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ORIGINAL STUDY

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Abstract

Background: **Over the past few decades, increased alcohol consumption has had deleterious effects on human health. Alcoholic fatty liver disease (AFLD) is becoming a major global challenge, as the currently approved drugs for AFLDs are subject to several side effects. This has broadened the scope of the use of natural compounds as therapeutics. Recent advances in nutraceuticals as therapeutics have shed light on flavonoids such as Quercetin. It is a natural antioxidant of multiple dietary origins and has been extensively studied for its beneficial role as an anti-inflammatory and anti-cancer agent.**

Objective: **Based on this framework, in the proposed study, we investigated the therapeutic role of Quercetin in Ethanol-induced liver damage using the Swiss Albino mice model and the hepatic cell line HepG2.**

Methodology: **WST-1 assay was performed to access the effect of Quercetin on cell proliferation. The impact of Ethanol on the body and liver weights of mice was measured, and liver injury was determined by H&E staining and TMS. The mRNA expression levels of inflammatory genes (TNF-α, IL-6, and IL-1β) and SND1, a significant unit of the RNA-induced silencing complex (RISC), were analyzed. The liver enzyme levels were also measured.**

Results: **Our experimental results showed that HepG2 cells treated with ethanol had a lower proliferation rate, which was later mitigated by treatment with quercetin. In the mice model, a considerable reduction in body weight was detected after ethanol treatment. Conversely, there was a significant elevation in liver weight and enzyme activity. All of these effects were ameliorated by Quercetin treatment. Immunohistochemistry data revealed an improvement in the inflammation and fibrosis characteristics in liver tissues of the Quercetin-treated group. Decreased expression of inflammatory markers and SND1 levels were also observed in the Quercetin-treated group.**

Conclusion: **Based on our results it may be concluded that Quercetin demonstrated hepatoprotective activity in both ethanol-treated HepG2 cell line and ethanol-induced liver injury in mice model. Here, we elucidated a novel and possible therapeutic role of Quercetin in Alcohol-Related Liver Disease (ARLD) by targeting the RISC machinery.**

Keywords: ARLD, Quercetin, Inflammation, Fibrosis, RISC, SND1

1. Introduction

The liver is an indispensable organ of the human body crucial for diverse metabolic processes. The breakdown of dietary matter into useable forms, storage of these byproducts, detoxification of harmful substances, bile production, and blood cleansing are important functions of the liver [1]. Despite its vital functions, the liver can be damaged by several factors, including excessive alcohol consumption, high-calorie diet, viral infections, and certain medications [2,3]. In severe cases, liver damage can lead to liver failure, a potentially life-threatening condition.

Liver diseases are rapidly arising as a health concern worldwide. Chronic consumption of

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alcohol, an ascending trend in alcohol use, and an increase in the levels of per capita alcohol consumption have increased the frequency of Alcohol related liver disease (ARLD) worldwide [4]. Alcohol use disorder is a widespread substance use disorder globally [5]. The percentage of alcohol consumers continues to increase in the Western Pacific and Southeast Asian regions, including China and India [6]. In India, the common cause of cirrhosis (34.3%) is attributed to alcohol, and 20% of all liver disease victims, regardless of cause, are present-day alcohol consumers [7].

Owing to its exceptional metabolic machinery, the liver is a major target organ for the toxic effects of alcohol in the body. ARLD is a broad term encompassing a range of liver conditions caused by excessive alcohol consumption. Alcoholic steatosis is an early sign of alcoholic fatty liver disease. Failing to control or reverse the condition, individuals with alcoholic steatosis are susceptible to steatohepatitis, fibrosis, cirrhosis, and in a few cases hepatic carcinoma [8].

Quercetin, a flavonoid with multiple dietary origins, is commonly present in the aglycone- and glycoside-derived forms [9]. It is a natural antioxidant that has beneficial health effects. Ongoing investigations have supported the anticancer properties of Quercetin, both *in vivo* and *in vitro*. Several investigations have implied that Quercetin may play a vital role in therapeutic and preventive strategies for alcoholic liver disease [9–12]. The molecular process promoting the curative action of Quercetin on ARLD has yet to be fully elucidated.

The RNA-induced silencing complex (RISC) is a ribonucleoprotein complex that is an important mediator of RNA interference [13]. Staphylococcal nuclease and Tudor domain-containing 1 (SND1) is an important component of RISC; it is a protein that modulates a profound range of tasks, including gene expression [14]. It promotes tumor attributes, such as migration, invasion, proliferation, metastasis, and angiogenesis [15]. Furthermore, it influences the lipid metabolism, stress responses, and inflammation. The involvement of SND1 in diverse molecular tasks makes it unique to other oncogenes that are comparatively steadier in their function.

The relationship between SND1 and the development of non-alcoholic fatty liver disease is well known [16]. However, the role of SND1 in ARLD remains unclear. Owing to the limitations of available treatment options, it is imperative to determine the roles of novel regulators. In the current study, we assessed the therapeutic effect of Quercetin in the management of ARLD by targeting the RISC machinery.

2. Materials and methodology

2.1. Materials

Quercetin (≥95% (HPLC), solid), TRIzol reagent, sodium palmitate, oleate, and Oil Red O stain were purchased from Sigma-Aldrich. MEM medium, penicillin/streptomycin (Penstrep), glutamax, sodium pyruvate, and trypsin were purchased from HiMedia Laboratories Pvt., Ltd. FBS was procured from Gibco. WST-1 reagent was purchased from Takara Bio. The antibodies were purchased from Santa Cruz Biotechnology. The liver enzyme diagnostic kits were procured from Agappe Diagnostics Ltd. The PVDF membrane and RIPA lysis buffer were obtained from Merck Millipore. The chemiluminescence detection kit, WesternBright ECL was procured from Advansta.The cDNA synthesis and SYBR green kits were purchased from Thermo Fisher Scientific. HepG2 cells were purchased from NCCS Pune, India.

2.2. Cell culture

HepG2, human hepatoma cell line was grown in Minimum Essential Medium Eagle (MEM) supplemented with 10% FBS, 1% penicillin, 1% glutamax and 1% sodium pyruvate. The culture was maintained in a humidified incubator at 37 °C and 5% CO2. HepG2 cells were seeded in 96 well culture plate (1×10^4 cells/well) and treated with ethanol for 24 h. The cells were incubated with Quercetin for 24 h. 10 μl of premixed WST-1 reagent was further added to the wells. The plate was incubated for 2 h. The absorbance was measured at 450 nm using a PerkinElmer Multimode Plate Reader.

2.3. Experimental animals and study design

The methodology of the rodent model study was validated by the Institutional Animal Ethics Committee. Male Swiss albino mice (n = 30), weighing 19–25 g, were purchased from M/s. Adita Biosys, Tumkur, and accustomed for a week ahead of all the *in vivo* studies. The mice were housed in polypropylene cages (n = 5/cage) and maintained at 25 ± 3 °C. The relative humidity of the room was controlled at 45–55% with a light and dark cycle of 12 h each (artificial photoperiod). The mice were fed a rodent chow diet purchased from M/s. Adita Biosys and mineral water ad libitum. The study was designed for a period of three weeks and the mice were arbitrarily segregated into three groups (Fig. 1). Group I (control group (C); n = 10) was fed rodent chow diet. Group II (Ethanol group (E);

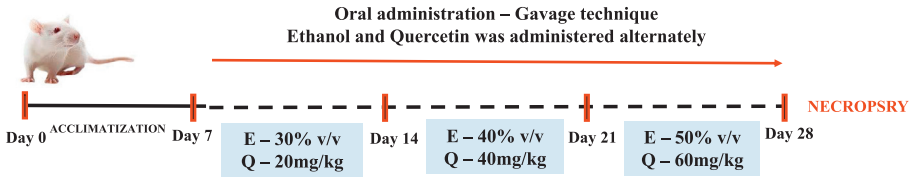

Fig. 1. Schematic representation of the in vivo experimental methodology.

n = 10) was orally administered ethanol on alternate days (30%v/v, week 1; 40%v/v, week 2; 50% v/v, week 3; diluted with water). Group III (Quercetin treatment group (E + Q); n = 10) received Ethanol (30%v/v, week 1; 40%v/v, week 2; 50% v/v, week 3; diluted with water) and Quercetin (20 mg/kg, week 1, 40 mg/kg − week 2; and 60 mg/kg, week 3; diluted with 0.1% DMSO). At the end of the experiment, the mice were sacrificed under light anesthesia by cervical dislocation. All organs were excised, fixed in buffered formalin (10%), frozen in liquid nitrogen, and stored at − 80 °C for further analysis. Blood samples were drawn through retro orbital vein and the centrifuged serum was stored at −80 °C.

2.4. Biochemical analysis

The activities of alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) were measured according to the manufacturer's instructions using kits from Agappe Diagnostics, Ltd.

2.5. Histopathological analysis

For histopathological studies, 5 μm thick sections of liver tissue were utilized. Hematoxylin and eosin staining was performed to analyze the inflammation. Hepatic fibrosis was estimated using Masson's trichome stain. All pathological changes were examined using a stereozoom microscope with a CCD Olympus camera (cellSens Dimensions 1.12).

2.6. Western blot

The bicinchoninic acid assay was performed for protein quantification. Proteins (30 μg) from the tissue lysates were separated by 10% SDS-PAGE. Further it was transferred to a PVDF membrane, and incubated with SND1 antibody overnight. Secondary antibodies used were Anti-mouse antibodies. Visualization of membranes was through chemiluminescence detection using Chemidoc Uvitec Alliance Q9.

2.7. Quantitative PCR

From frozen liver samples, total RNA was extracted using TRIzol reagent. mRNA expression was assessed using the SYBR green kit on a Rotor Gene Q (Qiagen) PCR system. Values were normalized to the housekeeping β-actin mRNAs, and the results were expressed as fold change relative to the control group.

Primer sequences used are as follows:

TNF-α forward: 5′- ATGGCCTCCCTCTCATCAGT-3′

TNF-α reverse: 5′- TTTGCTACGACGTGGGCTAC-3′

IL-6 forward: 5′- GTCCTTCCTACCCCAATTTCCA-3′

IL-6 reverse: 5′- TAACGCACTAGGTTTGCCGA-3′

IL-1β forward: 5′- TGCCACCTTTTGACAGTGATG-3′

IL-1β reverse: 5′- AAGGTCCACGGGAAAGACAC-3′

SND1 forward: 5′- GGTGGACTACATTAGACCAGCC-3′

SND1 reverse: 5′- AGACCTTTGCTGACAAGAGCCTC-3′

2.8. Statistical analysis

Statistical significance was assessed by One-Way ANOVA with Bonferroni's Multiple Comparison Test using GraphPad calculator 5.0, and significance was set at $P < 0.005$. The mean ± SEM values were used to determine the significance among the different groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. All data are shown as n = 5.

3. Results

3.1. Quercetin ameliorates ethanol-induced injury in hepatic cell line HepG2

As a preliminary study, we validated the efficacy of Quercetin on the proliferation of Ethanol-treated HepG2 cell lines. The cells were then treated with ethanol (50 mM and 60 mM) for 24 h. The cells were

further incubated with various concentrations of Quercetin (5 μM, 10 μM and 20 μM) for 24 h (Fig. 2A). At the end of the treatment period WST-1 cell proliferation assay was performed. Ethanol treatment notably inhibited HepG2 cell proliferation as observed in E 50 mM and E 60 mM. This effect was mitigated in Ethanol-treated cells supplemented with Quercetin (Figure 2B, C).

3.2. *Quercetin ameliorates the changes in body weight and liver weight of ethanol-administered Swiss albino mice*

It is well established that chronic consumption of alcohol leads to hypertrophy of the liver in humans and mice [8]. Based on this, we further assessed the effect of Quercetin on the body and liver weights of Ethanol-administered mice. Three groups of mice (n = 10) aged 5–7 weeks were randomly assigned. After acclimatization, the mice were alternately administered increasing concentrations of Ethanol and Quercetin for a period of three weeks. At the end of the treatment period, the E group showed a considerable decrease in body weight (Fig. 3A, B). Conversely, there was an increase in liver weight of E group compared to the C group counterparts (Fig. 3C, D). These changes were alleviated in the E + Q group.

3.3. *Quercetin mitigates liver enzymes activity in ethanol-treated mice*

Prolonged alcohol consumption affects the activity of liver enzymes [17]. Accordingly, we assessed the role of Quercetin in mitigating the activity of serological liver disease markers ALT, AST and ALP in Ethanol-treated mice model. After a treatment period of three weeks, the mice were euthanized and serum samples were used to analyze enzyme activity. The E group showed significantly higher ALT and AST activities (Fig. 4A, B), and decreased ALP activity as opposed to C group (Fig. 4C). This variation in the liver enzyme activities were ameliorated in the E + Q group.

3.4. *Quercetin improves inflammation and fibrosis features in ethanol-induced liver tissue injury*

Chronic alcohol consumption promotes hepatic lesions. Hepatic inflammation is the primary cause of hepatic tissue damage and a common cause of liver diseases [18]. Therefore, we assessed the role of Quercetin in improving inflammation and fibrotic changes in liver tissues of due to Ethanol treatment. Hematoxylin and eosin (H&E) staining was performed to analyze the inflammatory characteristics (Fig. 5A). Liver tissue sections of E group mice

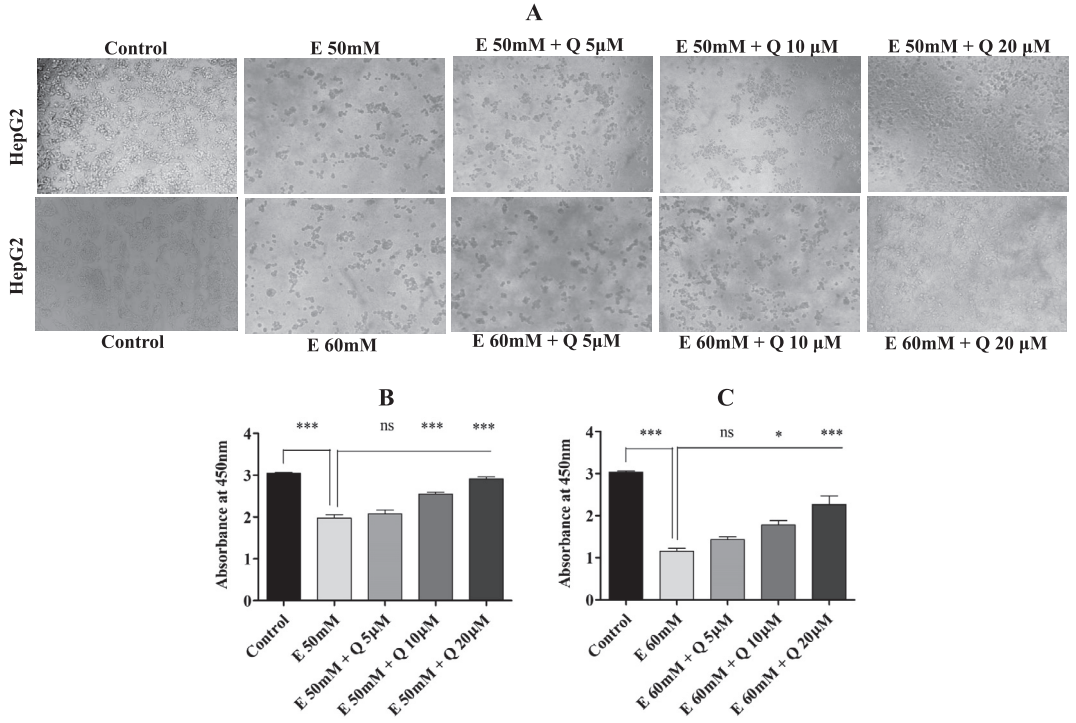

*Fig. 2. **Ethanol inhibited the proliferation of HepG2 cells, and these changes were ameliorated by Quercetin treatment.** A) Assessment of proliferation of HepG2 cells, absorbance was measured at 48 h. B) Quercetin mitigated the proliferation rate in Ethanol-treated (50 mM) HepG2 cells. C) Quercetin mitigated the proliferation rate in Ethanol-treated (60 mM) HepG2 cells.*

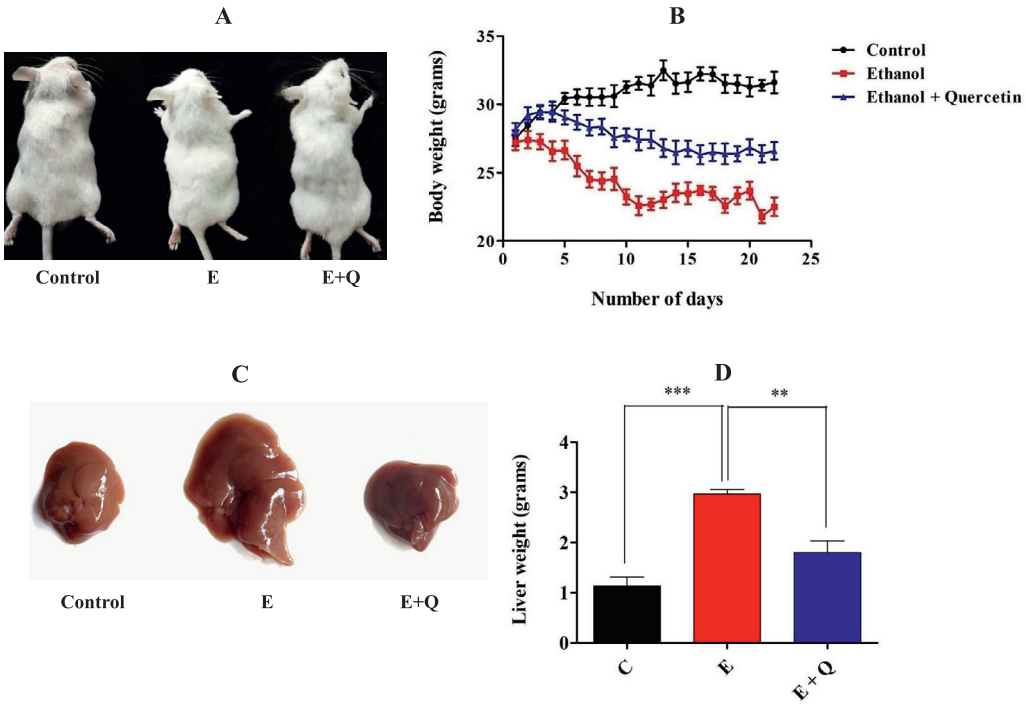

Fig. 3. ***Quercetin treatment improved the body and liver weights change of mice caused due to Ethanol administration.*** *A) Representative image of body weight differences in the mice groups. B) Graphical representation of the changes in body weights of mice throughout the treatment period. C) Representative image of liver weight differences in the mice groups. D) Graphical representation of the changes in liver weights of mice.*

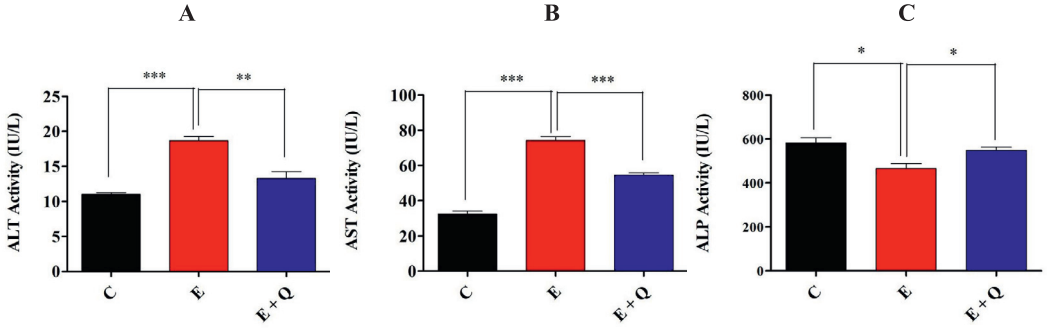

Fig. 4. ***Quercetin treatment alleviated the liver enzyme activity which was altered due to Ethanol treatment.*** *A) ALT. B) AST. C) ALP.*

showed inflammatory cell infiltration. Quercetin treatment mitigated the inflammatory characteristics of the liver tissues. This was further validated by measuring the mRNA levels of inflammatory markers such as TNF-α, IL-1β, and IL-6. The E group showed an increase in the expression of all inflammatory markers compared to the control. Quercetin treatment notably alleviated the expression of these markers (Fig. 5B – D). Additionally, Masson's trichrome staining was also performed to evaluate liver fibrosis features. A moderate level of collagen fibers formation was observed in E group and over again Quercetin ameliorated these features (Fig. 6).

3.5. Quercetin targets RISC machinery to alleviate ethanol-induced hepatic injury

SND1, an important component of RISC, is a multifaceted protein that controls a complex range of cellular processes. In this investigation, we attempted to elucidate the efficacy of SND1 as a potential therapeutic target for Quercetin in combating ARLD. Protein and mRNA expression levels were measured. The E + Q group showed a significant decrease in SND1 protein expression compared to the E group (Fig. 7A). Arbitrary values obtained from western blot data showed elevated expression of SND1 in E group compared to that in

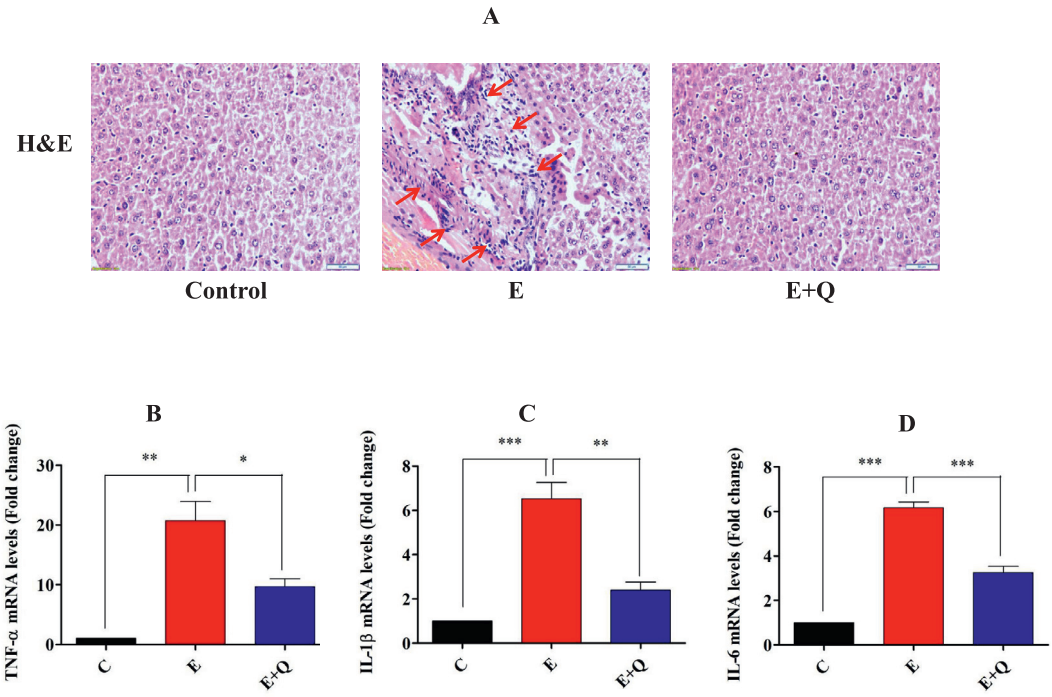

Fig. 5. Effect of Quercetin on Ethanol-induced liver inflammation. A) Representative images of H&E stain. B) TNF-α. C) IL-1β. D) IL-6.

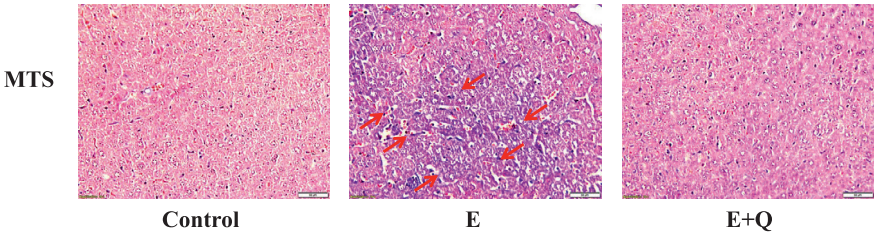

Fig. 6. Effect of Quercetin on Ethanol-induced liver fibrotic changes. Representative images of Masson's Trichome stain.

the healthy counterparts of C group (Fig. 7B). Upon assessing the mRNA levels of SND1, E + Q group showed a drastic reduction the expression of SND1 when compared to the E group (Fig. 7C).

4. Discussion

The liver is a vital organ found exclusively in vertebrates that performs several important biological functions, including detoxification. This is the primary organ involved in alcohol metabolism. Alcohol is majorly metabolized by an enzyme found in liver cells, alcohol dehydrogenase (ADH). ADH degrades alcohol to acetaldehyde, which is further converted to acetate by aldehyde dehydrogenase. Finally, acetate is metabolized and exits the body as CO_2 and H_2O [19]. This normal metabolic pathway is disrupted in chronic alcohol consumption and poses a serious threat to health. Despite substantial progress in understanding the etiology of alcohol-related liver disease, only a few medicines have been authorized to treat ARLD. Abstinence from alcohol and treatment sessions are indicated in the early stages, and medications are generally prescribed for patients with severe alcoholic hepatitis owing to numerous negative effects.

However, in recent years, there has been an inclination towards the use of natural compounds for the management of chronic conditions. Flavonoids are one such category of compounds that are gaining importance as nutraceutical agents. Quercetin is one of the most extensively studied flavonoids and a well-known antioxidant [20]. Its potential effects on liver health and disease management have been well explored. Several reports have shown that Quercetin may have a protective effect on the liver of alcohol-treated mice by reducing inflammation and liver damage.

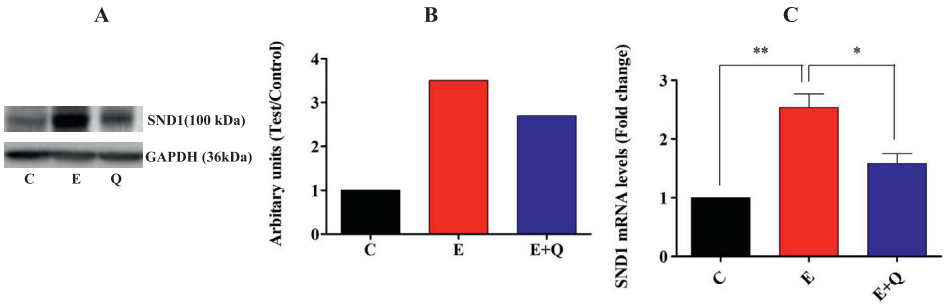

*Fig. 7. **Quercetin improves Ethanol-induced liver injury by targeting RISC machinery.** A) Validation of SND1 expression through western blot. B) Arbitrary units of western blot of SND1 protein. C) mRNA expression levels of SND1.*

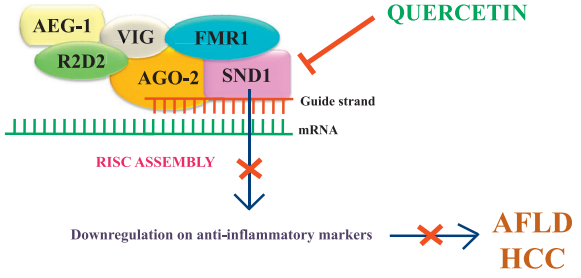

Fig. 8. SND1 is a therapeutic target for Quercetin mediated amelioration of Ethanol-induced hepatic injury.

Accordingly, our experimental data supports the hepatoprotective properties of Quercetin against Ethanol-induced liver injury. The proliferation rate of Ethanol-treated HepG2 cells was improved upon treatment with Quercetin. We also observed that Quercetin successfully alleviated the changes in body and liver weights caused by ethanol treatment. It also mitigated Ethanol-induced changes in liver enzyme activity. Immunohistochemical data supported the hepatoprotective activity of Quercetin against Ethanol-induced inflammation and fibrosis.

It is composed of small RNA molecules and proteins and acts as an RNA-directed endonuclease that cleaves specific messenger RNA (mRNA) molecules, thereby inhibiting protein synthesis and gene expression. Recently, The RISC complex has emerged as a potential therapeutic target for various diseases, including cancer, viral infections, and neurodegenerative diseases [21,22]. By targeting specific components of the RISC complex, it may be possible to regulate gene expression and modulate the cellular processes involved in disease development and progression. This is the first study to demonstrate the possible novel therapeutic role of Quercetin in treating ARLD by targeting SND1, an important component of RISC. We observed an increase in SND1 expression along with other inflammatory markers in the Ethanol-treated mice group. This effect was successfully alleviated in the Quercetin-treated group (Fig. 8).

Globally, the prevalence of ARLD is increasing. The current treatment options available for ARLD have several shortcomings. Identification of novel targets aids the development of new modalities for the effective treatment of this complex spectrum of diseases. In summary, our study demonstrated the hepatoprotective effect of Quercetin against Ethanol-induced liver injury by targeting SND1, an important component of the RISC machinery. However, the therapeutic potential of targeting SND1 or RISC components is still in its early stages, and further clinical research is needed to fully understand its effects and determine the safety and efficacy of these strategies.

Declaration of competing interest

None.

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