered naringenin. These findings are consistent with the biochemical and cellular evidence that indicates that naringenin supplementation protects against ethanol-induced liver damage.

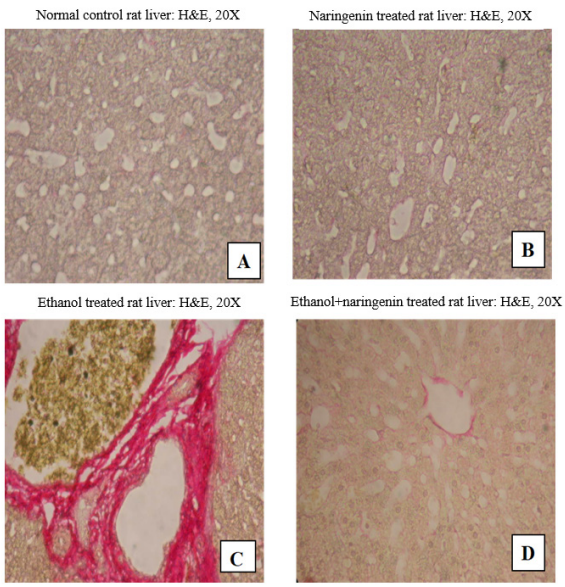

Fig. 6. Staining of collagen

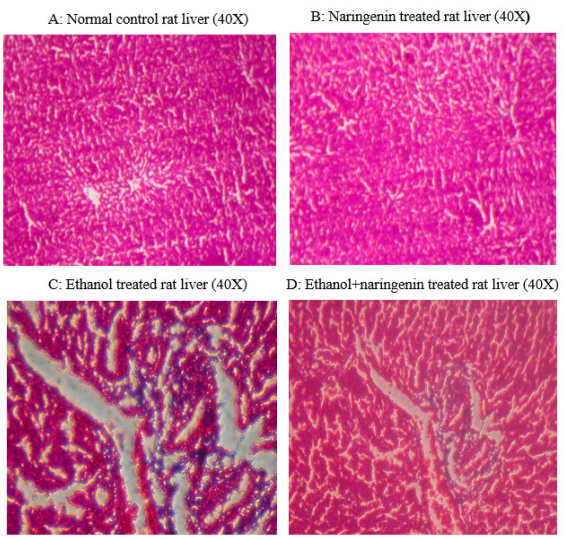

Fig. 7. Trichrome staining for collagen

Figure 7A shows the liver of the control rat, utilizing trichrome staining (red for collagen fibers), exhibits minimal collagen accumulation in the portal triad and central vein. Figure 7B presents rats exhibit collagen buildup and fatty changes in hepatocytes, while Figure 7C shows the livers show slight collagen accumulation around the veins, while hepatocytes remain within normal range after treatment.

Discussion

In our investigation, we observed elevated collagen levels and SMA expression in the livers of rats ingested ethanol. Liver disease injury caused by obstruction of the bile ducts has been associated with elevated levels of liver collagen.[35,36] In this context, it was shown that SMA-positive immunological cells are located primarily located in the portal ducts and fibrous septa align with collagen circulation.[37] Furthermore, SMA expression has been reported that SMA expression rises in the livers of rats fed ethanol, along with increased collagen levels in these livers. Elevated levels of collagen and SMA expression could be attributed to a higher count of SMA-positive cells in the liver. Furthermore, injured hepatocytes serve as significant sources of reactive oxygen intermediates, which are recognized for their ability to induce paracrine activation of stellate cells.[38-40]

In rats fed ethanol, naringenin supplementation markedly decreased the expression of collagen and SMA in liver. This could be due to reduced collagen levels, decreased oxidative stress, and suppressed HSC activation. Furthermore, rats treated with naringenin were found to exhibit reduced SMA expression levels in their livers.[41,42] The amount of naringenin used in this study could not reach the levels suggested for human treatment. Furthermore, the length of the study was inadequate to observe the full range of effects and long-term outcomes of naringenin treatment. Injection of ethanol can influence the level of liver fibrosis in the rat model. Variations in ethanol-induced fibrosis could influence the relevance of the findings. Not all aspects of a healthy liver might be reflected in the specific biomarkers and endpoints used to assess hepatic fibrosis and the efficacy of treatment. More studies will be conducted to determine the pharmacokinetic properties of naringenin.

Conclusion

The study suggests that naringenin positively impacts alcoholic liver fibrosis by regulating MMPs, TIMPs, and SMA, preventing ethanol-induced toxicity. Naringenin may improve liver histology and reduce fibrosis markers, potential therapeutic agent for liver fibrosis treatment. This study is limited by the inherent constraints of animal models, dosage parameters, and the need to further explore the safety profile and underlying mechanisms to establish naringenin's efficacy and safety of naringenin in human liver fibrosis. Additional research is needed, including clinical trials and studies addressing long-term effects and optimal therapeutic conditions.

Acknowledgments

The authors express gratitude to the department for research space and thank Almighty Lord for support.

Declarations

Funding

The authors did not receive funding.

Author contributions

Conceptualization, J.C.; Methodology, J.C.; Software, U.S.; Validation, J.C. and S.N.; Formal Analysis, J.C.; Investigation, J.C.; Resources, U.S.; Data Curation, U.S. and J.C.; Writing – Original Draft Preparation, U.S.; Writing – Review & Editing, K.M.; Visualization, J.C.; Supervision, S.N.

Conflicts of interest

No competing interests declared.

Data availability

Data sets from this study are available upon reasonable request to the author.

Ethics approval

Animal procedures ethically approved by Annamalai University (Reg.No: 160/1999/CPCSEA/557).

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