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Fisetin alleviates thioacetamide-induced hepatic fibrosis in rats by inhibiting Wnt/β-catenin signaling pathway

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ABSTRACT

Background: Liver fibrosis is a chronic wound-healing response to liver injury of various origins and represents a major health problem. The current study endeavored to investigate the repressing effect of fisetin on hepatic fibrosis induced by thioacetamide (TAA) in rats.

Objective: The current study endeavored to investigate the repressing effect of fisetin on hepatic fibrosis induced by thioacetamide (TAA) in rats.

Materials and methods: Rats were injected with TAA (200 mg/kg) intraperitoneally twice per week for 6 weeks to induce liver fibrosis. Fisetin (50 and 100 mg/kg/day) or silymarin (50 mg/kg/day) were given orally on a daily basis along with TAA. Liver function parameters, oxidative stress, inflammatory and fibrogenic biomarkers as well as wnt3a, β-catenin, glycogen synthase kinase 3 (GSK-3β) and cyclin D1 were estimated. Histoapthological and immunohistochemical examinations were performed.

Results: Fisetin restored normal liver functions, increased reduced glutathione (GSH) level and decreased malondialdehyde (MDA), as well as inflammatory biomarkers including; tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6). Additionally, it lessened transforming growth factor β1 (TGF-β1), collagen I and tissue inhibitor of metalloproteinase-1 (TIMP-1) levels as well as elevated matrix metalloproteinase-9 (MMP-9) hepatic content. Furthermore, fisetin significantly suppressed wnt3a gene expression associated with decreased β-catenin and increased GSK-3β levels. Moreover, fisetin decreased the progress of histologic hepatic fibroplasia and diminished hepatic expression of α-SMA and cyclin D1.

Conclusion: Fisetin curbed liver fibrosis and exhibited superior activity over silymarin through inhibition of hepatic stellate cells (HSCs) activation and proliferation via suppressing the Wnt/β-catenin pathway, modulating MMP-9 and TIMP-1, and inhibiting multiple profibrogenic factors, besides its antioxidant and anti-inflammatory effects. Therefore, fisetin is a promising therapeutic candidate for hepatic fibrosis.

KEYWORDS
Fisetin; fibrosis; Wnt/β-catenin; GSK-3β; cyclin D1

Introduction

Hepatic fibrosis is a dynamic wound healing process that excessively accumulates extracellular matrix (ECM) proteins and is triggered by drugs, viral infection, metabolic disorders, biliary diseases, immune-mediated injury, and alcoholic and nonalcoholic steatohepatitis. Liver fibrosis can be reversed only in an early stage, but, in the late stage, it is irreversible and results in cirrhosis and possibly hepatocellular carcinoma. Safe and effective drugs are urgently needed as there are no specific pharmaceutical treatments indicated for clinical use.

HSCs play a central role in the development of fibrosis and are considered the primary ECM source in the fibrotic liver. In a quiescent state, the function of HSCs is vitamin A storage and metabolism. Following liver injury, HSCs become activated, lose their vitamin A content, and transform into proliferative and contractile myofibroblast-like cells expressing α-SMA and type I collagen.

HSC activation and proliferation are crucial steps in the progression of liver fibrosis; the inhibition of which has become the most important strategy for the treatment of hepatic fibrosis.

Previous studies have proved that increased activation of Wnt/β-catenin signaling might have a central role in fibrogenesis and have been implicated in several fibrosis diseases, including heart, lung, and kidney. Furthermore, it has been reported that Wnt/β-catenin is a fundamental signaling pathway to regulate the proliferation of HSCs in liver fibrosis, and some molecules in the Wnt/β-catenin signaling pathway are highly expressed and involved in the process of this disease. Therefore, Wnt/β-catenin signaling inhibition could decrease HSCs activation and proliferation and attenuate TAA-induced liver fibrosis.

TAA is an organosulfur compound that has been reported as a carcinogen. Metabolism of TAA in vivo generates free radical derivatives, TAA sulfoxide and TAA-S,S-dioxide, resulting in lipid peroxidation, ROS generation, and eventually centrilobular damage and liver injury.
Fisetin (3,7,3′,4′-tetrahydroxyflavone) is a flavonoid found in a wide variety of fruits and vegetables, such as strawberries, grapes, apples, persimmons, onions and cucumbers, with the highest concentrations in strawberries [11]. Fisetin has numerous beneficial biological activities, including, antioxidant, anti-inflammatory, anti-aging, anti-angiogenic, hypolipidemic, neuroprotective, and antitumor effects [12].

Studies have revealed that fisetin can relieve pulmonary fibrosis induced by bleomycin through suppression of TGF-β/Smad3 signaling and the inhibition of alveolar epithelium cell senescence via the AMP-activated kinase/NF-κB signaling cascade [13]. A previous investigation also reported that fisetin ameliorated hepatic inflammation and lipid deposition induced by high-fat diet via inhibition of TNF-α/receptor-interacting protein kinase 3 axis [14].

Silymarin is a standardized extract from milk thistle plant seeds that is used clinically as a "hepato-protective" agent for the treatment of liver diseases. It has the ability to scavenge free radicals, stabilize the cytoplasmic membranes and modulate enzymes associated with the development of cellular damage, fibrosis and cirrhosis [15]. The hepatoprotective effects of silymarin are accomplished through anti-oxidative, anti-fibrotic, anti-inflammatory, antitoxic, and anticancerous mechanisms of action [16].

In the current study, we endeavored to explore the protective effect of fisetin against liver fibrosis induced by TAA in rats compared to silymarin and the underlying molecular mechanisms.

Materials and methods

Drugs and chemicals

TAA was purchased from (Sigma-Aldrich, Co., St. Louis, MO). Fisetin was obtained from (Senolyfe, Dover, DE). Silymarin was obtained from Chemical Industries Development, Giza, Egypt.

Animals

Adult male albino Wistar rats weighing 150–200 g were obtained from the animal house belonging to the National Research Centre (Cairo, Egypt). Experimental animals were housed and maintained in an environmentally controlled room at (22–25 °C, 50–60% humidity with a 12h light/dark cycle) and received a standard laboratory diet and water ad libitum.

Ethical statement

The rats received human care, and the protocols of this study were performed in accordance with the ethical guidelines for the care and use of experimental animals approved by the Medical Research Ethics Committee (MREC) at the National Research Center (Reg. no. 20/038).

Experimental design

After an acclimatization period of one week, the animals were randomly divided into five groups, each of six rats and treated as follows:

Group 1 (control group): rats were injected intraperitoneally (i.p.) with sterile saline twice per week. Group 2 (TAA group): rats received TAA (200 mg/kg, i.p.) twice per week for 6 weeks [17]. Group 3 (silymarin-treated group): rats received silymarin (50 mg/kg) by gastric gavage [17] daily for 6 weeks concurrent with TAA. Groups 4 and 5 (fisetin low and high doses-treated groups): rats received fisetin (50 and 100 mg/kg) orally by gastric gavage [18] daily for 6 weeks concurrent with TAA respectively.

Collection of blood samples and preparation of liver homogenate

After 6 weeks, rats were weighed and blood samples were collected from the retro-orbital plexus, under anesthesia with i.p. injection of 50 mg/kg of ketamine (Sigma-Aldrich, St. Louis, MO). Blood was left to clot, and then centrifuged at 3000 rpm for 15 min. The serum was separated and stored at −20 °C for further use. Rats were then euthanized by cervical dislocation and livers were excised, washed with saline and weighed parts were placed in ice-cold phosphate-buffered saline (pH 7.4) and homogenized (MPW-120 homogenizer, Med Instruments, Warszawa, Poland) to prepare a 20% homogenate that was centrifuged for 5 min at 5000 × g using a cooling centrifuge (Sigma and Laborzentrifugen, Osterode, Germany). The supernatant was stored at −80 °C till the time of analysis.

Assessment of liver function biomarkers

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, and albumin were determined using colorimetric kits from Biodiagnostic, Giza, Egypt. The total protein level was determined in liver homogenate using the biocinchonic acid (BCA) protein assay kit from JenWay, Bibby Scientific, Stone, UK.

Assessment of oxidative stress and inflammatory biomarkers in liver homogenate

Levels of MDA and GSH were determined using colorimetric kits from Biodiagnostic, Giza, Egypt. TNF-α and IL-6 levels were determined using ELISA kits from (Elabscience Biotechnology, Hubei, China) and (Sunlong Biotech Co., Ltd., Hangzhou, China), respectively, according to the manufacturer’s protocols.

Assessment of fibrotic biomarkers in liver homogenate

Levels of TGF-β1, collagen I, MMP-9, and TIMP-1 were determined using ELISA kits (NOVA, Beijing, China), (Sunlong Biotech Co., Ltd, Hangzhou, China) and (SinoGeneClon...
Biotech Co., Ltd., Hangzhou, China), respectively, according to the manufacturer’s protocols.

**Assessment of β-catenin and GSK-3β in liver homogenate**

β-catenin and GSK-3β levels were determined using ELISA kits (Elabscience Biotechnology, Hubei, China) and (Fine Biotech Co., Ltd., Hubei, China), respectively, according to the manufacturer’s protocols.

**Quantitative real-time polymerase chain reaction (qRT-PCR) for detection of Wnt3a gene expression**

According to the manufacturer’s protocol, total RNA was isolated from rat liver tissues using TRIzol reagents (Carlsbad, CA). The extracted total RNA was eluted in 40 μL nuclease-free water. Wnt3a PCR quantification was carried out using a Wnt3a PCR fluorescence diagnostic kit with a fast start DNA Master SYBR Green 1 (Roche Diagnostics, Basel, Switzerland) and a Rotor-Gene Q5 plex real-time rotary analyzer (CorbettLife Science, Carlsbad, CA). RNA was transcribed using the Prime (script™ RT) Reagent kit reverse transcription kit (TaKaRa Engineering Co., Ltd., Liaoning, China).

The primers were used as follows:

Forward 5'-ATGGGGGGAGGGGAGAGAT-3'
Reverse 5'-CGCCCCCATTTGATCTTTAAG-3'

The mRNA expression level of the Wnt3a gene was normalized to that of GAPDH as the housekeeping gene. Comparative Wnt3a mRNA expression was calculated based on CT value. The calculated fold differential expression in the target gene compared to normal sample counterpart was expressed as $2^{-\Delta\Delta\text{CT}}$ [19].

**Histological examination**

After 24 h of fixation in 10% buffered neutral formalin, paraffin sections were obtained by routine processing of the autopsy liver specimens of rats from various experimental groups. The specimens were washed in tap water, dehydrated in ethyl alcohol in serial dilutions then they were cleared in xylene and finally embedded in paraffin. Sectioning of the obtained paraffin blocks was performed at 4–5 μm thickness. The obtained tissue sections were stained with hematoxylin and eosin (H&E) [20].

**Immunohistochemical studies**

Immunohistochemical studies were performed using avidin–biotinperoxidase for detecting the expression of α-SMA and cyclin D1 on paraffinized liver sections of all groups according to the method described by Ali et al. [21]. Liver sections were incubated with monoclonal antibodies for α-SMA and cyclin D1 (1:200 and 1:100 dilutions respectively; Dako Corp, Carpinteria, CA) and avidin–biotin peroxidase (Vactastain ABC peroxidase kit, Vector Laboratories, Burlingame, CA) reagents for detection of antigen–antibody complex. Visualization of each marker expression was carried out by chromagen 3,3-diaminobenzidine tetra hydrochloride (DAB, Sigma Chemical Co., St. Louis, MO). The positive brown area of each marker expression was quantified using image analysis software (Image J, 1.46a, NIH, Bethesda, MA) as optical density in 7 high-power microscopic fields.

**Statistical analysis**

All data is presented as mean ± standard error of the mean (SE). One-way analysis of variance (ANOVA) was used to assess the data in this study, followed by Tukey’s multiple comparisons test. These statistical tests were conducted using GraphPad Prism software, version 8 (GraphPad Prism Inc., San Diego, CA). The difference was considered significant when $p < .05$.

**Results**

**Effect of fisetin on hepatic function biomarkers**

Injection of TAA (200 mg/kg, i.p) significantly elevated serum levels of ALT, AST, ALP, and total bilirubin by 3.8-, 2-, 2.8-, and 3.3-folds, respectively, in comparison with control rats. Additionally, TAA injection increased the liver index (liver weight/body weight%) by 66% compared to control rats. Albumin and total protein levels were significantly decreased with TAA injection by 38.3% and 30.37%, respectively, relative to control rats.

Concurrent treatment with silymarin (50 mg/kg) or fisetin (50 and 100 mg/kg) significantly reduced serum levels of ALT by 43.5%, 58.5%, and 75.5%, AST by 32%, 43%, and 68%, ALP by 30, 38, and 56%, total bilirubin by 44%, 53%, and 75%, and liver index by 28%, 27%, and 35%, respectively, and elevated the levels of albumin by 34, 27, and 46%, and total protein by 17%, 18%, and 27%, respectively, as compared to the TAA group (Table 1).

**Effect of fisetin on oxidative stress biomarkers**

Injection of TAA significantly elevated MDA level by 109% and reduced level of GSH by 73% compared to control rats. Concomitant administration of silymarin (50 mg/kg) or fisetin (50 and 100 mg/kg) noticeably reduced the elevated levels of MDA, AST, ALP, and total bilirubin by 3.8-, 2-, 2.8-, and 3.3-folds, respectively, and showed a significant elevation in GSH level by 45.6%, 78%, and 121%, respectively, compared with the TAA group (Table 2).

**Effect of fisetin on inflammatory cytokine production**

TAA injection significantly increased IL-6 and TNF-α in liver tissue by 1.43- and 1-folds relative to control. Whereas silymarin (50 mg/kg) and fisetin (50 and 100 mg/kg) noticeably reduced the hepatic IL-6 and TNF-α level by 52.5%, 48%, and 58.7% and 27.8%, 32.3%, and 45%, respectively, compared to the TAA group (Figure 1).
Effect of fisetin on hepatic fibrogenic biomarkers

TAA-intoxicated rats showed a significant elevation in the levels of TGF-β1 and collagen I by 40.5% and 90% compared to control. In contrast, concurrent treatment with silymarin (50 mg/kg) or fisetin (50 and 100 mg/kg) markedly decreased their levels by 21%, 17.5%, and 23.5% and 31%, 18.4%, and 33.6%, respectively, compared to TAA-injected rats (Figure 2(a,b)).

TAA caused a significant decrease in MMP-9 by 65% and an increase in TIMP-1 by 66.6% compared to the normal control group, while silymarin (50 mg/kg) or fisetin (50 and 100 mg/kg) increased MMP-9 hepatic content by 81%, 66%, and 140%, respectively, and reduced TIMP-1 by 19.5%, 8.4%, and 31%, respectively, compared to the TAA group (Figure 2(c,d)).

**Table 1.** Effect of fisetin on serum hepatic functions biomarkers and total protein in liver tissue.

<table>
<thead>
<tr>
<th>Normal control</th>
<th>TAA group</th>
<th>Silymarin (50 mg/kg)+TAA</th>
<th>Fisetin (50 mg/kg)+TAA</th>
<th>Fisetin (100 mg/kg)+TAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>55.9 ± 2.3</td>
<td>271.9 ± 25.4a</td>
<td>153.5 ± 22.7ab</td>
<td>113.5 ± 8.7b</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>79.7 ± 6.5</td>
<td>253 ± 21.2a</td>
<td>171.3 ± 23.8ab</td>
<td>144.7 ± 11.7b</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>169.3 ± 14.5</td>
<td>659.7 ± 13.3a</td>
<td>459.7 ± 17.5ab</td>
<td>408.3 ± 18.13ab</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>0.61 ± 0.05</td>
<td>2.68 ± 0.23a</td>
<td>1.51 ± 0.11ab</td>
<td>1.25 ± 0.043ab</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>4.17 ± 0.17</td>
<td>2.57 ± 0.112a</td>
<td>3.45 ± 0.157ab</td>
<td>3.27 ± 0.068ab</td>
</tr>
<tr>
<td>Total protein (mg/ml)</td>
<td>1.228 ± 0.06</td>
<td>0.855 ± 0.019a</td>
<td>1.003 ± 0.029a</td>
<td>0.963 ± 0.026a</td>
</tr>
<tr>
<td>Liver index</td>
<td>3.07 ± 0.08</td>
<td>5.08 ± 0.23a</td>
<td>3.68 ± 0.23b</td>
<td>3.73 ± 0.170b</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SE (n = 6) for each group. Statistical analysis was carried out by ANOVA followed by Tukey’s multiple comparisons test.

* Statistically significant from control group at p < .05

**Table 2.** Effect of fisetin on hepatic oxidative stress biomarkers.

<table>
<thead>
<tr>
<th>Normal control</th>
<th>TAA</th>
<th>Silymarin (50 mg/kg)+TAA</th>
<th>Fisetin (50 mg/kg)+TAA</th>
<th>Fisetin (100 mg/kg)+TAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (mg/g tissue)</td>
<td>9.96 ± 0.19</td>
<td>2.69 ± 0.19a</td>
<td>3.93 ± 0.18ab</td>
<td>4.81 ± 0.26ab</td>
</tr>
<tr>
<td>MDA (nmol/g tissue)</td>
<td>9.548 ± 0.45</td>
<td>19.97 ± 0.91a</td>
<td>11.55 ± 0.32ab</td>
<td>12.46 ± 0.24ab</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SE (n = 6) for each group. Statistical analysis was carried out by ANOVA followed by Tukey’s multiple comparisons test.

* Statistically significant from the control group at p < .05

**Effect of fisetin on hepatic Wnt3a, β-catenin, and GSK-3β**

TAA-intoxicated rats exhibited a significant increase in the mRNA expression level of Wnt3a as detected by qRT-PCR by 41.5% compared to the control group. Silymarin (50 mg/kg) or fisetin (50 and 100 mg/kg) significantly down-regulated the mRNA expression levels of Wnt3a by 21.5%, 18.3%, and 26.7% compared with the TAA group, as shown in Figure 3(a).

TAA caused a significant decrease in GSK-3β level by 47.5% and increase in β-catenin hepatic content by 64.5% as compared to normal control, while treatment with silymarin (50 mg/kg) or fisetin (50 and 100 mg/kg) significantly elevated GSK-3β level by 54%, 42%, and 79%, respectively, and decreased
hepatic β-catenin level by 35%, 33%, and 42%, respectively, as compared with the TAA group (Figures 3(b,c)).

**Fisetin explicitly inhibited the progress of hepatic fibrosis**

Liver sections of control rats displayed normal histological structures of central veins, portal areas, and hepatic parenchymal cells (Figure 4(a)). On the other hand, livers of rats in the TAA group showed severe fibroplasia started from the portal triads including; proliferation of bile duct epithelium, vascular congestion, infiltration of mononuclear inflammatory cells, and marked proliferation fibrous tissue (Figure 4(b)), which was peripherally extended as fibrous bands toward the parenchyma which caused marked parenchymal pseudo-lobulation (Figures 4(c,d)). Within those pseudo-lobules, the hepatic cells revealed vacuolar degeneration with eccentric nuclei, necrosis (Figure 4(e)), and apoptosis, as well as infiltration of mononuclear inflammatory cells alongside the fibrous septa (Figure 4(f)).

In regards to the livers of various treated groups, variable degrees of retraction of fibrous proliferation were noticed. The portal triads in the livers of silymarin-treated rats (Figures 4(g–i)) showed a moderate degree of fibrous proliferation with peripherally extended incomplete septa, accompanied with mild inflammatory cell infiltration and cholangiolar proliferation. Mild degenerative and necrotic changes in the hepatic cells were noticed. Regarding, the livers of low-dose fisetin administrated rats (Figures 4(j–l)) showed portal areas of fibroplasia but of mild intensity,
sometimes with peripheral extension and mild proliferation of bile duct epithelium and few inflammatory cell infiltration. The hepatic cells showed a moderate degree of degeneration, scattered necrosis, and apoptosis. While the livers of high dose fisetin-administrated rats (Figure 4(m–o)) revealed limitation of fibrous proliferation to the portal areas with minimal changes and good restoration of the hepatic cells.

**Immunohistochemistry analysis**

Livers of control rats showed mild regular α-SMA expression around the portal and central veins where the existence of myofibroblasts (Figure 5(a)) and negative expression of cyclin D1 (Figure 6(a)). Livers of TAA-administered rats revealed marked increased α-SMA expression in the portal areas, along with the extended fibrous septa (Figure 5(b,c)), pericellular and perisinusoidal spaces, as well as severe expression of cyclin D1 (Figure 6(b,c)). While deceased immunoexpression of both markers was observed in the silymarin-administrated group (Figures 5(d) and 6(d)). The administration of fisetin showed decreased the expression of both α-SMA (Figure 5(e,f)) and cyclin D1 (Figure 6(e,f)), particularly in the high-dose group. The quantitative analysis of the area percent of the positive brown color of α-SMA and cyclin D1 presented as the optical density revealed significant (p < .05) increased expression in the TAA-administered group compared to that of the other treated groups (Figures 5(g) and 6(g)).

**Discussion**

Liver fibrosis is a complicated process in which damage and cell death trigger inflammatory responses and immune cell recruitment. Chemokines, cytokines, and other growth factors
are secreted, which activate HSCs and enhance their proliferation and production of extracellular matrix components, and subsequently the fibrotic process [22].

The current work highlights the potential anti-fibrotic activity of fisetin against liver fibrosis induced by TAA in rats and the mechanisms underlying that activity.

In the present experimental model, hepatic fibrosis was induced by the injection of TAA intraperitoneally. The TAA group exhibited a significant increase in serum levels of ALT, AST, ALP, and total bilirubin with a reduction in serum level of albumin and hepatic content of total protein as compared to the normal control group. These results are in line with El-Baz et al. [17]. TAA is metabolized by cytochrome P450 2E1, producing ROS which attack lipids, proteins, and DNA, resulting in centrilobular necrosis and leakage of hepatic enzymes (ALT and AST) in serum, thus elevating their levels [9]. Moreover, TAA causes a considerable attenuation of hepatocyte synthetic function, including total protein and albumin, so decreasing their levels and detoxifying function that explains the elevation in serum bilirubin [23]. Furthermore, an increased level of ALP may be due to a pathological change in biliary flow [24]. The previous biochemical changes were confirmed by the observed histopathological alterations, which showed hepatocellular degeneration and necrosis in the TAA group. Additionally, excessive deposition of ECM in the fibrotic liver leads to a significant increase in

Figure 4. H&E-stained liver sections: (a) Liver of control rat shows normal histological structure. (b–f) Liver of TAA-administrated rat showing; (b) thickening of the portal tract with fibrous proliferation, inflammatory cells infiltration (thin arrow), proliferated bile duct epithelium, (c and d) marked parenchymal pseudolobulation, (e) vacuolar degeneration of the hepatic cells within the pseudo-lobules (thin arrow), and (f) marked apoptosis (thick arrow). (g and o) livers of; silymarin, fisetin (low dose) (j–l) and fisetin (high dose) (m–o) treated groups showing marked retraction of the fibrous tissue proliferation with its limitation to the portal areas and absence of parenchymal pseudolobulation and restoration of the hepatic cells, which all more obvious in fisetin (high dose) treated group. Signs on figures from g–o denote; incomplete septa (arrow), proliferated bile duct epithelium (dotted arrow), inflammatory infiltrates (thin arrow) in (i, k, l and o), and portal area (short arrow).
relative liver weight and the ratio of liver to body weight (liver index) [25]. This explains the recorded elevated liver index in the TAA group in our work.

Oral administration of fisetin concurrently with TAA injection was shown to be hepatoprotective, as indicated by the reduction of all liver enzymes and elevation of albumin and total protein levels. This outcome means that fisetin could protect hepatic cells from oxidative damage caused by TAA and reduce leakage of liver enzymes into the blood may be due to its powerful antioxidant activity [26]. A previous study showed that pretreatment with fisetin normalized liver enzyme levels in mice intoxicated with acetaminophen [27], confirming our results. Fisetin has also been shown to improve the functional status of the liver in ammonium chloride-induced hyper-ammonic rats [28].

Fisetin significantly decreased liver index compared to the TAA group, indicating that fisetin reduced ECM deposition in the liver [14].

Concurrent administration of silymarin significantly reduced serum transaminases and ALP levels, as well as increased albumin and total protein levels. Those findings are in line with those of Jalali et al. [29]. That effect can be ascribed to the ability of silymarin to stabilize the hepatocyte membrane, regulate membrane permeability, and prevent toxins from entering into hepatic cells [29].

In the present work, TAA caused oxidative stress in the rat liver, verified by a significant depletion of GSH level and a significant elevation in MDA level. This result is in agreement with Sukalingam et al. [30]. Excessive ROS production, induced by TAA, overwhelms the natural intracellular antioxidant capacity and thus increases oxidative stress, lipid peroxidation, and MDA levels. In addition, ROS accumulation triggers inflammatory signals and activates HSCs [31]. In the current study, fisetin displayed antioxidant properties by increasing GSH level and reducing MDA level, revealing that fisetin could alleviate lipid peroxidation by acting as a scavenger of ROS due to its electron donating capacity and neutralization of reactive oxygen species which is the result of the presence of a hydroxyl group at C-3', C-4' at B ring and a hydroxyl group at C-7 of A ring [12]. Another study showed that fisetin attenuated oxidative stress and boosted antioxidant defense in the diabetic heart by suppressing lipid peroxidation and restoring endogenous antioxidants [32].

Silymarin-treated rats showed restoration of GSH level and a reduction in the MDA level. The antioxidant activity of silymarin can be explained by its ability to scavenge free radicals, inhibit lipid peroxidation, and preserve GSH [33].
The increased release of proinflammatory cytokines such as TNF-\(\alpha\) and IL-6 by Kupffer cells and stimulated HSCs is one of the early signs of hepatic fibrosis. Many investigators have reported that TNF-\(\alpha\) and IL-6 have both proinflammatory and profibrotic effects [34,35]. In the present work, liver inflammation induced by TAA injection was revealed by a significant elevation in levels of TNF-\(\alpha\) and IL-6 in liver tissue. This result agrees with that of Elnfarawy et al. [36]. The inflammation caused by TAA may be attributed to increased ROS that stimulates the activation of the transcription factor nuclear factor kappa-B, which up-regulates inflammatory cytokines production, such as IL-6 and TNF-\(\alpha\) [9]. Also, hepatocyte damage induced by TAA leads to the release of inflammatory mediators that activate defensive and M1 macrophages. This in turn stimulates the production of proinflammatory cytokines, including IL-6, by Th1 cells [23]. Meanwhile, the concurrent treatment with fisetin caused a significant decrease in hepatic content of TNF-\(\alpha\) and IL-6 compared to the TAA group, which is in accordance with Zhang et al. [37]. The anti-inflammatory activity of fisetin may be attributed to the blockade of TNF receptor 1/TNF receptor-associated factor 2/nuclear factor kappa B (TNFR-1/TRAF-2/NF-\(\kappa\)B) signaling, which in turn decreases the synthesis of down-stream proinflammatory cytokines, such as IL-6 and TNF-\(\alpha\) [38].

Silymarin significantly decreased TNF-\(\alpha\) and IL-6 levels, this is in agreement with Ali et al. [33] who attributed the anti-inflammatory activity of silymarin to inhibition of Toll like receptor 4 gene expression, which suppresses NF-\(\kappa\)B that consequently down-regulates IL-6 and TNF-\(\alpha\) expression.

TGF-\(\beta\)1 is the major cytokine involved in the progression of liver fibrosis and also regulates cell growth and differentiation [39]. In normal hepatic tissues, TGF-\(\beta\)1 expression is mainly in Kupffer cells and hepatic sinusoidal endothelial cells, while the expression is relatively lower in HSCs. While, after hepatic injury, TGF-\(\beta\)1 level increases and binds to TGF-\(\beta\) receptor on HSCs surface, gradually activating HSCs, and then stimulating the production of collagen and \(\alpha\)-SMA, further stimulating the development of fibrosis [40]. In the present study, levels of TGF-\(\beta\)1, collagen I, and \(\alpha\)-SMA immune expression were significantly up-regulated in the TAA group; this is in line with Hsieh et al. [41]. These findings were supported by our histopathological observation of severe fibroplasia and marked parenchymal pseudolobulation in the TAA group. However, levels of TGF-\(\beta\)1, collagen I, and \(\alpha\)-SMA immune expression were significantly down-regulated in fisetin-treated groups, ameliorating ECM accumulation induced.

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**Figure 6.** Immunohistochemical analysis of cyclin D1. (a) control group showing negative expression, (b and c) TAA administrated group showing marked expression, (d) silymarin treated group showing moderate expression in scatted hepatic cells, (e and f) fisetin low and high doses-treated groups showing marked dose-related decreased expression, (g) quantitative image analysis of the area percent of the positive brown color of cyclin D1 presented as the optical density. Data are expressed as the mean \(\pm\) SE (\(n = 6\)) for each group. Statistical analysis was carried out by ANOVA followed by Tukey’s multiple comparisons test. *Statistically significant from the control group at \(p < .05\). **Statistically significant from the TAA group at \(p < .05\).
by TAA, as evidenced by the limitation of fibrous proliferation in histopathological examination. The latter brings to light that fisetin curbed the trans-differentiation of HSC to active myofibroblasts and consequently decreased ECM accumulation. Our results are in harmony with those of Zhang et al. [13].

The imbalance between the synthesis and degradation of ECM is a characteristic feature of liver fibrosis [42]. Physiologically, numerous matrix metalloproteinases (MMPs) control the secretion and deposition of ECM and can degrade ECM components. However, during fibrosis, tissue inhibitors of MMPs (TIMPs) are up-regulated in response to chronic liver injury and greatly inhibit MMPs [43]. In the present study, TAA decreased MMP-9 and increased TIMP-1 levels, a result which is in agreement with El-Baz et al. [44]. While fisetin concurrently administrated, could restore MMP-9 level and reduced TIMP-1 level to modulate ECM remodeling during liver injury. This effect may be due to the expression of high levels of MMPs by restorative macrophages during fibrosis resolution [45].

It was reported that fisetin up-regulated the expression of MMP-9 in stimulated hepatocellular carcinoma cells [46] and significantly reduced the hepatic expression of TIMP-1 gene in hepatic fibrosis in diet-induced obese mice [47].

Silymarin concurrent treatment reduced the levels of TGF-β1, collagen I, TIMP-1, and α-SMA immune expression, as well as increased level of MMP-9. These findings confirm the ability of silymarin to suppress hepatic fibrosis through inhibition of the conversion of HSC to active myofibroblasts and reduction of ECM deposition [44].

The canonical Wnt/β-catenin is a key signaling pathway regulating the proliferation of HSCs in the process of liver fibrosis [48]. Cyclin D1 is a significant target gene in the Wnt signaling pathway that induces cell entry into the S-phase and G2/M-phase, thus controlling the proliferation and differentiation of cells [49]. The activation of Wnt signaling is associated with abnormal expression of β-catenin and cyclin D1 [50]. Upon activation of Wnt signaling, Wnt ligands bind the seven-transmembrane domain receptor Frizzled (FZD) and the single-transmembrane-domain receptor low-density lipoprotein-receptor-related protein (LRP)5/6 to form a heterotrimer (Wnt-FZD-LPR5/6) that inhibits phosphorylation of β-catenin through recruitment of Disheveled and inhibition of GSK-3β [51]. This leads to the accumulation of β-catenin in the cytoplasm, which then enters the nucleus and binds with transcription factors such as T-cell factor (TCF)/lymphoid enhancer factor (LEF) to activate the expression of cyclin D1 and stimulates cell proliferation [52]. It was proven that the release of cytokines (e.g. IL-6 and TNF-α) and the recruitment of inflammatory cells were stimulated by the canonical Wnt/β-catenin signaling [53]. Furthermore, Wnt/β-catenin signaling can promote the function of HSCs and TIMP-1 as well as reduce MMPs activation that results in the development of liver fibrosis [54].

The current work revealed that TAA significantly increased gene expression of Wnt3a, level of β-catenin and immune-expression of cyclin D1, but significantly decreased the level of GSK-3β in liver tissue, which runs parallel with the results of Abd Elhameed et al. [55] and Abdel-Bakky et al. [56]. The concurrent administration of fisetin in our work significantly reduced Wnt3a gene expression, β-catenin level, and cyclin D1 immune-expression and significantly increased the level of Gsk-3β, a result which is in harmony with Syed et al. [57] who found that fisetin inhibited the growth of human melanoma cells via suppression of Wnt/β-catenin signaling. In the same line, fisetin was found to significantly reduce β-catenin and pGSK-3β levels in epithelial-mesenchymal transition induced by cigarette smoke extract in airway epithelial cells [58]. In addition, fisetin inhibited thyroid tumor cell proliferation by down-regulating the expression of cyclin D1 [59]. The inhibitory effect of fisetin on Wnt/β-catenin signaling may be the result of increased protein expression of Wnt endogenous inhibitors, including Dickkopf and Wnt inhibitory factor-1. Additionally, the cytosolic level Axin was increased and the phosphorylation of Gsk-3β was decreased by fisetin, resulting in a reduction in nuclear β-catenin levels. Furthermore, the functional cooperation between β-catenin and the transcription factor LEF-1/TCF-2 was interrupted by fisetin, leading to a reduction in β-catenin targets such as cyclin D1 [57].

Silymarin down-regulated Wnt3a gene expression, β-catenin level, and cyclin D1 immune-expression and increased the level of Gsk-3β. Our results are in line with Vaid et al. [60] who found that silymarin increased degradation and reduced the nuclear accumulation of β-catenin as well as enhanced the levels of casein kinase 1α, glycogen synthase kinase-3β and phosphorylated β-catenin in melanoma cells.

In this study, fisetin outperformed silymarin in terms of liver function improvement, substantial antioxidant effects via GSH stores replenishment, anti-inflammatory effects, modulation of MMP-9 and TIMP-1, and inhibition of multiple profibrogenic factors. Fisetin also demonstrated a distinguished decline in collagen deposition and considerable inhibition of HSC proliferation and activation via inhibition of Wnt/β-catenin signaling.

Conclusion

The present study revealed that fisetin could be a promising protective and therapeutic agent for liver fibrosis via: (1) inhibiting HSCs activation and proliferation through the suppression of the Wnt/β-catenin signaling pathway; (2) expressing anti-oxidant, anti-inflammatory, and anti-fibrotic effects; and (3) modulating MMP-9 and TIMP-1 pathway.

Author contributions

Amany A. El-Fadaly and Abeer Salama designed the experiments, analyzed the data, performed the experiments, wrote and revised the manuscript. Mohamed F. Abdelhameed performed the experiments and revised the manuscript. Sahar S. Abd El-Rahman performed histopathological and immunohistopchemical examinations, analyzed the data, wrote and revised the manuscript. A. Ramadan, Wafaa El-Eraky and Nehal A. Afffi supervised and revised the manuscript.
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