

The Potential Protective Effect of Rosuvastatin and Chrysin Combination against Hepatic Fibrosis Induced by Ethanol

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ABSTRACT

Objectives Alcohol-induced liver fibrosis is a great worldwide concern. Rosuvastatin is an anti-hyperlipidemic drug and chrysin is a natural flavonoid. This study examined the potential protective actions of rosuvastatin, chrysin, and their combination against ethanol-induced liver fibrosis based on their anti-inflammatory, antioxidant, and anti-fibrotic effects using silymarin as a reference.

Methods Male rats were given ethanol (25%, vol./vol.) (1 ml/100 g/day, P.O.) thrice weekly for seven consecutive weeks. Silymarin (100 mg/kg, P.O.), chrysin (100 mg/kg, P.O.), rosuvastatin (10 mg/kg, P.O.), or a combination of chrysin and rosuvastatin were administered thrice weekly for seven weeks.

Results Data showed that rosuvastatin and chrysin alone or in combination decreased liver fibrosis initiated by ethanol as hepatic enzymes (ALT, AST, ALP, GGT) were decreased and a decline in 4-HYP; and TGF- β 1 levels.

Conclusion This study declared for the first time the hepatoprotective role of rosuvastatin and chrysin combination against liver fibrosis initiated by ethanol via TGF- β 1/Smad pathway, decreasing inflammation, oxidative stress, and fibrosis.

Keywords: Alcohol, Chrysin, Fibrosis, Liver, Rosuvastatin.

1. INTRODUCTION

Repetitive and excessive alcohol consumption is considered a worldwide problem.¹ It causes damage to many organs all over the body, especially the liver.² As per the World Health Organisation (WHO), over 3 million fatalities worldwide (or 5.3% of all deaths) were attributed to hazardous alcohol intake in 2018.³ Worldwide, alcohol use ranks as the seventh most important risk factor for both disability and early mortality. It negatively impacts a person's health, contributing to the development of cancer (both liver and non-liver

neoplasms) as well as gastrointestinal and cardiovascular disorders. Geographic variations exist in alcohol-related morbidity and death, with the WHO European Region seeing the greatest impact.⁴ Europe has the largest per capita alcohol consumption, closely followed by the countries in the Region of the Americas. Alcoholic liver disease progresses from liver steatosis, then if not treated to alcoholic hepatitis which leads to liver fibrosis, and then to liver cirrhosis which is the most progressive and irreversible liver injury.⁵ Alcohol-induced liver fibrosis results from the metabolism of ethanol and its oxidation to acetaldehyde which is the most important intermediate in alcohol-induced fibrinogenesis as it induces hepatic stellate cells (HSCs) and subsequent extracellular

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matrix proteins production (as fibronectin, proteoglycan, collagen type I, III and IV, and laminin).⁶ Also, stimulation of HSCs turns them from quiescent cells storing vitamin A into myofibroblast-like cells, which have contractile, chemotactic, proliferative, and inflammatory effects.⁷ Acetaldehyde can impact transforming growth factor- β (TGF- β) signaling directly by inducing TGF- β expression and transformation of latent TGF- β complexes into the active form.⁸ TGF- β 1 has a tremendous intermediary outcome in the progression of hepatic inflammation and fibrogenesis.⁹

Statins are potent 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors used for hypercholesterolemia.¹⁰ Several epidemiological studies conducted in the past several years have demonstrated that statins offer advantages beyond those associated with atherosclerotic disease primary or secondary prevention. These are known as the pleiotropic effects, and they have been observed in several illnesses, including pancreatitis, erectile dysfunction, acute renal injury, chronic obstructive pulmonary disease, and chronic liver diseases (CLD).¹¹ Reductions in portal pressure, hepatic microvascular dysfunction, liver sinusoidal endothelial cells (LSEC), fibrogenesis, and sensitivity to endotoxin-mediated liver damage have all been linked to CLD and may be involved in preventing or postponing the development of cirrhosis.¹² It was hypothesized that statins may help with this by lowering oxidative stress and, consequently, the activation of inflammatory cells.¹³ Rosuvastatin has anti-inflammatory properties as it decreases the pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), and interleukin-1 β (IL-1 β).¹⁴ Also, it has anti-oxidant and cardioprotective effects as it diminishes TNF- α , IL-6, iNOS expression, and malondialdehyde (MDA) along with an elevation in superoxide dismutase (SOD).^{15,16} Rosuvastatin decreases the expression of Smad3 and TGF- β 1.¹⁷

Flavonoids are plant secondary metabolites, their physiological mechanisms make them used in the treatment of many medical disorders.¹⁸ Chrysin is found as a natural flavonoid in honey and propolis. It has many critical actions, such as antioxidant, anti-inflammatory, anti-cancer, and antiviral actions.¹⁹ Also, it can reverse liver fibrosis by inhibiting HSCs stimulation by its effect on the TGF- β 1/Smad pathway, as it remarkably reduced TGF- β 1 Smad-2 and Smad-3 levels.²⁰ Chrysin has crucial importance in the transcription of many inflammatory cytokines and enzymes such as COX-2, TNF- α , IL-1 β , and NF- κ B.²¹ It stabilizes the ratio between the oxidants and anti-oxidant levels as it inhibits MDA, NO, and iNOS expression and increases SOD and GSH anti-oxidant levels.²² This current study discussed the possible protective actions of Rosuvastatin and Chrysin alone or in combination against hepatic fibrosis prompted by ethanol in rats. Moreover, the potential underlying mechanisms were investigated.

2. METHODS

2.1. Drugs and Chemicals

Ethanol was bought from Adwic Chemicals Co. (Cairo, Egypt). Silymarin was obtained from Pharma Care Egypt Pharmaceuticals (Cairo, Egypt) and Chrysin was purchased from (Alfa Aesar, UK), they were dissolved in a vehicle consisting of DMSO, PEG, and saline. Rosuvastatin was dissolved in normal saline, it was bought from El-Obour Modern Pharmaceutical Industries Co. (Egypt). PEG and DMSO were bought from Loba Chemie (Mumbai, India). Drug solutions were freshly prepared directly before administration.

2.2 Experimental Design

Male albino Sprague Dawley rats (150-180 g) were purchased from the National Research Center (Giza, Egypt). They were allowed to acclimatize for 7 days in the laboratory with an open supply of water and chow. The animal experiments comply with ethical principles and guidelines for the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and are accepted through the committee of Research Ethics, Faculty of Pharmacy, Tanta University, Egypt (code: TP/RE/3/23 p-0013). Rats were distributed randomly into six different groups (n = 8 / group):

1. Normal control group: rats were administrated vehicle (DMSO, PEG, and Saline).
2. EtOH group: rats were administrated Ethanol (25%, vol./vol.) (1 ml/100 g/day) three times per week for seven weeks by oral administration.²³
3. Sil+EtOH: rats were administrated Silymarin (100 mg/kg) + EtOH for seven weeks by oral administration.²⁴
4. Chry+EtOH: rats were administrated Chrysin (100 mg/kg) + EtOH for seven weeks by oral administration.²⁵
5. Ros+EtOH: rats were administrated Rosuvastatin (10 mg/kg) + EtOH for seven weeks by oral administration.²⁶
6. Chry+Ros+EtOH: rats were administered Chrysin+Rosuvastatin+EtOH as described above.

After twenty-four hours from the last dose, rats were sacrificed. Blood and liver samples were taken.

2.3 Sample Collection

Samples of blood were obtained through cardiac puncture and were allowed to coagulate at room temperature for 20 minutes, then centrifuged for 20 minutes at 4,000 rpm to collect the serum. The serum was then stored at -20°C for measurement of these biochemical parameters (alanine transaminase (ALT), aspartate aminotransferase (AST), gamma glutamine transferase (GGT), and alkaline phosphatase (ALP)).

The liver of each rat was washed in normal saline and split into 2 parts: The first part of the liver was directly saved in liquid nitrogen and saved at a temperature of -80°C to determine TGF-β1 and 4-hydroxyproline (4-HYP) content. The other part of the livers was stored at 10% fixative buffered formalin for histopathological examination.

2.4. Measurement of Serum Hepatic Markers

Alanine transaminase was measured by the ALT assay kit (Catalog No. K752-100), AST was measured by assay kit (Catalog No. K753-100), and ALP was measured by assay kit (Catalog No. K412). Likewise, GGT was determined according to the assay kit (Catalog No. ab241029).

2.5. ELISA Technique for Determination of TGF-β1 and 4-HYP

TGF-β1 and 4-HYP content in liver tissues were estimated by the ELISA technique. Rat TGF-β1 ELISA kit (Catalog No. 201-11-0779), and 4-HYP ELISA kit (Catalog No. 201-11-2303) were purchased from (Sunred Biological Technology Company, Shanghai, China) and were used as the manufacturer’s protocol.

2.6. Histopathological Examination

Liver samples were left for 24 hours in 10% neutral buffered formalin solutions. Then, tissue was embedded in paraffin blocks with 3-5 μm thickness. The characteristics of histopathological features were analyzed under an Olympus

light microscope (Olympus CX21, Tokyo, Japan).

2.7. Statistical Analysis

The data was determined by using Graph Pad Prism software edition 5. Data were presented as mean ± SD. One-way analysis of variance test (ANOVA) followed by Tukey–Kramer multiple comparison was used to compare different groups. Statistical significance was accepted at P-value <0.05.

3. RESULTS

3.1. Effects of Chry, Ros, and Their Combination on Serum Level of Hepatic Markers

As shown in Figure 1 (a, b, c, and d), rats that were given EtOH showed significant elevation in serum ALT, AST, ALP, and GGT levels by 111.4%, 74.9%, 120%, and 103.1%, respectively, compared to the control group. The administration of Sil+EtOH leads to a significant decline in serum ALT, AST, ALP, and GGT levels by 8.4%, 2.5%, 8.8%, and 12.4%, respectively, in comparison to the EtOH group. Administration of Chry, Ros, and their combination + EtOH showed a significant decrease in serum ALT by (23%, 35%, and 51.2, respectively), AST by (15.3%, 25.4%, and 36.7%, respectively), ALP by (23%, 35.1%, 47.2%, respectively), GGT by (23.4%, 30.8%, 45.2%, respectively) in comparison to EtOH treated group.

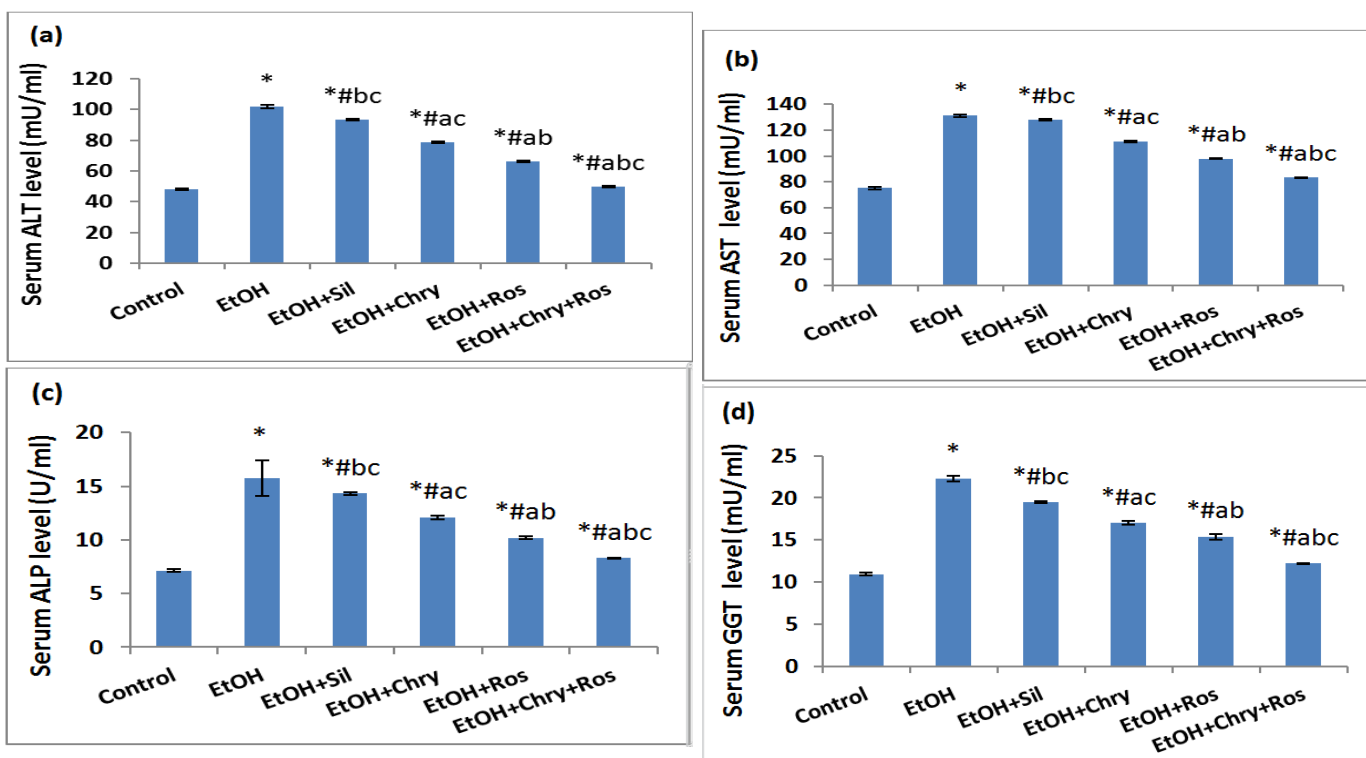


Figure 1. Effects of Chry, Ros, and their combination on serum level of hepatic markers. (a) ALT, (b) AST, (c) ALP, and (d) GGT. Data are presented as mean ± SD (n=8). *Significant difference from control group at P<0.05. #Significant difference from EtOH group at P<0.05. ^a Significant difference from the EtOH+Sil group at P<0.05. ^b Significant difference from EtOH+Chry group at P<0.05. ^c Significant difference from EtOH+Ros group at P<0.05. EtOH: Ethanol, Sil: Silymarin, Chry: Chrysin, Ros: Rosuvastatin.

3.2. Effects of Chry, Ros, and Their Combination on Hepatic TGF- β 1 Content

Administration of EtOH led to a significant elevation of hepatic TGF- β 1 by 4.28-fold in comparison with a control group (Figure 2). Also, treatment with Sil+EtOH caused a decrease in hepatic TGF- β 1 by 15.8%. Treatment with Chry, Ros, or Chry+Ros+EtOH significantly reduced TGF- β 1 content by (21.1%, 31.6%, and 50%, respectively) compared to EtOH treated group.

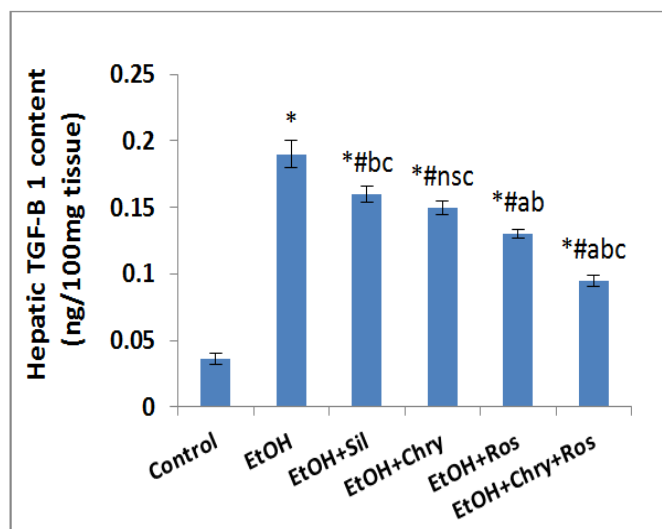


Figure 2. Effects of Chry, Ros, and their combination on hepatic TGF- β 1 content.

Data are presented as mean \pm SD (n=8). *Significant difference from the control group at $P < 0.05$. #Significant difference from EtOH group at $P < 0.05$. ^a Significant difference from the EtOH+Sil group at $P < 0.05$. ^b Significant difference from EtOH+Chry group at $P < 0.05$. ^c Significant difference from EtOH+Ros group at $P < 0.05$. ns non-significant at $P < 0.05$. EtOH: Ethanol, Sil: Silymarin, Chry: Chrysin, Ros: Rosuvastatin

3.3. Effects of Chry, Ros, and Their Combination on Hepatic 4-HYP Content

Hepatic 4-hydroxyproline content was significantly increased in the EtOH group by 2.3-fold compared to the control group. Whereas treatment with Sil, Chry, and Ros+EtOH, and the combination group, caused significant decreases in 4-HYP content by (16.3%, 31.5%, 42.5%, and 52%, respectively) compared to the treated group with EtOH. Ros+Chry+EtOH has a significant effect compared to Sil, Chry, and Ros+EtOH groups (Figure 3).

3.4. Effects of Chry, Ros, and Their Combination on Hepatic Histopathology

By observing hematoxylin and eosin-stained slides of liver tissue as appeared in (Figure 4), the control group (Figure 4 a) showed normal histological architecture consisting of central vein (CV) with radiating cords of

hepatocytes surrounding it, Normal hepatocytes with moderately acidophilic cytoplasm and central rounded pale-stained nuclei were seen together with binucleated cell in a certain area. On the other hand, a liver section in the EtOH-treated group showed dilated central vein (CV), rarefaction, and vacuolations of the cytoplasm of nearly all hepatocytes and pyknotic deeply stained nuclei in some hepatocytes (Figure 4 b).

Administration of EtOH+Sil showed mild improvement of histological architecture except for irregular dilated central vein (CV), vacuolations of the cytoplasm of some hepatocytes (V), pyknotic deeply stained nuclei in few cells, and scattered inflammatory infiltration (Figure 4 c). However, administration of EtOH+Chry revealed moderate improvement of histological architecture with radiating cords of hepatocytes of acidophilic cytoplasm and pale stained nuclei except for mildly dilated congested central vein (CV) (Figure 4 d). Also, the administration of EtOH+Ros showed marked improvement of histological architecture consisting of a central vein (CV) with radiating cords of hepatocytes containing central rounded pale nuclei, but the central vein was mildly congested, and the binucleated cell was seen (Figure 4 e). Remarkably, the administration of EtOH+Chry+Ros showed more or less normal histological architecture consisting of a central vein (CV) with radiating cords of hepatocytes of moderately acidophilic cytoplasm and central rounded pale-stained nuclei surrounding it. Binucleated hepatocytes were seen (Figure 4 f).

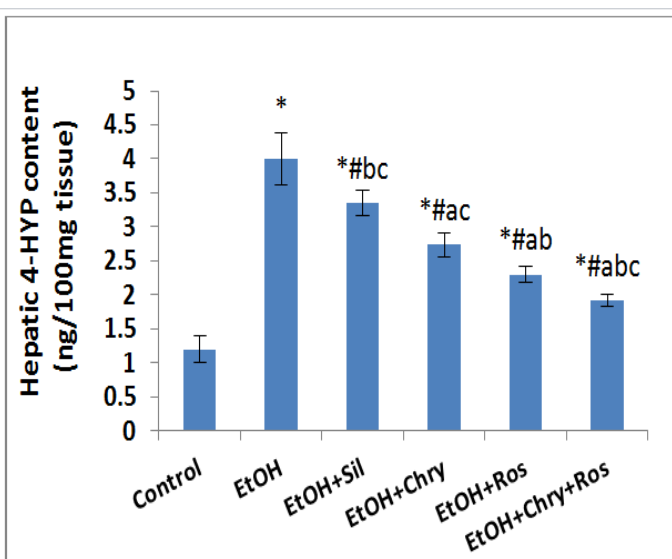


Figure 3. Effects of Chry, Ros, and their combination on hepatic 4-HYP content.

Data are presented as mean \pm SD (n=8). *Significant difference from the control group at $P < 0.05$. #Significant difference from EtOH group at $P < 0.05$. ^a Significant difference from the EtOH+Sil group at $P < 0.05$. ^b Significant difference from EtOH+Chry group at $P < 0.05$. ^c Significant difference from EtOH+Ros group at $P < 0.05$. EtOH: Ethanol, Sil: Silymarin, Chry: Chrysin, Ros: Rosuvastatin

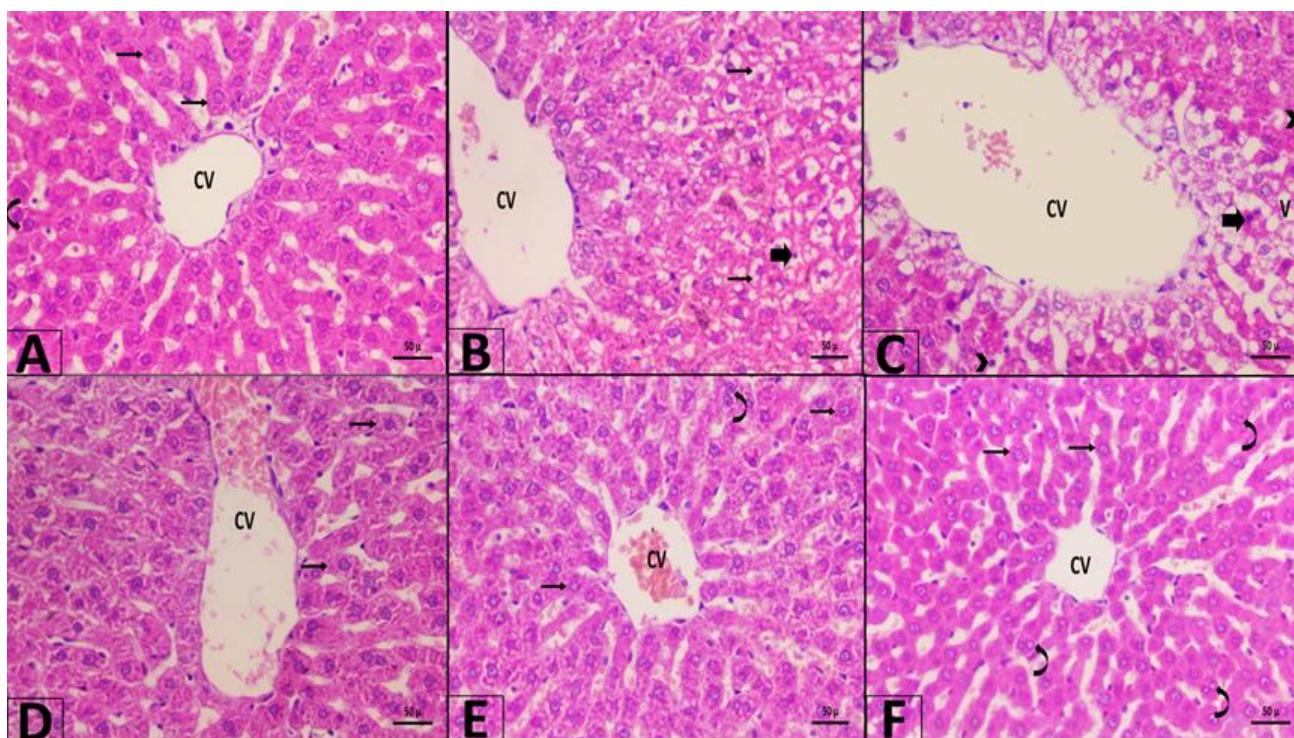


Figure 4. Effects of Chry, Ros, and their combination in liver sections stained by H&E staining (400x, scale bar=50 µm). **A)** Normal histological architecture consisting of a central vein (CV) with radiating cords of hepatocytes surrounding it. Normal hepatocytes with moderately acidophilic cytoplasm and central rounded pale-stained nuclei were seen (thin arrows) together with binucleated cells in certain areas (curved arrows). **B)** EtOH group, showed dilated central vein (CV), rarefaction of the cytoplasm of nearly all hepatocytes (thin arrows), and pyknotic deeply stained nuclei in some hepatocytes (thick arrows). **C)** EtOH+Sil, showed mild improvement of histological architecture except for irregular dilated central vein (CV), vacuolations of the cytoplasm of some hepatocytes (V), pyknotic deeply stained nuclei in few cells (thick arrow), and scattered inflammatory infiltration (arrowheads). **D)** EtOH+ Chry showed moderate improvement of histological architecture with radiating cords of hepatocytes of acidophilic cytoplasm and pale stained nuclei (thin arrows) except for mildly dilated congested central vein (CV). **E)** EtOH+ Ros group showed marked improvement of histological architecture consisting of a central vein (CV) with radiating cords of hepatocytes containing central rounded pale nuclei (thin arrows) but the central vein was mildly congested and binucleated cell was seen (curved arrow). **F)** The EtOH+Chry+Ros group, showed more or less normal histological architecture consisting of a central vein (CV) with radiating cords of hepatocytes of moderately acidophilic cytoplasm and central rounded pale-stained nuclei (thin arrows) surrounding it. Binucleated hepatocytes were seen (curved arrows). EtOH: Ethanol, Sil: Silymarin, Chry: Chrysin, Ros: Rosuvastatin.

4. DISCUSSION

Alcoholic liver disease is regarded as a crucial risk to public health. Drinking more than 40 gm of alcohol per day for years could cause liver fibrosis and then cirrhosis which is the critical end-fatal stage of liver injury that is only treated by liver transplantation.²⁷ In this study, treatment with rosuvastatin and chrysin alone or combined ameliorated alcohol-induced liver fibrosis. Ethanol treatment elevated serum ALT, AST, ALP, and GGT enzyme activities in comparison to the control as noted in a previous study.²⁸ However, chrysin and rosuvastatin alone or combined led to a decrease in serum ALT, AST, ALP, and GGT enzymes to nearly the normal levels in comparison with the ethanol group. Those results come in consistent with later studies that showed chrysin decreased serum ALP, ALT, GGT, and AST enzymes.²⁹⁻³¹ Also, many other studies declared that statins ameliorated the serum level of ALP, ALT, GGT, and AST in other hepatic disorders.^{32,33}

Oxidative stress and ROS production have been linked to alcoholic liver disease as they lead to cellular damage and necrosis.³⁴ In this study, the overproduction of MDA and NOx over the antioxidants as SOD may lead to oxidative stress that has a pivotal role in liver inflammation and may advance to liver fibrosis and cirrhosis. While the usage of chrysin and rosuvastatin alone or in combination led to significant results against oxidative stress, as they decreased MDA and NOx, nevertheless, also increased the antioxidant activity of SOD. Likewise, numerous previous studies indicated the role of chrysin and statins in declining oxidative stress and enhancing antioxidant activity.^{35,36}

Hydroxyproline is an important amino acid found in collagen (which is the main component of ECM).³⁷ Its presence in ECM generated by activated HSCs reserves the dependability and function of liver cells. Hydroxyproline level in liver tissues implicates a limiting factor that can control the progression of liver fibrogenesis.³⁸ The current study demonstrated a remarkable increase in hepatic 4-HYP in an ethanol-treated group together with increased patches of

fibrosis in liver sections. This result is consistent with other previous studies.^{18, 39} However, after treatment with rosuvastatin and chrysin as monotherapy or combined, a significant decrease in hepatic 4-HYP contents was noted together with decreased patches of fibrosis in liver sections. This shows the antifibrotic action of these drugs as they prohibit ECM deposition in the liver. These results come in agreement with others.⁴⁰

Transforming growth factor- β 1 is a cytokine that has essential participation in organizing many cellular signaling processes and ECM components.⁴¹ TGF family consists of 33 members. TGF- β 2 is crucial in biliary fibrosis induction, and TGF- β 1 is the most critical isoform in the stimulation of liver fibrosis.⁴² Similarly, its downstream molecules Smad2, Smad3, Smad4, and Smad7 are implicated in fibrosis.⁴³ TGF- β 1 signaling is set to be a critical target of liver fibrosis as its stimulation led to a series of critical actions such as the differentiation of fibroblast into contractile myofibroblast, elevating the expression of α -SMA and also the synthesis of ECM.⁴⁴

In addition, TGF- β 1/Smad2/3 is the main signaling pathway that regulates ECM synthesis, accumulation, and degradation during liver fibrosis.⁴⁵ TGF- β 1 binds to its receptor which phosphorylates Smad2/3 and forms a complex that translocates to the nucleus of the HSCs. This process causes collagen gene expression and induces liver fibrosis.⁴⁶ TGF- β and platelet-derived growth factor (PDGF) are two very crucial cytokines associated with HSCs activation and proliferation.⁴⁷ PDGF is a growth factor that induces the proliferation of HSCs. It has four distinct subunits, known as PDGF-A, -B, -C, and -D, where the PDGF-B is the most common factor related to HSCs differentiation.⁴⁸ The administration of ethanol in the present study elevated TGF- β 1. However, the administration of rosuvastatin and chrysin caused a noteworthy decline in TGF- β 1 contents. The results are consistent with the latter studies.^{24, 49}

Also, TGF- β can use independent Smad pathways, as nuclear factor kappa-light-chain enhancers of activated B cells (NF- κ B), which is capable of regulating the Smad pathway and interfering with the responses mediated by TGF- β .⁵⁰ NF- κ B regulates DNA transcription responsible for inflammatory responses and apoptosis, also its dysfunction may lead to autoimmune and inflammatory disorders.⁵¹

4. CONCLUSION

This study proved for the first time that rosuvastatin has a significant hepatoprotective action versus hepatic fibrosis induced by ethanol in rats. Moreover, it ensured the protection of the combination of rosuvastatin and chrysin was superior compared to each drug alone. Ethanol stimulated the production of TGF- β 1 and overproductions of ROS, inflammation, and fibrosis progression. These effects are prohibited by rosuvastatin, chrysin, and their combination.

AUTHORS' CONTRIBUTION

Nageh Ahmed El Mahdy: conceptualization, visualization, review, and supervision. Amgd Alaa El-Sisi: writing - original draft, conceptualization, investigation, formal analysis, and performing literature searches. Sally El-Sayed Abu-Risha: writing - original draft, methodology, formal analysis, data curation, editing manuscript, review, and supervision.

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DECLARATION OF CONFLICTING INTEREST

The authors declare no conflict of interest.

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