



Apigenin role against thioacetamide-triggered liver fibrosis: Deciphering the PPAR γ /TGF- β 1/NF- κ B and the HIF/FAK/AKT pathways

Rehab F. Abdel-Rahman^{1*}, Hany M. Fayed¹, Marwan Abd Elbaset Mohamed¹, Alyaa F. Hessin¹, Gihan F. Asaad¹, Sahar AbdelRahman², Abeer A.A. Salama¹, Mahmoud S. Arbid¹, Hanan A. Ogaly³

¹Department of Pharmacology, Medical Research and Clinical studies Institute, National Research Centre, Dokki, Giza 12622, Egypt

²Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt

³Department of Biochemistry, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt

ARTICLE INFO

Article Type:

Original Article

Article History:

Received: 2 October 2022

Accepted: 24 December 2022

Keywords:

Malondialdehyde

Transforming growth factor beta 1

Tumour necrosis factor alpha

Alpha-smooth muscle actin

Hydroxyproline

Liver fibrosis

Rats

ABSTRACT

Introduction: Liver tissue malfunction is a severe worldwide health concern that arises from various chronic liver conditions. The goal of this investigation was to look into the anti-fibrotic effect of apigenin (APG), an antioxidant found in various plants, versus thioacetamide (TAA)-triggered hepatic scarring in rats and the potential mechanisms behind it.

Methods: TAA was administered thrice weekly (100 mg/kg, i.p.) for two weeks to produce hepatic scarring. APG was administered after TAA for 14 days (5 or 10 mg/kg, orally). Thereafter, hepatic liver enzymes, inflammatory markers, fibrotic indicators, and histopathological changes were evaluated.

Results: TAA increased the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), reduced albumin and total protein, elevated hepatic level of lipid peroxidation, focal adhesion kinase (FAK), hypoxia-inducible factor-1 α (HIF-1 α), and inflammatory cytokines, decreased interleukin-10 (IL-10), reduced hepatic expression of peroxisome proliferator-activated receptor gamma (PPAR γ) and nuclear factor-erythroid factor 2-related factor 2 (Nrf2), and elevated serine-threonine protein kinase (AKT) expression. Furthermore, TAA increased hepatic contents of collagen I, connective tissue growth factor (CTGF), hydroxyproline, and alpha-smooth muscle actin. On the other hand, APG evaded these changes and mitigated the harmful effects of TAA in a dose-dependent way. Histopathological and immunohistochemical observations reinforced these biochemical outcomes.

Conclusion: APG can potentially alleviate liver fibrosis mediated via FAK and HIF1 inhibiting signaling pathways.

Implication for health policy/practice/research/medical education:

Our research showed that apigenin has the potential to be a therapeutic medication for hepatic fibrosis. However, further studies are needed to establish the clinical applicability of APG treatment in active liver fibrogenesis human cases.

Please cite this paper as: Abdel-Rahman RF, Fayed HM, Mohamed MA, Hessin AF, Asaad GF, AbdelRahman S, et al. Apigenin role against thioacetamide-triggered liver fibrosis: Deciphering the PPAR γ /TGF- β 1/NF- κ B and the HIF/FAK/AKT pathways. J Herbm Pharm. 2023;12(2):202-213. doi: 10.34172/jhp.2023.21.

Introduction

Liver illnesses are a prominent reason for fatality and comorbidity worldwide, accounting for two million fatalities (2.4% of all deaths) and 1.5% of disability-adjusted life yearly. Most cases of chronic liver disease are brought on by one of three things: viral hepatitis B or C infection, liquor-related hepatic disease, or non-liquor lipidemic hepatic disease (1). These disorders implicate the

destruction and regeneration of liver parenchyma leading to hepatic fibrosis and cirrhosis. Cirrhosis can be fatal as a result of complications related to portal hypertension, hepatocellular carcinoma (HCC), and liver failure, leaving a liver transplant as the only therapy (2). New hepatitis C drugs may slow the progression of fibrosis, but there is still no definite treatment for fibrosis (3). Egypt is the country with the highest hepatitis C virus prevalence worldwide.

*Corresponding author: Abdel-Rahman, Email: rehabs2001@yahoo.com, rf.abdelrahman@nrc.sci.eg

As per the information from the Egyptian Health Issues Survey “14.7% of the people aged 15–59 years had an active hepatitis infection, which decreased to 7% in 2015 and was noticeably higher than global levels” (4).

Liver fibrosis is a progressive process of repetitive and prolonged liver damage that occurs when hepatic stellate cells (HSCs) are activated and changed into expansive and contractile myofibroblasts (5). The production of myofibroblasts is enhanced, and the breakdown of extracellular matrix components is slowed, which results in fibrogenesis (6). However, research from both human trials and animal models clearly shows that liver fibrosis is a progressive response that may be controlled by slowing or speeding up its advancement. As a result, knowing the molecular processes behind these events is critical for developing antifibrotic drugs (7).

New studies have shown that a collection of transcription factors known as hypoxia-inducible factors (HIFs) may be important in the advancement of liver fibrosis (8). HIFs are a group of transcriptional regulators that have evolved to have a physiological sensitivity to low oxygen levels. HIFs comprise an oxygen-reliant α component, “HIF-1 α , HIF-2 α , or HIF-3 α ”, and a fundamental formed β component. The HIF-1 α is responsible for cellular responses to hypoxia (9). In response to hypoxia, HIF-1 α transfers from the cell cytoplasm to the nucleus, where it binds to the nuclear protein HIF1 β and then triggers the activation of target genes involving matrix metalloproteinases, platelet-derived growth factor, transforming growth factor (TGF), and pro-inflammatory/fibrotic genes like VEGF (10). HIF-1 α promotes hepatic steatosis, necroinflammation, and fibrogenesis by controlling the genes responsible for the production of lipids within the hepatocyte, activation of HSCs, initiation of free fatty acid oxidation, and activating Kupffer cell. Overexpression of HIF-1 α has been connected to non-liquor lipidemic hepatic disease, hepatic inflammatory disease, and scarring in several animal models (11).

Focal adhesion kinase (FAK) is a tyrosine kinase in the cytoplasm that is involved in various cellular events, including cell proliferation, survival, migration, and invasion (12). Multiple profibrotic stimuli can activate the FAK pathway. Its downstream signalling is implicated in two key pathways, mTOR/P70S6K and ERK1/2, which can trigger alpha-smooth muscle actin (α -SMA)-positive myofibroblast differentiation in addition to collagen synthesis, reflecting liver fibrosis development (13). Consequently, the pharmacological inhibition of HIF-1 α and FAK pathways could be an effective therapeutic strategy against liver fibrosis.

Apigenin, 4',5,7-trihydroxyflavone (APG), is an antioxidant found in many fresh foods, such as parsley, celery, onions, apples, and spices. APG's sedative, antihypertensive, anti-inflammatory, antioxidant, and anticancer properties have gotten a lot of recognition. APG has also been shown to reduce cell viability in

prostate cancer, breast cancer, melanoma, and leukemia cells. Its antitumor effect has been linked to a number of mechanisms, comprising cell cycle arrest due to the alteration of cell cycle proteins, apoptotic induction, fatty acid synthesis inhibition, reactive oxygen species generation, and survival signalling inactivation (14). FAK kinase activity was proved to be suppressed by APG via phosphorylation, resulting in a substantial downregulation of the FAK-dependent downstream pathway (15). APG has also been demonstrated to inhibit the production of HIF-1 α in human pancreatic cancer cells (16). APG's low intrinsic toxicity has sparked interest as a health-promoting drug. APG's potential as an anti-fibrotic medication that targets HSCs has only been established in one in vitro study (17).

Therefore, we postulate that APG may be repositioned as an anti-fibrotic therapeutic option against liver fibrosis through modulation of HIF-1 α and FAK pathways. Here, we employed a rat model of hepatic fibrosis produced in the lab to assess APG's anti-fibrotic effects and clarify the potential involvement of downregulation of HIF-1 α and FAK pathways in mediating these effects.

Materials and Methods

Animals

Albino male rats weighing between 180 and 210 g were brought from the “Animal House at the National Research Centre, Cairo, Egypt”. Rats were preserved under normal circumstances of ambient conditions with equal day and night intervals. All tests and methods were approved by “the Ethics Committee of Animal Care and Use of the National Research Centre (NRC) with approval number MREC-19-214”.

Chemicals

Sigma-Aldrich in the United States provided thioacetamide (TAA). APG was obtained from Swanson, North Dakota, USA, and all other substances used during the study were of the uppermost diagnostic level accessible.

Experimental design

The rats were split up into four different groups randomly (6 animals per group) after a one-week acclimatization period, as per the following system:

- Group 1: The negative control group: Rats received i.p. 1% Tween 80 in distilled water (DW), thrice weekly for two weeks, and daily received the vehicle orally for another two weeks.
- Group 2: The TAA intoxicated group: Rats received TAA (100 mg/kg, i.p.) three times weekly for 2 consecutive weeks, and daily received the vehicle orally for another two weeks.
- Groups 3 and 4: Rats orally received APG (5 and 10 mg/kg, respectively; dissolved in DW daily) (18) for 14 days subsequent to the 2 weeks of TAA injection.

Blood and tissue samples

After 4 weeks, blood specimens were taken from every rat's tail vein under anesthesia with mild midazolam (10 mg/kg). The acquired blood samples were processed to get the serum, frozen at -20°C until additional biochemical analysis. Under the influence of IP ketamine-xylazine (K, 100 mg/kg; X, 10 mg/kg), each animal was euthanized by cervical dislocation, and its hepatic tissue was swiftly detached, washed in iced saline, dried, and weighed immediately after blood was drawn. Specimens from the left segment of the rats were separated and deposited in 10% formol for histological and immunohistochemical analyses. At the same time, the other portion of the liver with a known weight was stored at -80°C for future analysis.

Examining the biochemical profile of the blood

Serum activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, and total protein were estimated by colorimetric method with the aid of Biodiagnostic kits.

Homogenization of hepatic tissue

According to Mansour et al (19), liver homogenate of 20% w/v was prepared. Hepatic tissue was homogenized with ice-cooled saline using a homogenizer; "Medical equipment, MPW-120, Poland". In order to get rid of cellular waste, the homogenate was centrifuged at 3000 rpm/10 minutes at 4°C in a cooling centrifuge; "Laborzentrifugen, 2k15, Sigma, Germany". For subsequent biochemical examination, the aliquot was stored at -80°C.

Analysis of oxidative stress markers

Substances of reduced glutathione in the liver (GSH) and malondialdehyde (MDA) were performed chemically.

Assessment of fibrotic markers and proinflammatory indicators

Liver contents of hypoxia-inducible factor 1-alpha (HIF1- α), FAK, tumour necrosis factor-alpha (TNF- α), nuclear factor-kappa B (NF- κ B), interleukin-10 (IL-10), interleukin 1 beta (IL-1 β), TGF- β 1, α -SMA, collagen 1 (Col 1), hydroxyproline (Hyp), and connective tissue growth factor (CTGF) were determined with the aid of ELISA kits as per to the pamphlet of "Sunlong Biotec Co. LTD, Zhejiang, China".

Histological examination

According to Bancroft & Gamble methodology (20), liver specimens collected from various groups were fixed for 24 hours in 10% buffered neutral formalin. Next, the fixed specimens were subjected to a routine processing for obtaining paraffin blocks. The later blocks were sectioned serially into 4–5 μ m thick sections to be evaluated histologically and immunohistochemically. H&E and Van Gieson's stains were used to examine tissue sections from each group. Additionally, the Ishak scoring system

was used for the evaluation of fibrosis and the extension of the necro-inflammatory lesions. For fibrosis it ranged from F0 = no fibrosis to F6 = cirrhosis and for necro-inflammatory lesions, focal lytic necrosis, apoptosis, and focal inflammation it ranged from F0 (absent) to F4 (severe). The positive red area of Van Gieson's stain, fibrosis indicator was quantified using "image analysis software ImageJ, 1.46a, NIH, USA" with optical density in seven different high-magnification microscopical regions.

Immunohistochemical examination

Immunohistochemical detection of the expression of Akt and Nrf2 was carried out on paraffin (21). Hepatic sections were preserved with antibodies for Akt Abcam (ab32038) as well as Nrf2 (ab92946) (Abcam, Cambridge, USA) at dilutions of 1:200 and 1:100 respectively. Then avidin-biotin peroxidase, Vectastain ABC peroxidase kit (Vector Laboratories reagents for detection of antigen-antibody complex) were used for the analysis of Akt overexpression by Chromagen 3, 3-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co). The positive brown area of each marker's expression was quantified using image analysis software (ImageJ, 1.46a, NIH, USA), and the optical density in seven different high-magnification microscopical regions was performed.

qRT-PCR analysis for Nrf2, PPAR- α , and NF- κ B expression in the liver tissues

TRIzol™ Reagent supplied by Invitrogen, USA (Cat. No. 74104) was used for total RNA extraction from frozen liver samples. A UV spectrophotometer was used to measure the concentration and purity of the isolated RNA. Then, using a SuperScript IV VILO kit supplied by Invitrogen, reverse transcription of the extracted RNA was done to produce complementary DNA (cDNA). Nrf2 and PPAR- α genes were amplified from cDNA using Luminaries Color HiGreen Low ROX Real-Time PCR System supplied by Thermo Scientific and the following primers sets:

Nrf2 forward CACATCCAGACAGACACCAGT;
Nrf2 reverse CTACAAATGGGAATGTCTCTGC (XM_006234398.3); PPAR α forward TTCGGAACTGCAGACCT; PPAR α reverse TTAGGAACTCTCGGGTGAT (NM_013196.1); NF- κ B forward CTGGCAGCTCTTCTCAAAGC; NF- κ B reverse CCAGGT-CATAGAGAGGCTCAA (XM_006233360.3); β -actin forward AT GGTGGGTATGGGTCAG; and β -actin reverse CAATGCCGTGTTCAATGG (NM_031144.3) (22). All were used following the manufacturer's instructions. Expression levels were normalized to β -actin as the endogenous control gene in each examined sample. Relative mRNA expression was calculated using the comparative cycle threshold (Ct) ($2^{-\Delta\Delta Ct}$) equation according to Schmittgen and Livak. Melting curves were checked to confirm the specificity of PCR-amplified products.

Statistical analysis

As per the procedure of Elbaset et al, before proceeding with the statistical analysis, data values were checked for normality using the Shapiro test. The results were displayed as means \pm SD. The data analysis operation was performed by One-way ANOVA followed by the Tukey post hoc test. GraphPad Prism software (version 9, USA) was employed to perform the statistical analysis and to establish the represented graphs. The significance level was set to ($P \leq 0.05$) for all statistical tests (23).

Results

Effect of APG on liver weight and liver injury markers

Relative liver weight was appreciably increased in the TAA group related to the normal rats. Meanwhile, treatment with APG significantly alleviated the increase in the relative liver weight of TAA rats during the treatment course (data not shown).

After TAA administration, the hepatic enzymes (ALT and AST) leaked into the blood from the damaged hepatic cells, manifested by a significant elevation of their activities versus the negative control group. Similarly, the

intraperitoneal injection of TAA considerably reduced the concentrations of serum albumin and total protein, as well as the albumin/globulins (A/G) ratio, compared to the negative control group. Treatment with APG (5 and 10 mg/kg) significantly reduced hepatic enzymes levels (ALT and AST). It restored the concentrations of serum albumin, total protein, and A/G ratio to almost normal levels versus the TAA group (Figure 1).

Liver oxidative stress indicators and their impact on APG treatment

A substantial elevation in hepatic MDA was shown in the TAA-treated rats versus the control group. An influential diminution accompanied this elevation in the liver's GSH content in comparison to the control group. These changes were successively restored in APG-treated groups, where APG prominent enhanced GSH content and reduced MDA, versus the TAA group, as shown in Table 1.

Effect of APG on inflammatory markers status in liver

The pro-inflammatory markers, TNF- α , IL-1 β , NF- κ B, and TGF- β , were discovered to be appreciably amplified

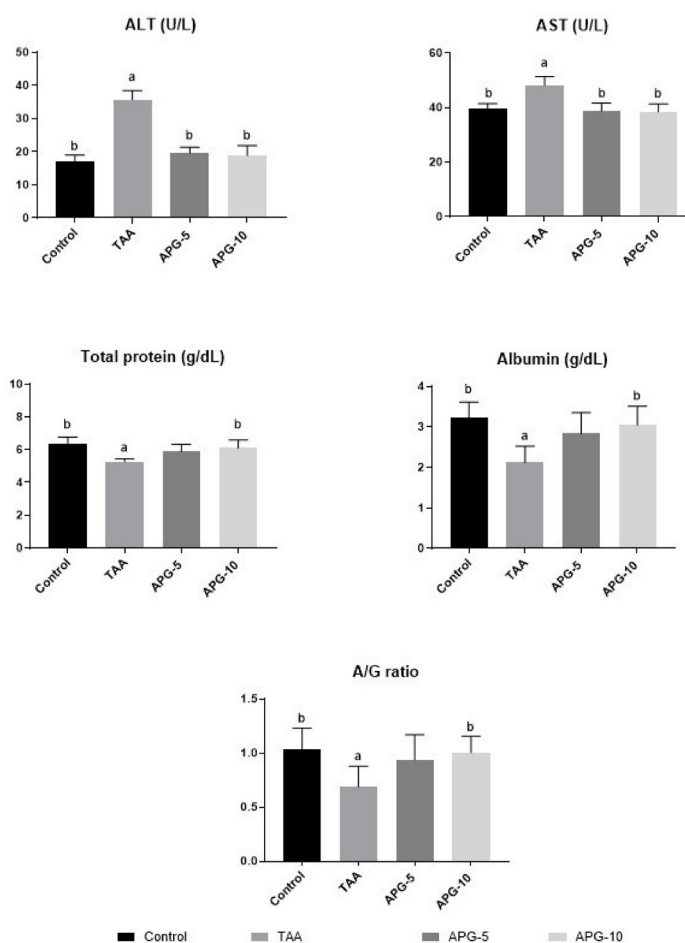


Figure 1. Effect of APG on liver injury markers. Values are expressed as mean \pm SD ($n = 6$ rats). Statistical analysis was carried out by one way ANOVA followed by Tukey's multiple comparisons test. ^a vs control group, ^b vs TAA group ($P \leq 0.05$). TAA (Positive control), APG-5 (Apigenin 5 mg/kg), APG-10 (Apigenin 10 mg/kg).

Table 1. Effect of APG on oxidative stress markers in a rat model of TAA-triggered liver fibrosis

Group	Hepatic GSH ($\mu\text{Mol/g}$ tissue)	Hepatic MDA (nMol/g tissue)
Control	23.2 \pm 0.48 ^b	47.2 \pm 1.87 ^b
TAA	10.3 \pm 0.42 ^a	111.7 \pm 6.95 ^a
APG-5	20.1 \pm 0.67 ^b	44.2 \pm 1.35 ^b
APG-10	22.0 \pm 1.44 ^b	45.2 \pm 0.65 ^b

Values are expressed as mean \pm SD (n = 6 rats). Statistics was carried out by one way ANOVA followed by Tukey's multiple comparisons test. ^a vs control group, ^b vs TAA group ($P \leq 0.05$). TAA (Positive control), APG-5 (Apigenin 5 mg/kg), APG-10 (Apigenin 10 mg/kg). GSH: reduced glutathione, MDA: malondialdehyde.

in TAA-intoxicated rats versus the normal group (Table 2). In addition, TAA caused a significant decrease in the anti-inflammatory cytokine IL-10, related to the normal group. The treatment with both doses of APG significantly decreased the pro-inflammatory marker levels. It significantly increased the anti-inflammatory cytokine versus the normal rats, representing the mitigation of liver inflammation with APG treatment (Table 2).

Effect of APG on α -SMA, COL1, CTGF, and Hyp

Our present study observed a remarkable increase in collagen 1, CTGF, hydroxyproline, and alpha-smooth muscle deposition in the TAA-treated rats related to the

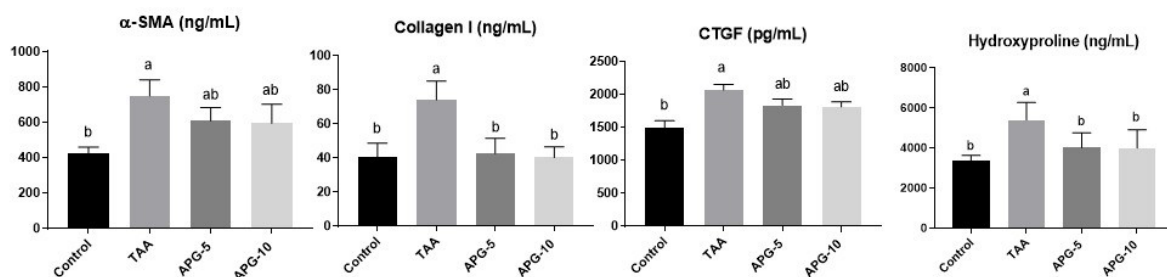
normal control. Meanwhile, APG-treated rats presented minimal collagen 1, CTGF, and α -SMA expression levels throughout the experiment compared with TAA-treated rats (Figure 2).

Impact of APG on HIF1- α and FAK pathways in liver

TAA appreciably upregulated HIF1 and FAK above the normal control. Relative to the sole TAA-treated group, APG-treated groups depicted a substantial downregulation in HIF1 and FAK expression (Table 3).

Effect of APG on histological and immunohistochemical examination

Livers of the control rats exhibited normal histological architecture with normal hepatic parenchymal cells, central veins, and portal areas (Figure 3A), while livers of rats administrated TAA (Figure 3B) revealed extensive portal fibrosis and inflammation with a marked expansion of portal areas by fibrous proliferation, inflammatory cells, and multiple proliferated bile ductules. The hepatic architecture was divided into multiple pseudo-lobules by extended fibrous tracts from the portal areas as bridging fibrous tracts dissecting the bordered hepatic cells. Those fibrous tracts contained proliferated bile ductules, inflammatory cells (lymphocytes, plasma cells, and a few macrophages), oval cells, and scattered hepatocytes. Within the pseudo-lobules, the hepatic cells showed macro and some microvesicular vacuolation,

**Figure 2.** Effect of APG on α -SMA, COL1, CTGF, and Hyp. Values are expressed as mean \pm SD (n = 6 rats). Statistical analysis was carried out by one way ANOVA followed by Tukey's multiple comparisons test. ^a vs control group, ^b vs TAA group ($P \leq 0.05$). TAA (Positive control), APG-5 (Apigenin 5 mg/kg), APG-10 (Apigenin 10 mg/kg). α -SMA: alpha smooth muscle actin, COL1: collagen 1, CTGF: connective tissue growth factor, Hyp: hydroxyproline.**Table 2.** Effect of APG on inflammatory markers status in a rat model of TAA-triggered liver fibrosis

Group	IL-10 (pg/mL)	TNF- α (ng/L)	IL-1 β (pg/mL)	NF- κ B (pg/mL)	TGF- β (pg/mL)
Control	313.7 \pm 18.63 ^b	259.3 \pm 14.43 ^b	282.7 \pm 22.58 ^b	4170.6 \pm 287.94 ^b	4077.1 \pm 285.58 ^b
TAA	185.6 \pm 15.10 ^a	863.6 \pm 63.09 ^a	797.9 \pm 37.40 ^a	22633.3 \pm 2073.37 ^a	8293.5 \pm 633.81 ^a
APG-5	283.7 \pm 19.06 ^b	314.0 \pm 11.38 ^b	355.7 \pm 29.60 ^b	5726.8 \pm 510.01 ^b	5863.3 \pm 242.27 ^{ab}
APG-10	292.0 \pm 21.91 ^b	296.2 \pm 25.04 ^b	307.8 \pm 20.71 ^b	3929.8 \pm 549.45 ^b	5641.3 \pm 495.56 ^b

Values are expressed as mean \pm SD (n = 6 rats). Statistics was carried out by one way ANOVA followed by Tukey's multiple comparisons test. ^a vs control group, ^b vs TAA group ($P \leq 0.05$). TAA (Positive control), APG-5 (Apigenin 5 mg/kg), APG-10 (Apigenin 10 mg/kg).

IL-10: interleukin-10, TNF- α : tumour necrosis factor-alpha, IL-1 β : interleukin 1 beta, NF- κ B: nuclear factor-kappa B, TGF- β : transforming growth factor β .

Table 3. Effect of APG on HIF1- α and FAK in a rat model of TAA-triggered liver fibrosis

Group	Hepatic HIF1- α (pg/mL)	Hepatic FAK (pg/mL)
Control	7411.6 \pm 336.97 ^b	10038.7 \pm 297.30 ^b
TAA	9791.1 \pm 334.38 ^a	14498.9 \pm 249.64 ^a
APG-5	7754.1 \pm 238.36 ^b	13111.9 \pm 264.24 ^{ab}
APG-10	7436.6 \pm 125.73 ^b	10234.8 \pm 372.61 ^b

Values are expressed as mean \pm SD (n = 6 rats). Statistics was carried out by one way ANOVA followed by Tukey's multiple comparisons test. ^a vs control group, ^b vs TAA group ($P \leq 0.05$). TAA (Positive control), APG-5 (Apigenin 5 mg/kg), APG-10 (Apigenin 10 mg/kg). HIF1- α : hypoxia-inducible factor-1 α , FAK: focal adhesion kinase.

few cells with ballooning degeneration with cytoplasmic reticulation and eccentric nuclei, necrosis, and along the fibrous septa, there is apoptosis and infiltrate of granulocytes inflammatory cells. Occasionally, portal veins and lymphatics were markedly dilated.

Regarding the livers of the APG-treated groups at 5 mg/kg (Figure 3C) and 10 mg/kg (Figure 3D) showed marked retraction of portal ad parenchymal fibroplasia with a moderate degree of fibrosis limited to the portal areas with sometimes a peripheral extension of incomplete septa particularly in some rats from the low dose group. The low-dose group had mild portal tracts fibroplasia with a sometimes peripheral extension of incomplete septa and minor propagation of biliary epithelium and infiltration of very limited granulocytes. Hepatocellular degeneration, dispersed necrosis, and apoptosis were of a lower magnitude. At the same time, the livers of the high-

dose group showed minimal fibrous proliferation with minimal changes in the hepatic cells.

Modified Ishak scoring for the degree of fibrosis showed the highest degree of liver fibrosis substantially in TAA-treated rats (Figure 3E). At the same time, the APG-treated groups, particularly the high-dose group, showed a lower fibrosis score than the TAA group. Figure 4 shows the histomorphometric analysis of Van Gieson's staining presented as an area percent corresponding to the results of H&E.

Immunohistochemistry analysis

Decreased expression of Akt was seen in the livers of normal rats (Figure 5) and mild diffuse expression of Nrf2 (Figure 6). Livers of TAA-administrated rats revealed marked expression of Akt with noticeably decreased expression of Nrf2 compared to the control ad treated groups. In comparison, decreased expression of Akt and noteworthy increased expression of Nrf2 were observed in APG-administrated groups, particularly at 10 mg/kg dose administration. The optical density represented a quantification of brown staining of Akt and Nrf2 and was expressed as area %.

Effect of APG on Nrf2, PPAR- α and NF- κ B mRNA expression levels

In the current study, the transcriptional levels of Nrf2, PPAR- α , and NF- κ B protein expression in the hepatic tissue were estimated by qRT-PCR. The relative expression of PPAR- α and Nrf2 mRNAs was significantly

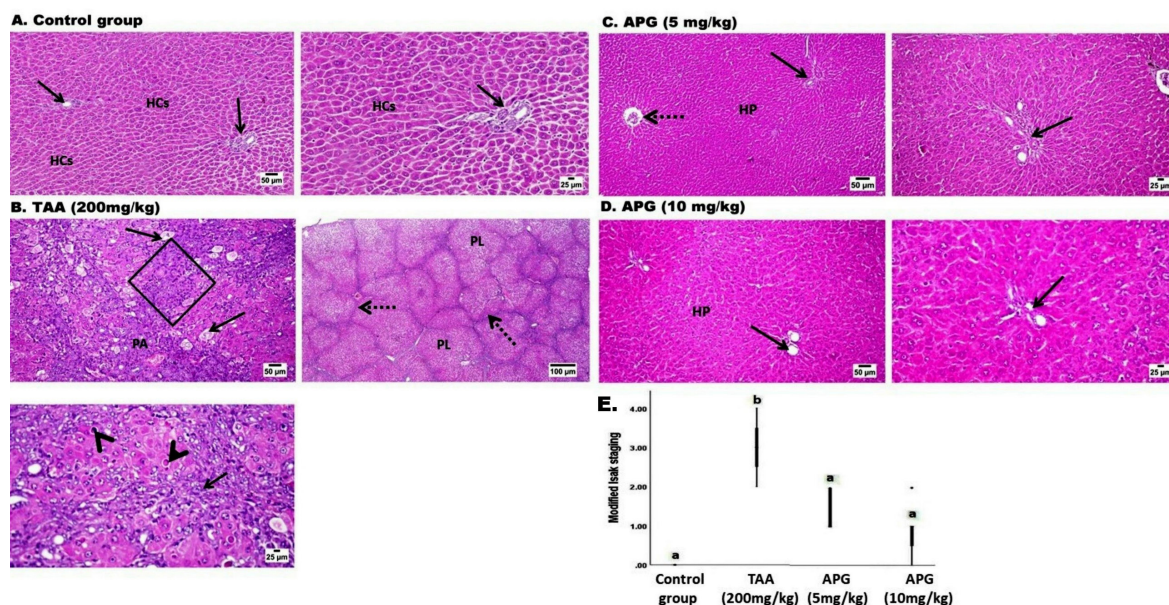


Figure 3. H&E-stained micrographs of liver sections; (A) Liver of control rat showing normal histological structure of portal areas (arrow) and hepatic cells (HCs). (B) Liver of TAA-administrated rat showing expansion of the portal areas (PA) with fibrous proliferation, inflammatory cells, and proliferated bile duct with peripheral extension of fibrous tracts (square) with bridging fibrosis (dotted arrow) and parenchymal pseudo-lobulation (PL), hepatocellular vacuolar degeneration, ballooning degeneration (arrow), and apoptosis (arrowhead). (C and D) Livers of APG (5 and 10 mg/kg respectively) treated rats showing marked retraction of the fibrous tissue proliferation with its limitation to the portal areas (arrow), absence of parenchymal pseudo-lobulation, and restoration of the hepatic cells (HP). TAA (Positive control), APG-5 (Apigenin 5 mg/kg), APG-10 (Apigenin 10 mg/kg).

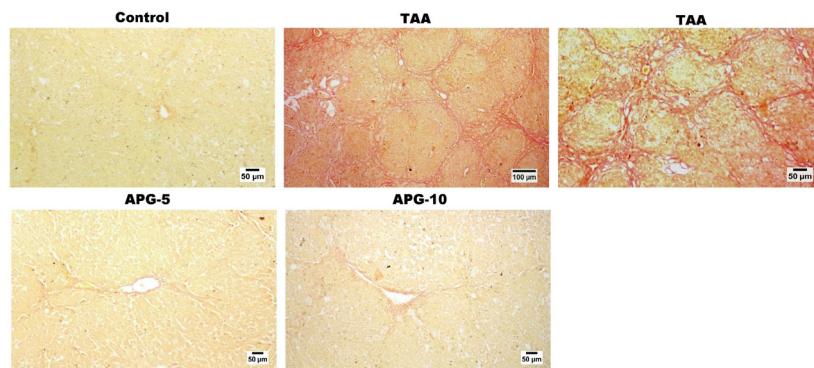


Figure 4. Photomicrographs Van Gieson's stained liver sections; Control group, showing the average amount of fibrous tissue at the portal areas. TAA group shows the distortion of the hepatic architecture with extensive bridging fibrosis (notice red fibrous tissue) connecting portal areas with pseudo-lobulation. APG-treated groups showed conspicuously limited portal fibroplasia and parenchymal fibrosis. TAA (Positive control), APG-5 (Apigenin 5 mg/kg), APG-10 (Apigenin 10 mg/kg).

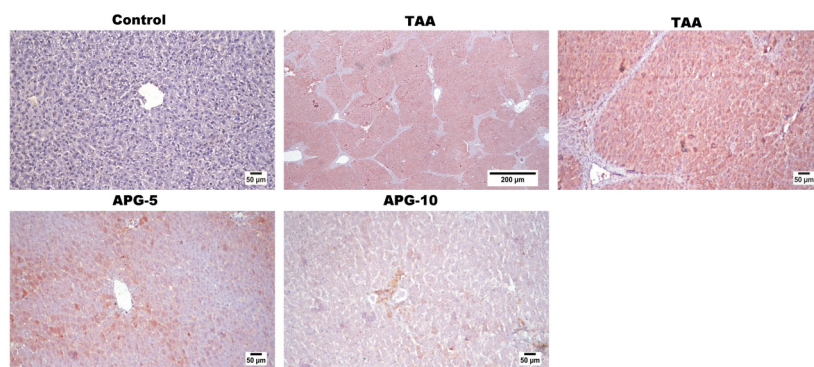


Figure 5. Immunohistochemical photomicrographs of serine-threonine protein kinase (Akt) expression in liver sections. Control rat showing negative expression. TAA group showing marked Akt expression among the hepatic cells. APG low and high doses treated groups showed marked dose-related decreased hepatocellular Akt expression. Quantitative image analysis of the area percent of the positive brown color of Akt presented as the optical density. TAA (Positive control), APG-5 (Apigenin 5 mg/kg), APG-10 (Apigenin 10 mg/kg).

reduced in the liver tissue of the TAA-exposed group to approximately 31% and 27% of their normal control levels, respectively ($P \leq 0.001$). Compared to the TAA group, APG administration in the TAA group appreciably boosted the gene activity of PPAR- α (48% and 49%) and Nrf2 (65% and 80%) (Figure 7). On the other hand, TAA induced a significant elevation in the NF- κ B mRNA level to 292% of its normal control level. Administration of APG (5 mg/kg and 10 mg/kg) caused a substantial downregulation in hepatic NF- κ B mRNA level to 182% and 147% of their normal control levels, respectively.

Discussion

Liver fibrosis is a long-term liver injury that causes a persistent wounding-healing response. Despite liver fibrosis having few outward signs in its early stages, the mortality risk increases rapidly as it progresses to cirrhosis and HCC (24). APG is a type of dietary flavonoid found in celery, thyme, parsley, onions, and chamomile. APG has several biological benefits, include anti-inflammatory, antioxidant, and anticancer actions (15). Accordingly, the

present investigation aimed to discover the antifibrotic activity of APG versus liver fibrosis triggered via TAA.

APG has been reviewed for its ability to suppress inflammation, oxidation, apoptosis, and tumor properties. Nevertheless, the probable inhibition of the HIF-1 α and FAK pathways by APG in TAA-mediated liver fibrosis in rats is unidentified. Here, we explored the role of APG against TAA-induced liver fibrosis, particularly its effect on oxidative stress, inflammation, HIF-1 α , and FAK pathways to clarify the mechanism by which APG exerts its effects. The obtained results confirmed that the antifibrotic effects of APG were correlated with the down-regulation of TGF- β 1, HIF-1 α , and FAK pathways, respectively, which mediate the progression of liver fibrosis.

Toxins and xenobiotics detoxification is one of the liver's main functions. In the current work, liver fibrosis was induced in rats using the TAA method. TAA, a potent hepatotoxic agent, is metabolized by Cytochrome 450 enzyme in the liver and is transformed into toxic compounds, TAAS-oxide and TAAS-dioxide, via oxidative

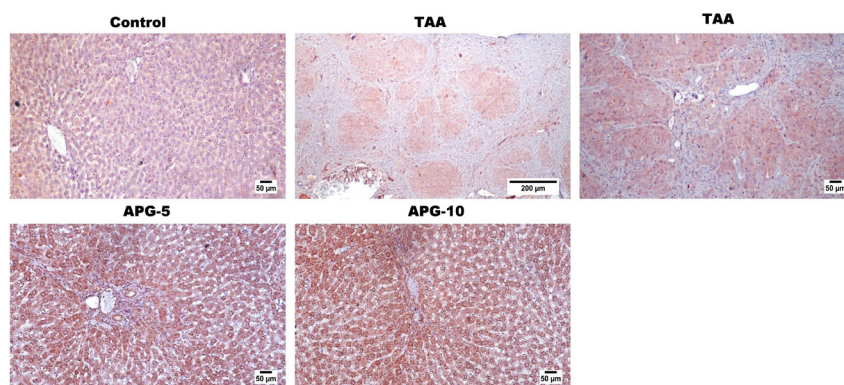


Figure 6. Immunohistochemical Photomicrographs of Nrf2 (Nuclear factor-erythroid 2 related factor 2) expression in liver sections. The Control group showed mild expression in hepatic parenchymal cells. TAA group showed moderately increased expression of Nrf2 amongst the hepatic cells in the pseudo-lobules. APG low and high doses treated groups showed marked dose-related increased Nrf2 expression in the hepatic cells. Quantitative image analysis of the area percent of the positive brown color of Nrf2 presented as the optical density. TAA (Positive control), APG-5 (Apigenin 5 mg/kg), APG-10 (Apigenin 10 mg/kg).

chains (25). Liver necrosis, fibrosis, cirrhosis, and finally, HCC were induced upon TAA administration which is caused by free radical-mediated lipid peroxidation (25). TAA's highly toxic reactive intermediate (TAA S-dioxide) affects the integrity and stability of cellular membranes, increasing permeability and causing enzymes like AST and ALT to leak out (26). Our findings disclosed that serum ALT and AST activities were markedly increased, while serum albumin, total protein concentrations, and albumin/globulins (A/G) ratio were markedly reduced after TAA administration. Treatment with APG notably conserved hepatocyte integrity in a dose-dependent manner, as indicated by the reduced enzyme activities,

and restored serum albumin concentrations, total protein, and A/G ratio. These findings demonstrated that APG protects hepatic damage triggered by TAA.

Due to its high concentration of mitochondria, the liver is the body's primary source of reactive oxygen species (27). An imbalance between pro-oxidants and antioxidants produces oxidative stress in hepatocytes, resulting in antioxidant depletion or inactivation, ultimately causing a variety of liver disorders to emerge (28). The Nrf2 pathway, regarded as the master regulator of a cellular defense system against toxic assaults, is activated by oxidative stress. By activating the antioxidative response, Nrf2 controls the cellular response to oxidative stress (26).

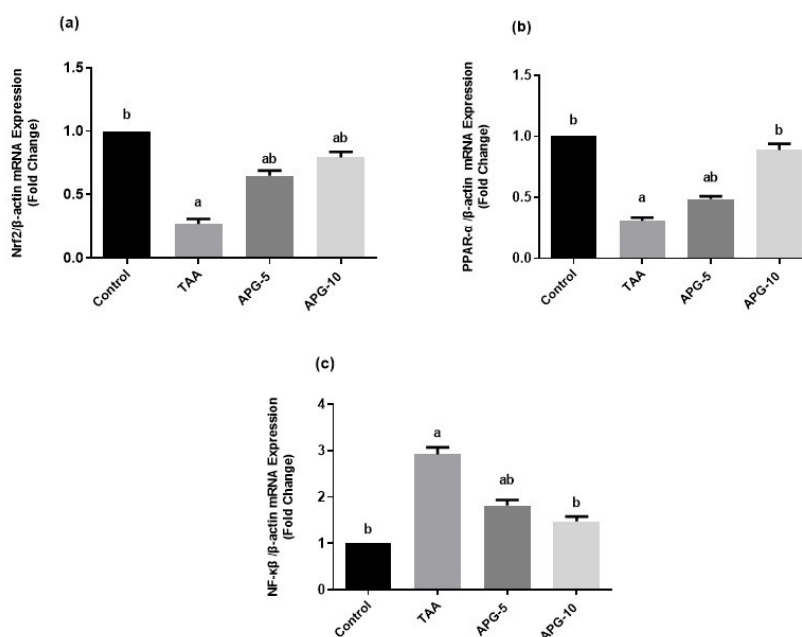


Figure 7. Effects of APG on mRNA expression of Nrf2 (A), PPARα (B), and NF-κB (C) in the liver tissues. TAA (Positive control), APG-5 (Apigenin 5 mg/kg), APG-10 (Apigenin 10 mg/kg), Nrf2: nuclear factor-erythroid factor 2 related factor 2, PPARα: peroxisome proliferator-activated receptor gamma, and NF-κB: nuclear factor-kappa B. ^a vs control group, ^b vs TAA group ($P \leq 0.05$).

Nrf2 dissociates from Kelch-like ECH-associated protein 1 (Keap1) under oxidative stress. It translocates to the nucleus, activating the expression of downstream factors, such as HO-1, NQO1, and other antioxidant enzymes (29). In the present study, TAA decreased hepatic Nrf2 expression and GSH content which was associated with the elevation of MDA level. Excessive free radicals formation after TAA intoxication caused a substantial upsurge in lipid peroxidation with the resultant formation of MDA and malfunction of the hepatic cells to counter ROS (5). Remarkably, APG treatment upregulated the gene activity of Nrf2 and GSH content linked with MDA level diminution. Our study agrees with another study, which showed that APG could alleviate the liver oxidative stress induced by acetaminophen (30). Additionally, previous studies have shown that APG exerted potent antioxidant properties via the activation of Nrf2 signaling (31). The results exhibited the protective properties of APG against TAA-induced liver oxidative stress.

The peroxisome proliferator-activated receptor gamma (PPAR γ) has been confirmed to regulate adipocyte differentiation. PPAR γ acts by inhibiting the NF κ B pathway, lowering the expression of proinflammatory cytokines, including TGF- β 1, TNF- α , and IL-6, alleviating liver damage. Downregulation of the PPAR gene has also been linked to hepatic disease progression (32).

In the same context, TAA caused a marked down-regulation of liver PPAR gene expression, which was restored by APG therapy. Our finding is parallel with those of Feng et al (33) who indicated that APG could ameliorate nonalcoholic fatty liver disease by regulating Nrf2 and PPAR γ in inhibiting oxidative stress.

Inflammation is a key pathogenic process contributing to liver damage and fibrogenesis (30). The upregulation of inflammatory mediators TNF- α , IL-8, IL-1 β , and IL-6 resulted in the activation of HSCs and the synthesis of ECM. Our results showed a prominent surge in TNF- α as well as IL-1 β , and the level of IL-10 was markedly reduced in the TAA model group. Our findings are in accordance with a previous study (26). TNF- α , a cytokine with broad effects, mediates inflammation and apoptosis and has a role in the pathophysiology of chronic liver inflammation and liver fibrosis by activating local HSCs into myofibroblasts (34). IL-10 acts as an anti-inflammatory and antifibrotic mediator. In order to delve deeper into the mechanism by which APG inhibits inflammation, we similarly inspected the levels of the NF- κ B and TGF- β 1, which are important for the expression of numerous pro-inflammatory cytokines. NF- κ B is a key regulator transcription factor that stimulates the transcription of proinflammatory genes, such as TNF- α and IL-10, after its translocation to the nucleus. Inhibiting liver fibrosis via NF- κ B and its cascade is seen as a viable strategy (35). TGF- β 1 is also one of the most essential cytokines for activating and promoting HSC transformation by triggering a cascade of intracellular signaling events, aggravating the fibrosis

process in the liver (36). TGF- β promotes HSCs to express markers of HSC activation such as α -smooth muscle actin (α -SMA), fibronectin, and CTGF (37). Our findings demonstrated that TAA significantly increased the levels of NF- κ B and TGF- β 1. TAA triggers the transcription of NF- κ B, which activates the production of proinflammatory cytokines, such as TNF α and IL-1 β , and the modulation of IL-10 anti-inflammatory cytokine (38). The anti-inflammatory activity of APG was demonstrated by lower levels of TNF- α , IL-1 β , NF- κ B, and TGF- β 1, as well as higher levels of IL-10, implying that APG reduces liver fibrosis by limiting the levels of inflammatory cytokines and exerting its protective effect by inhibiting the NF- κ B and TGF- β 1 pathways. The results of a previous study are parallel with our results (39).

Makino et al defined that CTGF is a type of matrix protein that is typically up-regulated in liver fibrosis and plays an important role in the disease (40). CTGF protein is expressed and secreted by hepatocytes, bile duct cells, and HSCs in the fibrotic liver, according to previous studies (41). CTGF is highly elevated and possesses a serious role in hepatic scarring. It is also a subsequent controller of TGF- β 1, guiding the ECM buildup to reach its ultimate stage (42). Activated HSCs cause increased proliferation and overexpression of α -SMA in liver fibrosis. They are a preliminary sign of liver fibrosis progression because they indicate the stimulation of HSCs to myofibroblast-like cells prior to collagen deposition. Furthermore, Col I is the most essential type of collagen, which play a critical role in the progression of liver fibrosis (43).

In this study, TAA intoxication caused a remarkable upsurge in Col I, CTGF, hydroxyproline, and α -SMA expression. These fibrotic markers were significantly ameliorated by APG treatment in a dose-dependent manner. Ricupero et al reported that the antifibrotic ability of APG might be credited to the repression of TGF- β 1. In lung fibroblasts, Ricupero et al found that APG suppressed myofibroblast proliferation, Col I and SMA expression, and decreased TGF- β -induced α -SMA expression (44).

Activation of HIF-1 α , a hypoxic transcription factor, promotes liver fibrosis. HIF-1 α activation is usually caused by blocking ubiquitination and proteasomal degradation via inhibiting O₂-dependent prolyl hydroxylation. In animal models, many researchers have shown the connection between HIF-1 α and liver fibrosis. Hypoxia activates HIF-1 α in isolated hepatocytes, according to Coppole et al, and this leads to an increase in numerous profibrotic mediators. Hypoxia was shown to activate HIF-1 α in isolated HSCs, resulting in increased profibrotic gene expression (45). In a bile duct ligation model of cholestatic liver damage, the deletion of HIF-1 resulted in reduced liver fibrosis via dysregulation of comparable profibrotic mediators. TAA, the hepatotoxic agent, has been shown to trigger the HIF-1 α - mTOR pathway in several preclinical studies of hepatic disorders as well as *in vitro* studies (46).

This parallels our results; the expression of HIF1- α was significantly upregulated in the TAA group. Furthermore, the inflammation was found to be associated with the elevated expression of HIF-1 α in preclinical studies of hepatic fibrosis (1). These results are compliant with our discoveries of elevated HIF-1 α levels and inflammatory biomarkers such as TNF- α , IL-1 β , NF- κ B, and TGF- β 1 in the hepatic fibrosis model. Treatment with APG significantly down-regulated the expression of HIF-1 α in hepatic tissue, indicating that APG's anti-scarring activity may result from the repression of the HIF-1 α pathway in a certain manner. Parallel with our findings, Semenza revealed that APG could target HIFs by increasing HIF-1 α degradation (47).

The protein tyrosine kinase superfamily includes FAK and nonreceptor cytosolic tyrosine kinases. FAK is involved in cellular signaling and promotes biological processes in cells and tissues, such as proliferation, migration, wound healing, and angiogenesis (15). FAK is a free signaling molecule in the cytoplasm of fibroblasts involved in TGF- β -mediated intracellular signal transduction. It has been shown that FAK regulates the proliferation, activation and apoptosis of HSCs. In addition, several studies suggested that substance suppression of the FAK/Akt pathway may be a possible inhibitor of hepatic fibrosis. In our study, FAK was significantly upregulated in the TAA group. Significant downregulation of FAK expression was observed in APG-treated groups, which is parallel with a previous study conducted by Wang et al, which showed that APG markedly decreased FAK kinase activity, resulting in a marked reduction in FAK phosphorylation (activation) (15). Additionally, APG was shown to inhibit TGF- β -mediated VEGF levels and prevent prostate cancer by controlling the FAK/Akt pathway, as demonstrated by Mirzoeva et al (48).

There is evidence indicating the role of hepatic PPAR- α as etiologic transcription factor in the molecular control of fibrogenesis. PPAR- α controls lipid physiology and energy balance (49). PPAR- α activation has reversed fibrosis by downregulating TGF- β 1, suppressing ECM remodeling genes and HSCs activation. Fenofibrate and Wy-14643, PPAR- α agonists, have been reported to mitigate hepatic fibrosis in TAA and methionine choline-deficient models (50). Moreover, Chen et al demonstrated that oleoylethanolamide, a PPAR- α agonist (51), effectively ameliorated hepatic fibrosis via a PPAR- α associated mechanism. An earlier investigation (52) illustrated that APG alleviated hepatic fibrosis in carbon CCl₄ as well as biliary obstruction model in rodents by activating p38/PPAR α pathways. Our findings found that APG significantly restored TAA-induced downregulated PPAR- α mRNA expression.

Conclusion

Taken together, it could be concluded that APG hinders TAA-triggered liver fibrosis in rats by, at least partly,

suppressing oxidative stress and profibrogenic and pro-inflammatory biomarkers. In addition, our results established a recent approach associating the anti-fibrotic mechanism of APG through inhibiting HIF1- α and FAK pathways. Overall, our research showed that APG has the potential to be a therapeutic medication for hepatic fibrosis. However, further studies are needed to establish the clinical applicability of APG treatment in active liver fibrogenesis human cases.

Authors' contribution

RFA, HMF, and MAM: Conceptualization and methodology. RFA, HMF, MAM, AFH, GFA, and AAA: Biochemical analysis. SA: Histopathological and immunohistochemical experiments. HAO: Molecular study. RFA and MAM: Data analysis. MSA: Supervision. RFA, HMF, MAM: Writing, reviewing and editing.

Conflict of interests

The authors have no conflicts of interest to declare.

Ethical considerations

Ethical issues regarding authorship, data acquisition, review, and analysis have been carefully observed by the authors. All the animal experimental procedures have been approved by the Ethics Committee of Animal Care and Use of the National Research Centre (NRC) (approval number" MREC-19-214).

Funding/Support

The authors thank the National Research Centre-Egypt for funding the research project no. 12060127.

References

1. Abdel-Rahman RE. Non-alcoholic fatty liver disease: epidemiology, pathophysiology and an update on the therapeutic approaches. *Asian Pac J Trop Biomed.* 2022;12(3):99-114. doi: 10.4103/2221-1691.338919.
2. Kennedy OJ, Fallowfield JA, Poole R, Hayes PC, Parkes J, Roderick PJ. All coffee types decrease the risk of adverse clinical outcomes in chronic liver disease: a UK Biobank study. *BMC Public Health.* 2021;21(1):970. doi: 10.1186/s12889-021-10991-7.
3. Berumen J, Baglieri J, Kisseleva T, Mekeel K. Liver fibrosis: pathophysiology and clinical implications. *WIREs Mech Dis.* 2021;13(1):e1499. doi: 10.1002/wsbm.1499.
4. Metwally AM, Elmosalami DM, Elhariri H, El Etreby LA, Aboulghate A, El-Sonbaty MM, et al. Accelerating hepatitis C virus elimination in Egypt by 2030: a national survey of communication for behavioral development as a modelling study. *PLoS One.* 2021;16(2):e0242257. doi: 10.1371/journal.pone.0242257.
5. Ramadan A, Afifi N, Yassin NZ, Abdel-Rahman RE, Abd El-Rahman SS, Fayed HM. Mesalazine, an osteopontin inhibitor: the potential prophylactic and remedial roles in induced liver fibrosis in rats. *Chem Biol Interact.* 2018;289:109-18. doi: 10.1016/j.cbi.2018.05.002.
6. Abd El-Rahman SS, Fayed HM. Targeting AngII/AT1R

- signaling pathway by perindopril inhibits ongoing liver fibrosis in rat. *J Tissue Eng Regen Med.* 2019;13(12):2131-41. doi: 10.1002/term.2940.
7. Trautwein C, Friedman SL, Schuppan D, Pinzani M. Hepatic fibrosis: concept to treatment. *J Hepatol.* 2015;62(1 Suppl):S15-24. doi: 10.1016/j.jhep.2015.02.039.
 8. Nath B, Szabo G. Hypoxia and hypoxia inducible factors: diverse roles in liver diseases. *Hepatology.* 2012;55(2):622-33. doi: 10.1002/hep.25497.
 9. Roth KJ, Copple BL. Role of hypoxia-inducible factors in the development of liver fibrosis. *Cell Mol Gastroenterol Hepatol.* 2015;1(6):589-97. doi: 10.1016/j.jcmgh.2015.09.005.
 10. Ju C, Colgan SP, Eltzschig HK. Hypoxia-inducible factors as molecular targets for liver diseases. *J Mol Med (Berl).* 2016;94(6):613-27. doi: 10.1007/s00109-016-1408-1.
 11. Paschetta E, Belci P, Alisi A, Liccardo D, Cutrera R, Musso G, et al. OSAS-related inflammatory mechanisms of liver injury in nonalcoholic fatty liver disease. *Mediators Inflamm.* 2015;2015:815721. doi: 10.1155/2015/815721.
 12. Siesser PM, Hanks SK. The signaling and biological implications of FAK overexpression in cancer. *Clin Cancer Res.* 2006;12(11 Pt 1):3233-7. doi: 10.1158/1078-0432.ccr-06-0456.
 13. Fan GP, Wang W, Zhao H, Cai L, Zhang PD, Yang ZH, et al. Pharmacological inhibition of focal adhesion kinase attenuates cardiac fibrosis in mice cardiac fibroblast and post-myocardial-infarction models. *Cell Physiol Biochem.* 2015;37(2):515-26. doi: 10.1159/000430373.
 14. Souza RP, Bonfim-Mendonça PS, Gimenes F, Ratti BA, Kaplum V, Bruschi ML, et al. Oxidative stress triggered by apigenin induces apoptosis in a comprehensive panel of human cervical cancer-derived cell lines. *Oxid Med Cell Longev.* 2017;2017:1512745. doi: 10.1155/2017/1512745.
 15. Wang H, Guo B, Lin S, Chang P, Tao K. Apigenin inhibits growth and migration of fibroblasts by suppressing FAK signaling. *Aging (Albany NY).* 2019;11(11):3668-78. doi: 10.18632/aging.102006.
 16. Melstrom LG, Salabat MR, Ding XZ, Strouch MJ, Grippo PJ, Mirzoeva S, et al. Apigenin down-regulates the hypoxia response genes: HIF-1 α , GLUT-1, and VEGF in human pancreatic cancer cells. *J Surg Res.* 2011;167(2):173-81. doi: 10.1016/j.jss.2010.10.041.
 17. Hicks DE, Goossens N, Blas-García A, Tsuchida T, Wooden B, Wallace MC, et al. Transcriptome-based repurposing of apigenin as a potential anti-fibrotic agent targeting hepatic stellate cells. *Sci Rep.* 2017;7:42563. doi: 10.1038/srep42563.
 18. Malik S, Suchal K, Khan SI, Bhatia J, Kishore K, Dinda AK, et al. Apigenin ameliorates streptozotocin-induced diabetic nephropathy in rats via MAPK-NF- κ B-TNF- α and TGF- β 1-MAPK-fibronectin pathways. *Am J Physiol Renal Physiol.* 2017;313(2):F414-F22. doi: 10.1152/ajprenal.00393.2016.
 19. Mansour DF, Nada SA, El-Denshary ES, Omara EA, Asaad GE, Abdel-Rahman RE. Milk whey proteins modulate endotoxemia-induced hepatotoxicity in rats. *International Journal of Pharmacy and Pharmaceutical Sciences.* 2015;7(5):65-71
 20. Suvarna KS, Layton C, Bancroft JD. Bancroft's theory and practice of histological techniques E-Book. Elsevier Health Sciences; 2018.
 21. Hegazy RR, Mansour DF, Salama AA, Abdel-Rahman RE, Hassan AM. Regulation of PKB/Akt-pathway in the chemopreventive effect of lactoferrin against diethylnitrosamine-induced hepatocarcinogenesis in rats. *Pharmacol Rep.* 2019;71(5):879-91. doi: 10.1016/j.pharep.2019.04.019.
 22. Ogaly HA, Alsherbiny MA, El Badawy SA, Abd-Elsalam RM, Guang Li C, Azouz AA. Gastroprotective effects and metabolomic profiling of Chasteberry fruits against indomethacin-induced gastric injury in rats. *J Funct Foods.* 2021;86:104732. doi: 10.1016/j.jff.2021.104732.
 23. ElBaset MA, Salem RS, Ayman F, Ayman N, Shaban N, Afifi SM, et al. Effect of empagliflozin on thioacetamide-induced liver injury in rats: role of AMPK/SIRT-1/HIF-1 α pathway in halting liver fibrosis. *Antioxidants (Basel).* 2022;11(11):2152. doi: 10.3390/antiox11112152.
 24. Sharawy N, Imam AA, Aboulhoda BE, Khalifa MM, Morcos GNB, Abd Algaleel WA, et al. Iron dyshomeostasis and time-course changes in iron-uptake systems and ferritin level in relation to pro-inflammatory microglia polarization in sepsis-induced encephalopathy. *Front Physiol.* 2022;13:953206. doi: 10.3389/fphys.2022.953206.
 25. Ayoub IM, El-Baset MA, Elghonemy MM, Bashandy SAE, Ibrahim FAA, Ahmed-Farid OAH, et al. Chemical profile of *Cyperus laevigatus* and its protective effects against thioacetamide-induced hepatorenal toxicity in rats. *Molecules.* 2022;27(19):6470. doi: 10.3390/molecules27196470.
 26. Abdel-Rahman RE, Fayed HM, Asaad GE, Ogaly HA, Hessin AF, Salama AAA, et al. The involvement of TGF- β 1/FAK/ α -SMA pathway in the antifibrotic impact of rice bran oil on thioacetamide-induced liver fibrosis in rats. *PLoS One.* 2021;16(12):e0260130. doi: 10.1371/journal.pone.0260130.
 27. Mansouri A, Gattolliat CH, Asselah T. Mitochondrial dysfunction and signaling in chronic liver diseases. *Gastroenterology.* 2018;155(3):629-47. doi: 10.1053/j.gastro.2018.06.083.
 28. Koyama Y, Taura K, Hatano E, Tanabe K, Yamamoto G, Nakamura K, et al. Effects of oral intake of hydrogen water on liver fibrogenesis in mice. *Hepatol Res.* 2014;44(6):663-77. doi: 10.1111/hepr.12165.
 29. Raslan M, Abdel Rahman R, Fayed H, Ogaly H, Fikry R. Metabolomic profiling of *Sansevieria trifasciata* hort ex. Prain leaves and roots by HPLC-PAD-ESI/MS and its hepatoprotective effect via activation of the NRF2/ARE signaling pathway in an experimentally induced liver fibrosis rat model. *Egypt J Chem.* 2021;64(11):6647-71. doi: 10.21608/ejchem.2021.78970.3877.
 30. Xu X, Li M, Chen W, Yu H, Yang Y, Hang L. Apigenin attenuates oxidative injury in ARPE-19 cells thorough activation of Nrf2 pathway. *Oxid Med Cell Longev.* 2016;2016:4378461. doi: 10.1155/2016/4378461.
 31. Zhang Y, Yang Y, Yu H, Li M, Hang L, Xu X. Apigenin protects mouse retina against oxidative damage by regulating the Nrf2 pathway and autophagy. *Oxid Med Cell Longev.* 2020;2020:9420704. doi: 10.1155/2020/9420704.
 32. Hardy T, Zeybel M, Day CP, Dipper C, Masson S, McPherson S, et al. Plasma DNA methylation: a potential biomarker for stratification of liver fibrosis in non-alcoholic fatty liver disease. *Gut.* 2017;66(7):1321-8. doi: 10.1136/

- gutjnl-2016-311526.
33. Feng X, Yu W, Li X, Zhou F, Zhang W, Shen Q, et al. Apigenin, a modulator of PPAR γ , attenuates HFD-induced NAFLD by regulating hepatocyte lipid metabolism and oxidative stress via Nrf2 activation. *Biochem Pharmacol.* 2017;136:136-49. doi: 10.1016/j.bcp.2017.04.014.
 34. Wang L, Huang QH, Li YX, Huang YF, Xie JH, Xu LQ, et al. Protective effects of silymarin on triptolide-induced acute hepatotoxicity in rats. *Mol Med Rep.* 2018;17(1):789-800. doi: 10.3892/mmr.2017.7958.
 35. Younis NS, Ghanim AMH, Elmorsy MA, Metwaly HA. Taurine ameliorates thioacetamide induced liver fibrosis in rats via modulation of toll like receptor 4/nuclear factor kappa B signaling pathway. *Sci Rep.* 2021;11(1):12296. doi: 10.1038/s41598-021-91666-6.
 36. Kang KH, Qian ZJ, Ryu B, Karadeniz F, Kim D, Kim SK. Hepatic fibrosis inhibitory effect of peptides isolated from *Navicula incerta* on TGF- β 1 induced activation of LX-2 human hepatic stellate cells. *Prev Nutr Food Sci.* 2013;18(2):124-32. doi: 10.3746/pnf.2013.18.2.124.
 37. Tu K, Li J, Verma VK, Liu C, Billadeau DD, Lamprecht G, et al. Vasodilator-stimulated phosphoprotein promotes activation of hepatic stellate cells by regulating Rab11-dependent plasma membrane targeting of transforming growth factor beta receptors. *Hepatology.* 2015;61(1):361-74. doi: 10.1002/hep.27251.
 38. Fernandes JC, Schemitt EG, Da Silva J, Marroni NP, Lima A, Ferreira RB. Combination of trans-resveratrol and ϵ -viniferin induces a hepatoprotective effect in rats with severe acute liver failure via reduction of oxidative stress and MMP-9 expression. *Nutrients.* 2021;13(11):3677. doi: 10.3390/nu13113677.
 39. Yue S, Xue N, Li H, Huang B, Chen Z, Wang X. Hepatoprotective effect of apigenin against liver injury via the non-canonical NF- κ B pathway in vivo and in vitro. *Inflammation.* 2020;43(5):1634-48. doi: 10.1007/s10753-020-01238-5.
 40. Makino Y, Hikita H, Kodama T, Shigekawa M, Yamada R, Sakamori R, et al. CTGF mediates tumor-stroma interactions between hepatoma cells and hepatic stellate cells to accelerate HCC progression. *Cancer Res.* 2018;78(17):4902-14. doi: 10.1158/0008-5472.can-17-3844.
 41. Ramazani Y, Knops N, Elmonem MA, Nguyen TQ, Arcolino FO, van den Heuvel L, et al. Connective tissue growth factor (CTGF) from basics to clinics. *Matrix Biol.* 2018;68-69:44-66. doi: 10.1016/j.matbio.2018.03.007.
 42. Wu Y, Wang W, Peng XM, He Y, Xiong YX, Liang HF, et al. Rapamycin upregulates connective tissue growth factor expression in hepatic progenitor cells through TGF- β -Smad2 dependent signaling. *Front Pharmacol.* 2018;9:877. doi: 10.3389/fphar.2018.00877.
 43. Xu Y, Liu W, Fang B, Gao S, Yan J. Artesunate ameliorates hepatic fibrosis induced by bovine serum albumin in rats through regulating matrix metalloproteinases. *Eur J Pharmacol.* 2014;744:1-9. doi: 10.1016/j.ejphar.2014.09.035.
 44. Ricupero DA, Poliks CE, Rishikof DC, Kuang PP, Goldstein RH. Apigenin decreases expression of the myofibroblast phenotype. *FEBS Lett.* 2001;506(1):15-21. doi: 10.1016/s0014-5793(01)02737-5.
 45. Coppole BL, Bai S, Burgoon LD, Moon JO. Hypoxia-inducible factor-1 α regulates the expression of genes in hypoxic hepatic stellate cells important for collagen deposition and angiogenesis. *Liver Int.* 2011;31(2):230-44. doi: 10.1111/j.1478-3231.2010.02347.x.
 46. Wu YL, Zhang YJ, Yao YL, Li ZM, Han X, Lian LH, et al. Cucurbitacin E ameliorates hepatic fibrosis in vivo and in vitro through activation of AMPK and blocking mTOR-dependent signaling pathway. *Toxicol Lett.* 2016;258:147-58. doi: 10.1016/j.toxlet.2016.06.2102.
 47. Semenza GL. Pharmacologic targeting of hypoxia-inducible factors. *Annu Rev Pharmacol Toxicol.* 2019;59:379-403. doi: 10.1146/annurev-pharmtox-010818-021637.
 48. Mirzoeva S, Kim ND, Chiu K, Franzen CA, Bergan RC, Pelling JC. Inhibition of HIF-1 α and VEGF expression by the chemopreventive bioflavonoid apigenin is accompanied by Akt inhibition in human prostate carcinoma PC3-M cells. *Mol Carcinog.* 2008;47(9):686-700. doi: 10.1002/mc.20421.
 49. Lefebvre P, Chinetti G, Fruchart JC, Staels B. Sorting out the roles of PPAR α in energy metabolism and vascular homeostasis. *J Clin Invest.* 2006;116(3):571-80. doi: 10.1172/jci27989.
 50. Toyama T, Nakamura H, Harano Y, Yamauchi N, Morita A, Kirishima T, et al. PPAR α ligands activate antioxidant enzymes and suppress hepatic fibrosis in rats. *Biochem Biophys Res Commun.* 2004;324(2):697-704. doi: 10.1016/j.bbrc.2004.09.110.
 51. Chen L, Li L, Chen J, Li L, Zheng Z, Ren J, et al. Oleoylethanolamide, an endogenous PPAR- α ligand, attenuates liver fibrosis targeting hepatic stellate cells. *Oncotarget.* 2015;6(40):42530-40. doi: 10.18632/oncotarget.6466.
 52. Ji P, Chen C, Hu Y, Zhan Z, Pan W, Li R, et al. Antiviral activity of *Paulownia tomentosa* against enterovirus 71 of hand, foot, and mouth disease. *Biol Pharm Bull.* 2015;38(1):1-6. doi: 10.1248/bpb.b14-00357.