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Apocynin ameliorates liver fibrosis events in vivo through modulation of oxidative stress, inflammatory, and apoptotic mediators

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Abstract

The liver has a tremendous regeneration potential, yet chronic liver injury poses a life-threatening condition if not managed appropriately. Apocynin, an NADPH oxidase inhibitor, has been a central focus of attention in recent years due to its significant antioxidant/anti-inflammatory potentials. In this study, we evaluated the acute toxicity and hepatoprotective effects of Apocynin against thioacetamide (TAA)-induced liver fibrosis in rats. Liver fibrosis was induced by 200 mg/kg TAA three times/week for two months, along with treatment with distilled water (positive control), silymarin (reference, 50 mg/kg), or apocynin (50 and 100 mg/kg/day). Hepatic tissues were screened for histopathological, biochemical, and immunohistochemical changes, while hepatic homogenate was examined for the antioxidant contents (catalase, CAT; superoxide dismutase, SOD) and MDA levels. Apocynin treatment showed significant hepatoprotective effects against TAA-hepatotoxicity, evidenced by reduced hepatic tissue alterations with a slight fibroplasia, reduction of hepatomegaly, less hepatic nodules/necrosis, and recovered hepatic function. Additionally, apocynin administration reduced oxidative stress by lowering pro-oxidants (MDA) and up-regulating antioxidants (SOD and CAT). Furthermore, the anti-apoptotic and anti-fibrotic effects of apocynin were confirmed by reduced pro-apoptotic P53 proteins and β-catenin (tissue proliferation/aggregation enhancer). Apocynin treatment ameliorated ECM generation (lowered collagen bundles/fibrous septa) and reduced inflammatory (less TNf-α and IL-6 cytokines) mediators, all of which restored liver functional parameters (ALT, AST, ALP, and albumin). Apocynin attenuated TAA-mediated liver fibrosis by its modulatory potentials on several cytoprotective mechanisms associated with the oxidative stress/inflammation, making it a viable therapeutic source for liver fibrosis.

Keywords *Apocynin*, Liver fibrosis, Thioacetamide, Antioxidants, Histopathology

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Introduction

Liver fibrosis (LF) is a chronic health disorder that occurs in progressive phases of all chronic liver diseases, characterized by tissue fibrosis, necrosis, and numerous regenerative hepatic nodules. As the 14th leading cause of mortality globally, it contributes significantly to 1.3 million deaths/year and 560.4/100,000 disability-adjusted life years (26.8% less DALYs in 2019 than that of 1990) [1]. Liver fibrosis has been labelled as a major death risk factor in patients with non-alcoholic fatty liver disease, alcoholism, chronic liver disease, hepatitis B and C infections, and autoimmune disorders, altogether causing more than two million annual deaths that account for 4% global deaths (1 in every 25 deaths); almost two-thirds of all liver-associated deaths occur in men [2]. Studies have demonstrated a sharp increase in mortality and morbidity rates for liver fibrosis, from a 1-year case-fatality rate to almost 80%. Eventually, LF and other chronic hepatic disorders become a substantial global/local burden on patients, healthcare, and governments [3]. Moreover, LFs are characterized by the proliferation/aggregation of extracellular matrix proteins (collagen) along with provoked immune and inflammatory responses toward chronic inductions [4]. At an early stage, LF begins with liver tissue damage caused by numerous causative and stress factors that promote prolonged inflammation and the conversion of hepatic stellate/perisinusoidal cells into cells similar to fibroblasts, which can generate tremendous collagen/laminin proteins. Consequently, these structural/functional changes reduce the liver's functional performance [3]. To reduce inflammation and fibrosis, the liver secretes many profibrogenic cytokines that regulate phases of chronic liver disease, which enhance the conversion of inflammation and fibrosis into fibrosis and liver carcinoma. Once the liver is exposed to stress factors, the Wnt/β-catenin signalling cascade will be activated, which promotes cellular proliferation/aggregation of hepatic stellate cells and oncogene-induced senescence. The latter cell releases numerous pro-inflammatory cytokines that can further deteriorate liver tissues. This cellular alteration could be overcome by ablation of growth control genes, p53 or its target gene Bax, and p21 [5].

Oxidative stress is a predominant key player in the initiation of most chronic liver disease, regardless of the causatives of the hepatic disorder. Once ROS/antioxidant imbalance reaches a certain point, the resultant oxidative stress modulates the expression of redox-sensitive transcription mediators (NF-kappa B and AP-1). Additionally, Cellular kinases (mitogen-activated protein kinase), NF-κB pathway, and PI3K/PTEN/Akt pathways are also controlled and modulated by hepatocyte oxidative stress [6]. The pathological cascade reaction promotes further oxidative stress/inflammation, which enhances more pro-apoptotic protein expression and extracellular matrix (ECM) collagen production by the hepatic injury tissues [7]. Increased ROS generation and electrophiles enhance the release of Nrf2 from sequestration and travel into the nucleus, where it provokes the production/transcription of cell defence genes, including superoxide dismutase, catalase, glutathione peroxidase, all of which can eliminate ROS molecules and minimize oxidative stress-mediated liver damage [8]. As a liver fibrosis inducer, thioacetamide (TAA) has been extensively used in study trials, which can provoke liver necrosis around the pericentral area after being activated by CYP450 2E1 enzyme, forming TAA-S-oxide and TAA-S-dioxide [9]. TAA (50–300 mg/kg) can promote reactive oxygen species, pro-oxidant molecules (MDA), and pro-inflammatory (tumour necrotic factor-α, interleukin-6) productions, all of which increase oxidative stress/inflammation rate, increase expression of intracellular signal transducers in the WNT/β catenin mechanisms, as well as increasing pro-apoptotic P53 and its target Bax protein (Bcl-2-like protein) [10, 11]. Altogether, these result in hepatic fibrosis/necrosis, liver injury, and dysfunction, which can be observed through significant alterations in plasma biochemicals, including alkaline phosphatase (ALP), albumin, Alanine aminotransferase (ALT), and total proteins [12].

Nature has been a remarkable source of healing compounds, offering numerous medicinal plants enriched with a variety of phytochemicals (phenolics, flavonoids, and terpenoids). Ethnobotanists have declared that using plant parts as therapeutics dates back hundreds of years, which can be served in various forms, including fresh, hydrodistilled, decoction, macerated, or by infusion. Moreover, plant-based compounds comprise a useful supply of active ingredients, with several bioactivities that can be given as treatment/supplementation for nearly all diseases/disorders [13]. Recent data from the WHO unveiled that nearly three-quarters of the world's nations use therapeutic herbs to manage health disorders, including liver diseases [14]. Since most of the available contemporary pharmaceuticals originate from herbal therapeutics, human remedies are therefore closely related to plant biodiversity. Modern medical studies revealed that traditional herbal therapeutic strategies for LF are multimodal, which can play an anti-cholestasis effect against multiple targets, including abnormal bile acid metabolism, inflammatory response, oxidative stress, necrosis, and liver fibrosis, with superior treatment advantages [15]. This creates a gap in having a single drug for the treatment of cholestatic liver disease/fibrosis. The available pharmaceuticals approved by the U.S. Food and Drug Administration for managing liver fibrosis/cholestasis are only ursodeoxycholic acid (UDCA) and oxybolic acid (OCA). However, both drugs

fail to meet the healthcare satisfaction due to their limited efficacy in the early stages of primary biliary cholangitis, and one-third of liver disease cases fail to respond. On the other hand, OCA was found to be effective in only half of the liver disease patients, not to mention their side effects, such as severe pruritus. While non-responders to both synthetic chemicals are at risk of having biliary fibrosis, end-stage liver disease, liver transplantation, or death [15–17]. This is also the main impetus for searching for better alternatives and complementary remedies with fewer side effects for LF/cholestasis.

Apocynin (4-hydroxy-3-methoxy-acetophenone) is a natural methoxy-substituted catechol that was first isolated from A. androsaemifolium and A. cannabinum, which are the most common Apocynum species widely employed as therapeutic plants by Native Americans [18]. Prepared as a therapeutic tea, Apocynum venetum is widely consumed by people of northern China and Japan for many health issues, including liver disorders, respiratory problems, hyperemia/dysentery, epilepsy/hemorrhoids, carbuncles, and chronic diarrhea [19]. Apocynin has been recognized as an NADPH-oxidase inhibitor, which is considered the main enzyme associated with the initial ROS generation in activated leukocytes. Once it reaches cells, Apocynin is processed by peroxidases and turned into more potent metabolites. Therefore, apocynin can suppress neutrophil oxidative stress/chemotaxis, which down-regulates neutrophil-induced cell damage [20]. It has been explored as a preventive of inflammation-related disorders, including asthma, cardiovascular diseases, arthritis, and lung injury [21, 22].

Given its potent antioxidant and anti-inflammatory properties, we hypothesized that Apocynin would attenuate liver fibrosis. Therefore, current investigations were geared to unveil the therapeutic effects of apocynin on thioacetamide-mediated liver fibrosis in vivo through depicting its modulatory effects on inflammatory, oxidative stress, and apoptotic pathways.

Materials and methods

Ethical approval

The animal handling procedures comply with the **ARRIVE** and EU Directive 2010/63/EU standards [23, 24]. The study protocol was confirmed by the ethical committee of Erbil Polytechnic University (No. 30 approved on Feb. 2, 2025).

Chemicals

The chemical inducer, TAA, and silymarin (reference drug) were purchased from Sigma-Aldrich (Merck, Germany). TAA was dissolved using 10% Tween 20, liquefied, and stored in dark vials at 8 °C for later use. The silymarin was prepared in distilled water, delivered in 50 mg/kg to experimental rats [25]. Apocynin (Cas no. 498–02-2) was purchased from Sigma-Aldrich. Apocynin was dissolved in 10% Tween 20 [26], which was prepared in 50 and 100 mg/kg and stored in dark vials for later experiments, and the dose selection was based on literature data on the effective dose of apocynin for liver disease [27]. The ELISA kits for estimating the SOD, CAT, and MDA were also purchased commercially from Sigma-Aldrich, China.

Acute toxicity experiment

Sprague Dawley male rats (10–12 weeks; 180–200 g in weight) were purchased from the Research Centre/Erbil Polytechnic University. Thirty healthy rats were randomly allocated to three cages in a mesh-wired model (to avoid coprophagia) for a 7-day adaptation period. Rats were fasted overnight, and on the next day, they received either 10% tween 20 (5 mL/kg), 250 mg/kg Apocynin, or 500 mg/kg Apocynin. Rats were fasted again for 3–4 hours after treatment, and they were regularly observed for any possible toxicity incidence until the end of the two-week trial. After 14 days and on the 15th day, all rats received 1 mL of anaesthesia (3 mg/kg xylazine and 30 mg/kg ketamine) and were sacrificed by decapitation using a sharp blade. The blood specimen and relevant organs were taken for biochemical and histopathological estimations [28].

Hepatoprotective trial

Experimental design

Healthy Sprague Dawley rats were provided by the research centre of Erbil Polytechnic University. The animals were kept in 5 mesh-wired plastic cages (6 animals each) at 25 °C temperature, 70% humidity, 12 h light/dark cycle with free access to ad libitum diet, and water. After the acclimatization period, the 5 group rats were treated as follows for the next two months (Fig. 1):

Group A served as a normal control and received oral doses of 10% Tween 20 and three injections/week of distilled water.
Group B served as the TAA control group and had oral doses of 10% Tween 20 and three injections i.p. at 5 mL/kg/week of TAA.
Group C (reference drug) received 50 mg/kg of silymarin orally and three injections i.p. at 5 mL/kg/week of TAA.
Group D rats had daily 50 mg/kg Apocynin orally and three injections i.p. at 5 mL/kg/week of TAA.
Group E rats had daily 100 mg/kg Apocynin orally and three injections i.p. at 5 mL/kg/week of TAA.

After a two-month period, rats were given 1 mL of anaesthesia [ketamine (30 mg/kg) and xylazine (3 mg/kg)], and sacrificed. The dissected liver and blood samples (by

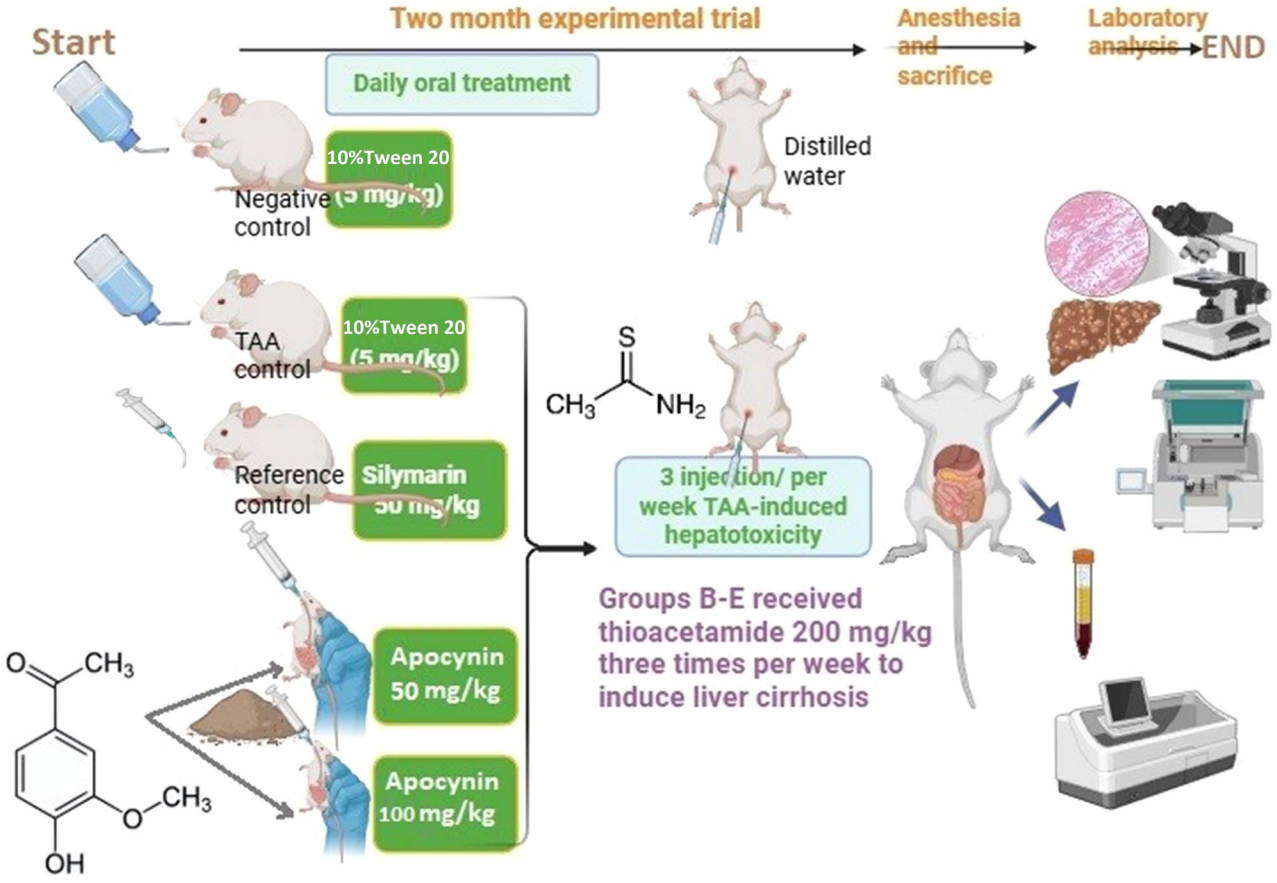

Fig. 1 Hepatoprotective experimental design for apocynin

cardiac puncture) were obtained for subsequent laboratory analysis [29].

Macroscopic viewing of the liver

The isolated liver from all experimental rats was placed in a cold normal saline container, and dried with filter papers for relevant gross evaluations, including weight and morphological characteristics. Additionally, the liver index was also determined for each experimental rat group using the equation below, as detailed previously [30].

$$Liver\ index\% = \frac{liver\ weight}{body\ weight} \times 100$$

Histology of the liver

The dissected liver lobes were placed in fixative solution (10% phosphate buffered formalin). After that, small pieces of liver tissue were transferred into cassettes for the tissue processing procedure using an automated machine (Histo-Tek, vpi, Sakura, Netherlands). After blocking the samples, 5 μm slices of liver tissues were sectioned and fixed on slides for microscopic observation using Haematoxylin, Eosin, and Masson Trichrome techniques (El [31]).

Immunohistochemistry

The rat monoclonal Anti-β-catenin/anti-P53 antibodies were bought from ViroMed Diagnostica (Sevilla, Spain). Sections of liver were fixed on poly-L-lysine-coated slides and placed in an oven (DON-HE Series, Infitek, Shandong, China) at 60 °C for 25 minutes. The slides were deepened in xylene for deparaffinization and dehydrated by a series of graded alcohols. The antigen was heated in a microwave as a heat-induced epitope retrieval procedure, and then mixed with a 10 mM sodium citrate buffer. For the staining procedure, we followed the recommendation of the producer's company. Briefly, endogenous peroxidase was blocked with 0.03% hydrogen peroxide and sodium azide for 5 min. Sectioned tissues were buffer-washed and placed in an incubator alongside antibodies (β-catenin, 1:400; P53, 1: 200) for 15 minutes. Slides were washed carefully and re-incubated for 15 min with streptavidin HRP. After incubation of tissue samples for 7 min with diaminobenzidine substrate chromogen, tissues were washed with buffer before haematoxylin counterstain (5 seconds). Finally, liver tissues were placed

in ammonia (0.037 mol/L) for 5 times, washed, and cover-slipped for the observational procedure for brown coloured cytoplasmic granules and nuclei as the positive signs for β-catenin and P53 expression [32].

Liver antioxidant

The sections of isolated liver tissues (1 gram) from both liver lobes were homogenized in 10 mL of ice-cold/distilled water using a Teflon homogenizer (5000 rpm, 15 min at − 4 °C). An amount of 10% homogenate was placed in a container containing the same amount of ice-cold 100 mM phosphate buffer (pH 7.4), followed by centrifugation at 11,000 g at 4 °C for 20 min using a Wincom ultracentrifuge (Wincom, Wuhan, China). The resultant supernatant was analysed for the SOD, CAT, and MDA contents [33].

Serum inflammatory cytokines

The present inflammatory response toward TAA hepatotoxicity was evaluated using commercial kits of (TNF-α, IL-6, and IL-10) chemicals, which followed the instructions found on the ELISA kit (Solarbio Life Science Co., Beijing, China).

Serum biochemicals

The serum specimens were investigated for ALT, ALP, AST, total protein, and albumin contents. The metabolic waste, total bilirubin, was estimated following protocols published elsewhere [34].

Statistical analysis

The present data are shown as mean ± SEM. The statistical analysis was conducted by SPSS, and One-way ANOVA (Post hock, Tukey's HSD) with a significance set at $p < 0.05$. The designed graphs were prepared with GraphPad Prism 9.03.

Results

Acute toxicity

Oral administration of Apocynin at 250 and 500 mg/kg to rats did not cause any noticeable toxic signs or physiological abnormalities during and after a two-week trial. Observation routine throughout the experiment did not find any obvious toxicological indications (skin/eye colour, salivation, irritation, locomotion, itching, diarrhoea, tremors, or convulsions). Supplemented rats had normal behaviour and consumed the usual amount of food and water, comparable to normal controls. The histopathological analysis of kidneys and liver tissues unveiled similar tissue layer organizations between Apocynin-treated rats and normal controls (Fig. 2). The serum analysis of functional kidney and liver parameters was found within normal values based on those of normal controls (available on request). The outcomes indicate a high safety margin for Apocynin, with a NOAEL determined to be 500 mg/kg in this model.

Hepatoprotectives of Apocynin

Body and liver masses

The present gross evaluation of the obtained liver organs was found to vary significantly due to different treatments (Table 1 and Fig. 3). The normal controls had a usual liver weight of 6.9 g and liver index values of 2.0%, which were found within the standard normal range. TAA control rats had significantly reduced body weight by 51.87% and increased liver index by 241.5% compared to normal controls. In contrast, silymarin or Apocynin oral administration (50 mg/kg and 100 mg/kg) maintained body, liver weight, and lowered liver index by 58.41%, 30.30%, and 44.50% compared to the value of TAA control rats. The outcomes show increased hepatoprotective effects of 50 and 100 mg/kg of apocynin, comparable to Silymarin, in terms of restoration of liver weightage and liver index in rats exposed to TAA hepatoxicity (Table 1).

Microscopic results

The liver tissue screening unveiled an extended range of hepatic alterations due to thioacetamide, plus different treatment strategies (Fig. 4). As expected, normal controls had usual liver tissue layers with no sign of inflammation or necrosis (Fig. 4A). Liver tissues of TAA control rats were seen with disorganized layers and extensive endothelial penetrations, an ambiguous nucleus, increased cytoplasmic vacuoles, and elevated inflammatory cell aggregations as clear signs of necrosis and fibrosis. Additionally, the formation of large fibrous septa disrupted the parenchymal layers that initiate the collagen bundles in the triangles of liver tissues, showing numerous micro- and macro-nodules in the liver tissues. The liver nodules were noticed inside connective tissue bundles that divided the liver into several lobules, followed by severe inflammation and hepatic necrosis (Fig. 4B). In contrast, silymarin or Apocynin oral administration resisted TAA hepatotoxicity, indicated by reduced inflammatory cell aggregation, less centrilobular tissue necrosis, and clearer round nuclei than TAA control rats. Apocynin-supplemented rats had more organized hepatic lobules, reduced vacuolization, and more distributed capillary veins across connective tissues than the TAA control rats (Fig. 4C–E). The outcomes indicated that despite the early onset of fibrosis in Apocynin-treated rats (Fig. 4Dand E), this did not progress into severe/liver cirrhosis, unlike the TAA control rats (Fig. 4B), denoting the hepatoprotective potential of this natural product.

The hepatic tissues observation using Masson's trichrome technique unveiled different levels of collagen content in liver tissues exposed to TAA and different

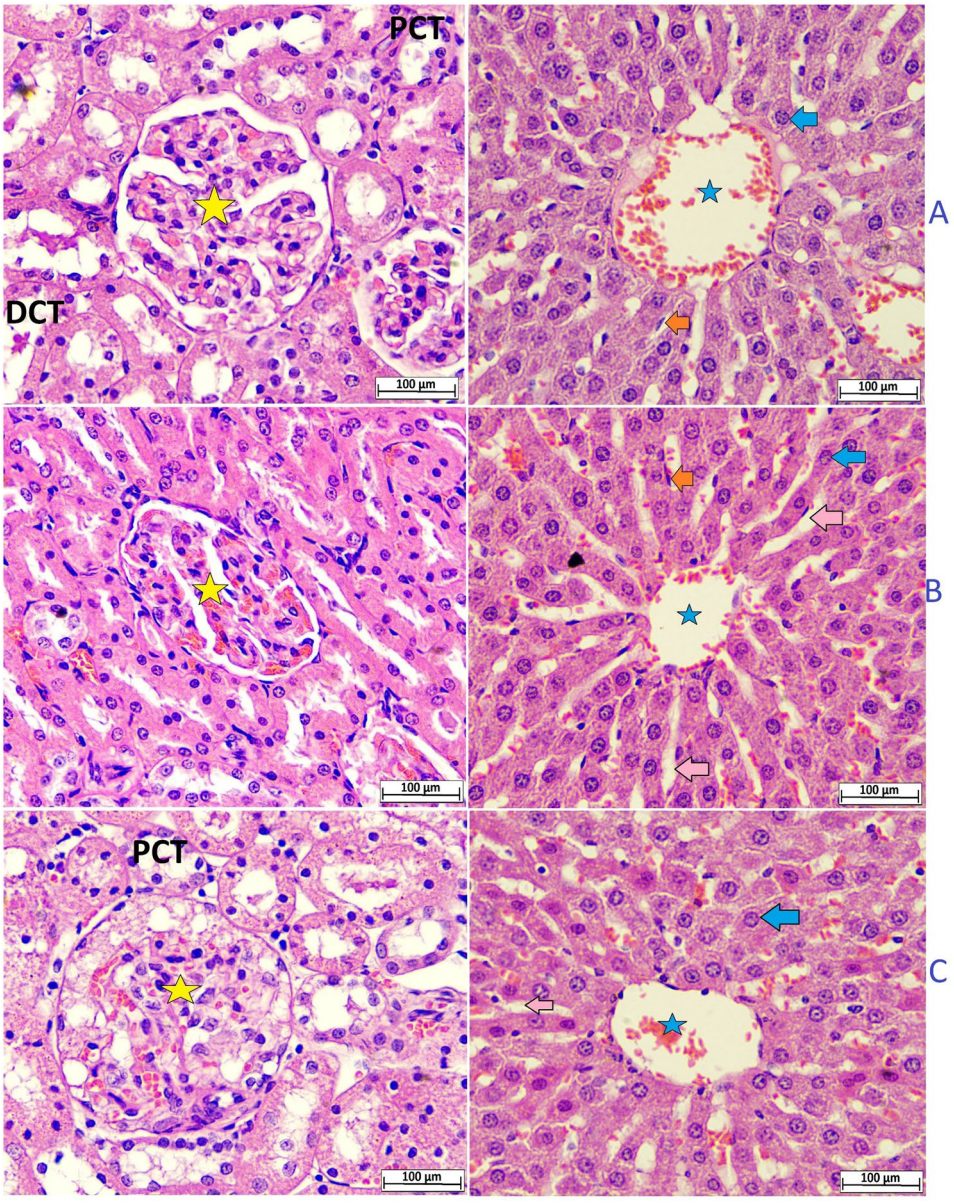

Fig. 2 Structural tissue arrangement of the liver and kidneys of rats in a toxicity trial. Group a, normal controls received 10% tween 20; group B had 250 mg/kg Apocynin; group C rats were supplemented with 500 mg/kg apocynin. The oral administration of apocynin did not cause any structural tissue alterations based on its comparison with normal controls. yellow star, glomerulus. The hepatic tissues are shown with a normal central vein (blue star), a kupffer cell (orange arrow), sinusoids (pink arrow), and sheets of hepatocytes (blue arrow); PCT, proximal convoluted tubules; DCT, distal convoluted tubules (40x)

treatments (Fig. 5). The hepatic tissues of normal controls did not show any evidence of collagen formation/leakage. The TAA control rats showed enlarged fibrous septa with elevated collagen deposition about the pericentral area, as a reaction of the liver to increased inflammation and severe hepatic injury. Silymarin or Apocynin-treated rats have liver tissues with moderate to reduced collagen deposition/tissue congestion around the hepatic central vein. Moreover, Apocynin or silymarin administration down-regulated mononuclear cell aggregation, fewer small/shrunken eosinophilic cells, and fewer scattered acidophil bodies, all of which denote less inflammation and less tissue alterations than TAA control rats. Liver tissues of Apocynin-treated rats had maintained regular tissue layers with almost normal lobular organization, noticed with fewer cytoplasmic vacuoles, fewer abnormal nuclei, and less necrotized tissue area than TAA control rats (Fig. 5).

Immunohistochemical protein expression

The present immunohistochemical expression of P53 genes (a regulator of TGF-β1 and hepatic satellite cells

Table 1 Effect of apocynin on body and liver estimations in rats exposed to taa hepatotoxicity

Groups	Body Weight (gm)	Liver weight (gm)	Liver Index LW/BW
A	343.5±5.2[a]	6.9±0.5 [a]	2.0
B	165.3±4.1[d]	11.3±0.9[d]	6.83
C	288.1±3.3[b]	8.2±1.1 [c]	2.84
D	218.3±4.8[c]	10.4±0.4 [b]	4.76
E	258.5±4.2[b]	9.76±0.8 [b]	3.79

Group A rats had distilled water; group B rats received TAA+ 10%tween 20; group C rats received TAA+ Silymarin; groups D and E rats received TAA+50 or 100 mg/kg Apocynin, respectively. Values shown as mean ± SEM ($n=6$). Means with different superscript letters within a column are significantly different ($p<0.05$)

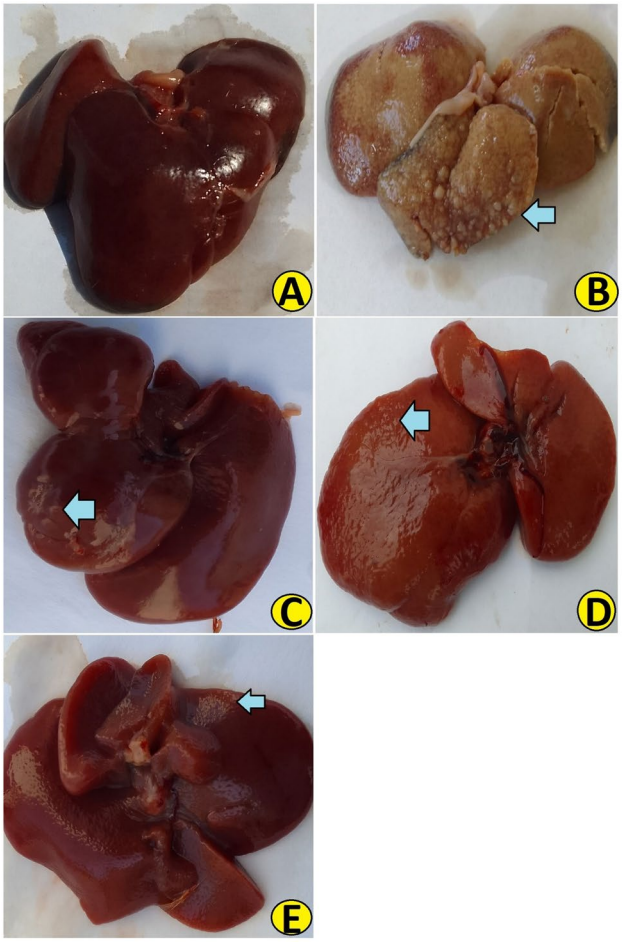

Fig. 3 Shows gross views of liver organs obtained from different treated rats. Group A rats had normal saline; group B rats received TAA+10% tween 20; group C rats received TAA+Silymarin; groups D and E rats received TAA+50 or 100 mg/kg apocynin, respectively. TAA injections caused significant hepatotoxicity and hepatomegaly with numerous nodules/benign lesions (blue arrow). While apocynin oral administration resisted TAA hepatoxicity, as denoted by lower gross alterations and fewer nodules than TAA control rats

(HSC)) was noticeably varied in liver tissues exposed to TAA hepatotoxicity plus different treatments Fig. 6A–E. TAA control rats exhibited increased P53 expression in their liver tissues, indicating increased apoptotic actions and myofibroblast differentiation and ECM production. In contrast, Apocynin oral administration decreased expression of P53 (Bax proteins), consequently provoking HSC and lowering apoptosis-mediated pathways in damaged tissues that aided in refolding of partially damaged proteins, thereby limiting TAA-mediated hepatic injury, with 100 mg/kg apocynin being more potent than 50 mg/kg (Fig. 6).

The effect of Apocynin on the onset of cellular propagation of hepatic tissues exposed to TAA hepatoxicity was demonstrated by presenting immunohistochemical β-catenin (nuclear receptor coactivator) expression in hepatic tissues using anti-β-catenin antibody (Fig. 7). The liver tissues of normal controls showed no sign of β-catenin, indicating a lack of cell proliferation or inflammatory-signalling cascades. TAA control rats were seen with increased β-catenin intensity in their liver tissues, meaning elevated cellular propagation, increased mitotic activity, and tissue necrosis, all of which highlight the dissemination of TAA-mediated liver intoxication. Apocynin oral administration at 50 mg/kg apocynin showed moderate improvement in the liver β-catenin expression, while 100 mg/kg apocynin supplementation reduced β-catenin expression, denoting superior protection, less mitotic actions, fewer necrotized hepatocytes' proliferation, and suppressed inflammatory pathways (Fig. 7). The outcomes present noticeable hepatoprotective actions of Apocynin that could be linked with suppressed effects on β-catenin expression, which was significantly up-regulated in severely injured/highly proliferating liver tissues of TAA control rats.

Apocynin effects on liver antioxidants and oxidative stress markers

TAA inoculation provoked oxidative stress in liver tissues, promoting further tissue inflammation and liver tissue injury. As seen in Fig. 8, normal controls exhibited increased endogenous antioxidants (SOD, 11.76 U/mL; CAT, 49.83 nmol/min/ml) and reduced pro-oxidant marker non-enzymatic (MDA, 1.58 nmol/mg) in their liver homogenates. TAA control rats unveiled oxidative stress tissue injury, indicated by the lowest antioxidants (SOD, 6.4 U/mL; CAT, 28.17 nmol/min/ml) and the highest pro-oxidant marker (MDA, 6.73 nmol/mg) in tissue homogenates. The ROS/antioxidant imbalance was less significant in Silymarin or Apocynin-treated rats, denoted by higher SOD (20.67, 10.60, 14.17 U/mL, respectively), CAT (43.67, 30.50, 28.83 nmol/min/ml, respectively), and lowered pro-oxidant MDA marker (2.65, 4.38, 2.30 nmol/mg, respectively) than TAA

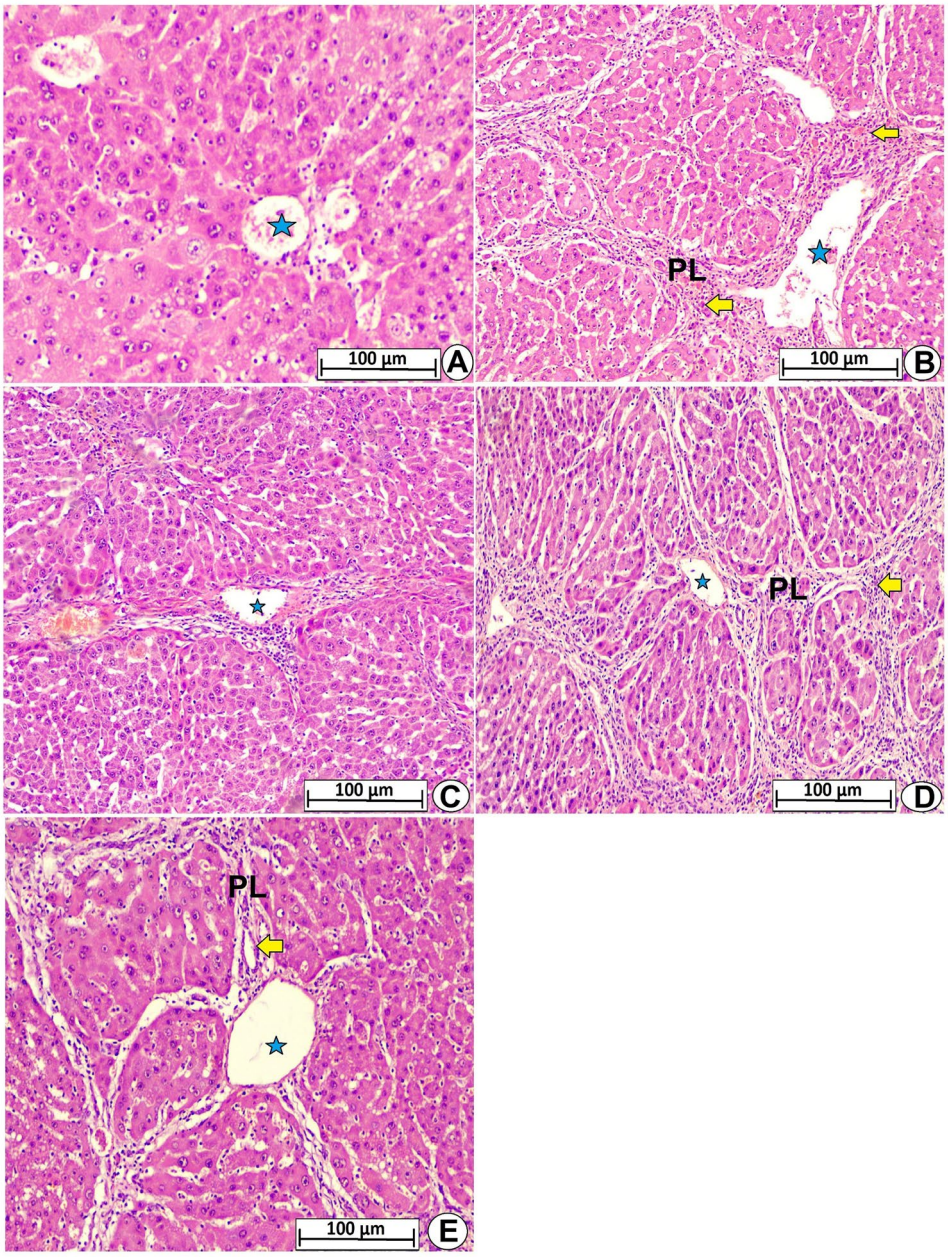

Fig. 4 Microscopic views of liver tissue exposed to TAA hepatotoxicity (Haematoxylin and eosin). Group A rats had distilled water; group B rats received TAA+10% tween 20; group C rats received TAA+Silymarin; groups D and E rats received TAA+50 or 100 mg/kg apocynin, respectively. TAA control rats showed increased cellular proliferation (PL) and an inflammatory area (yellow arrow). Apocynin administration resisted TAA-mediated hepatoxicity and maintained structural tissue integrity, indicated by less inflammation and lower cellular proliferation than TAA control rats. Rats had 100 mg/kg apocyninhad comparable liver tissue organizations as silymarin-treated rats (10 x). Blue arrow, central vein

control rats. The above data present noticeable antioxidant potentials of Apocynin, limiting oxidative stress-mediated tissue injury, which was significantly lower than that of TAA control rats.

Inflammatory cytokines

The systemic inflammation and immune system dysregulation, an integral part of fibrosis initiation, were significantly varied due to TAA hepatotoxicity and different treatment strategies. As expected, normal controls had no serum alterations in terms of inflammatory cytokines, denoted by maintained levels of TNF-α (100.2 pg/ml), IL-6 (98.4 pg/ml), and IL-10 (255.4 pg/ml) in their serum specimens. TAA control rats experienced severe inflammation based on altered serum cytokine contents, increased TNF-α (698.4 pg/ml); IL-6 (456.3 pg/ml); and a lower IL-10 cytokine levels (82.3 pg/ml) compared to normal controls. In contrast, Silymarin or Apocynin

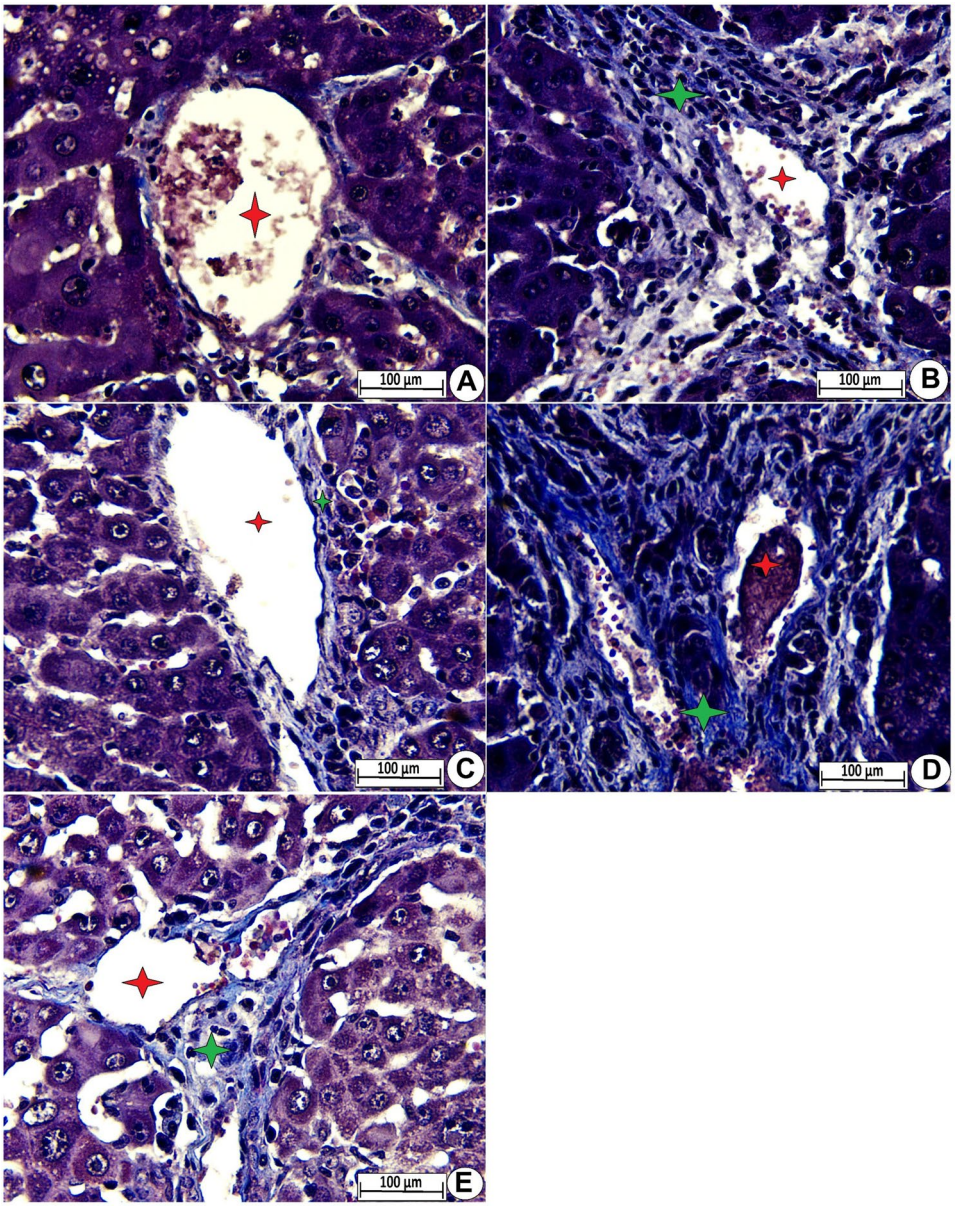

Fig. 5 Microscopic views of liver tissue exposed to TAA hepatotoxicity (Mason Trichrome, 20x). Group A rats had distilled water; group B rats received TAA+10% tween 20; group C rats received TAA+Silymarin; groups D and E rats received TAA+50 or 100 mg/kg apocynin, respectively. TAA control rats exhibited elongated bile ducts, large fibrous septa, and elevated deposition of collagen (green star) near the central vein (red star). Rats administered silymarin or apocynin had moderate tissue alterations, indicated by smaller fibrous septa, reduced collagen deposition, and inflammatory cell aggregations compared to TAA control rats

supplementation resisted TAA-mediated inflammation/hepatic injury, denoted by negative modulation of TNF-α (157.7, 230.8, 192.5 pg/ml, respectively), IL-6 (162.3, 201.5, 182.4 pg/ml, respectively), and up-regulated IL-10 (199.2, 164.4, 203.0 pg/ml, respectively) cytokines, which were statistically significant compared to values of TAA control rats. The data mentioned above could justify the anti-inflammatory potentials of Apocynin, which maintained cellular integrity and limited TAA-mediated liver injury (Fig. 9 A-E).

Effect of apocynin on liver biochemistry

The relevant serum biochemical parameters are significantly varied in rats exposed to TAA and silymarin or Apocynin treatments. Normal controls had liver functional indicators within the normal range, which was not the case for TAA control rats. Moreover, TAA control rats experienced severe enzyme leakage and poor liver synthetic production in terms of albumins/proteins as a result of TAA-mediated liver dysfunction. Such liver dysregulations and synthetic performance were restored following silymarin or Apocynin oral administration,

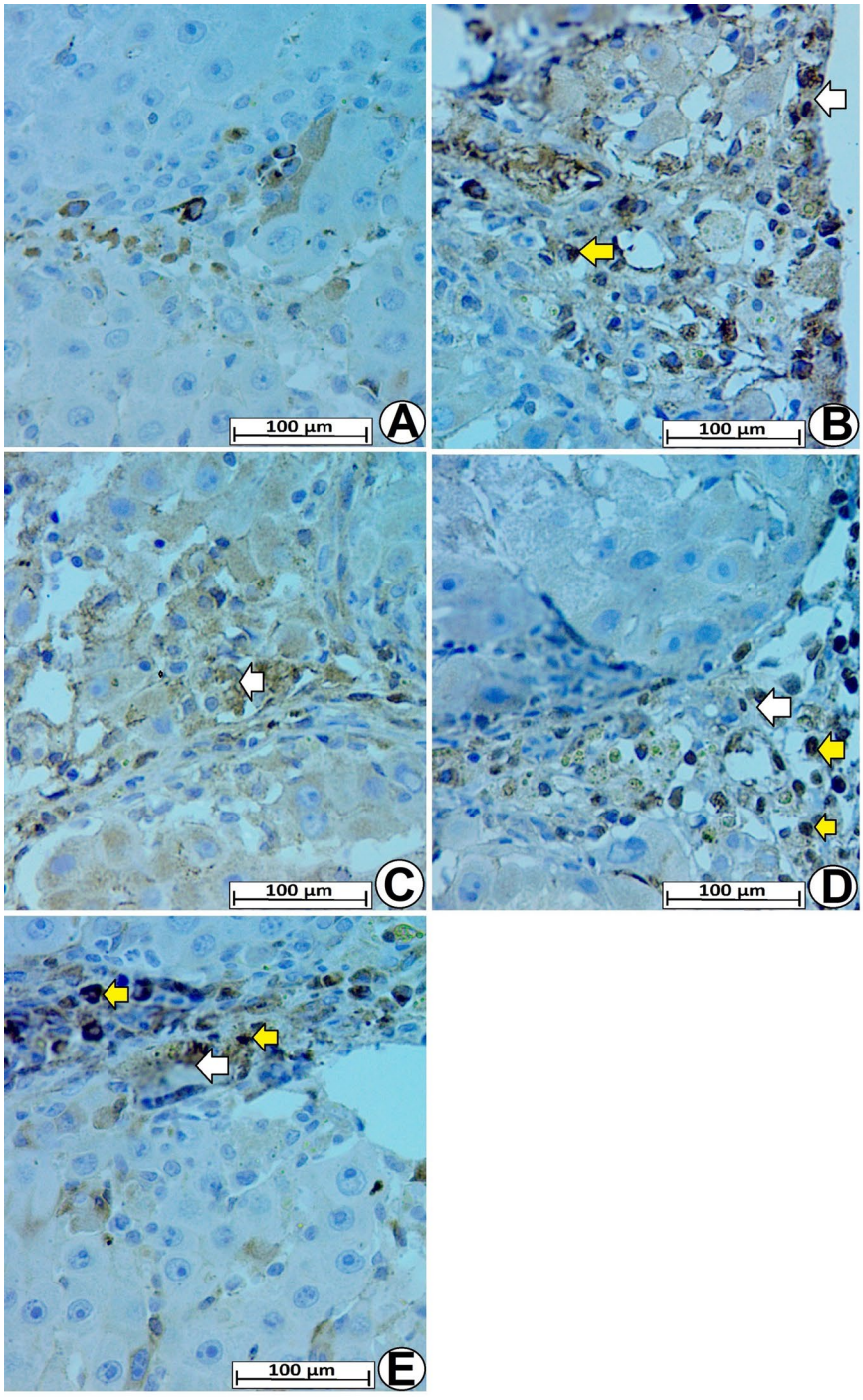

Fig. 6 Shows P53 protein expression in liver tissues exposed to TAA hepatotoxicity + treatments. Group A rats had distilled water; group B rats received TAA+10% tween 20; group C rats received TAA + silymarin; groups D and E rats received TAA+50 or 100 mg/kg apocynin, respectively. TAA control rats had elevated proapoptotic Bax appearance in their tissues, meaning increased apoptotic action in damaged areas; silymarin or apocynin-treated rats showed intermediate to low levels of P53 expression in their liver tissues, denoting a reduced apoptotic action and thus less liver tissue injury. Yellow arrow, inflammatory cells; white arrow, area of P53 expression

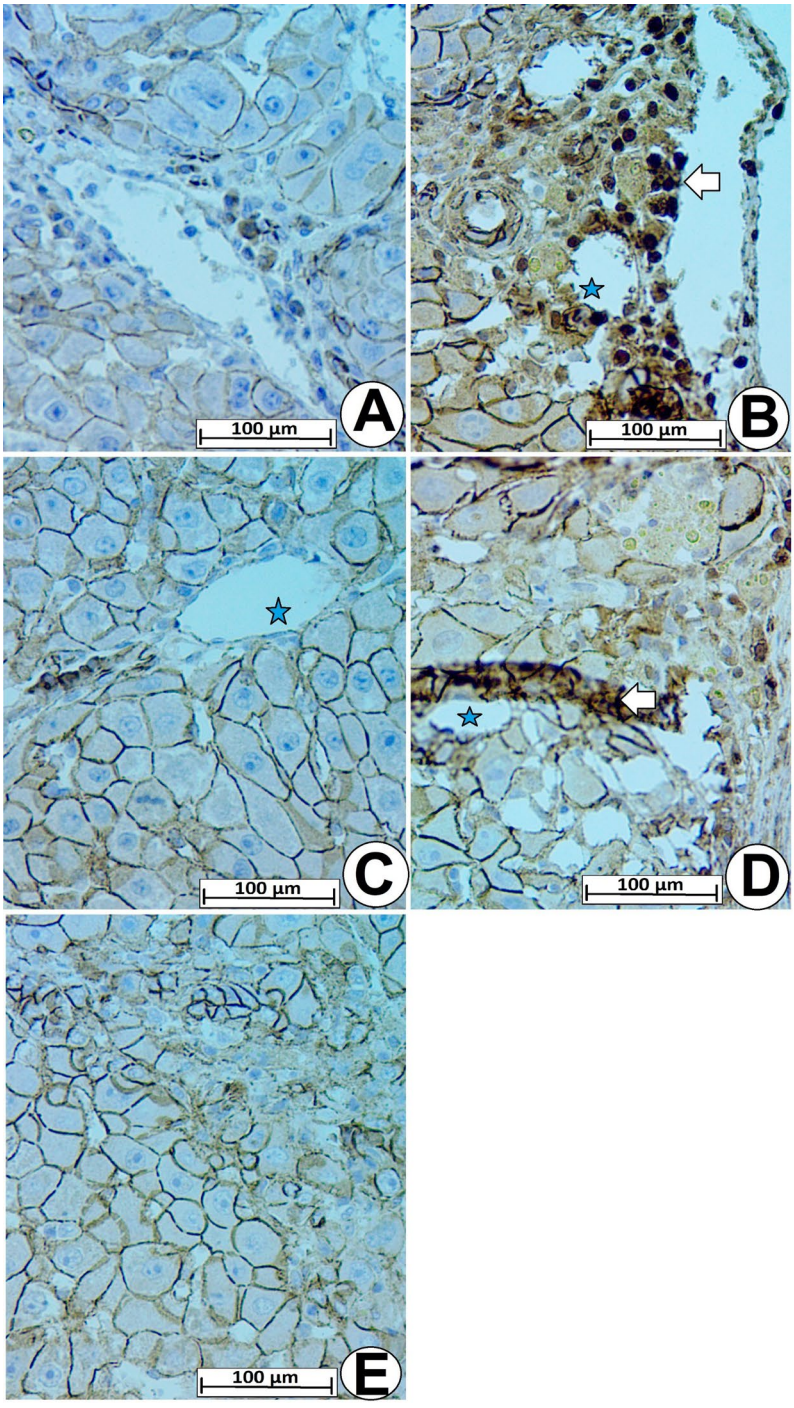

Fig. 7 Effect of apocynin on the β-catenin expression in liver tissues exposed to TAA hepatotoxicity + treatments. Group A rats had distilled water; group B rats received TAA+10% tween 20; group C rats received TAA + Silymarin; groups D and E rats received TAA+50 or 100 mg/kg apocynin, respectively. TAA control rats showed increased β-catenin expression (white arrow) in their liver tissues, denoting elevated cellular proliferation, which further deteriorated liver tissues. Apocynin supplementation at 50 and 100 mg/kg showed significant resistance against TAA hepatotoxicity, indicated by moderate to minor β-catenin proteins in liver tissues that were notably less than those of TAA controls. Blue star, central vien

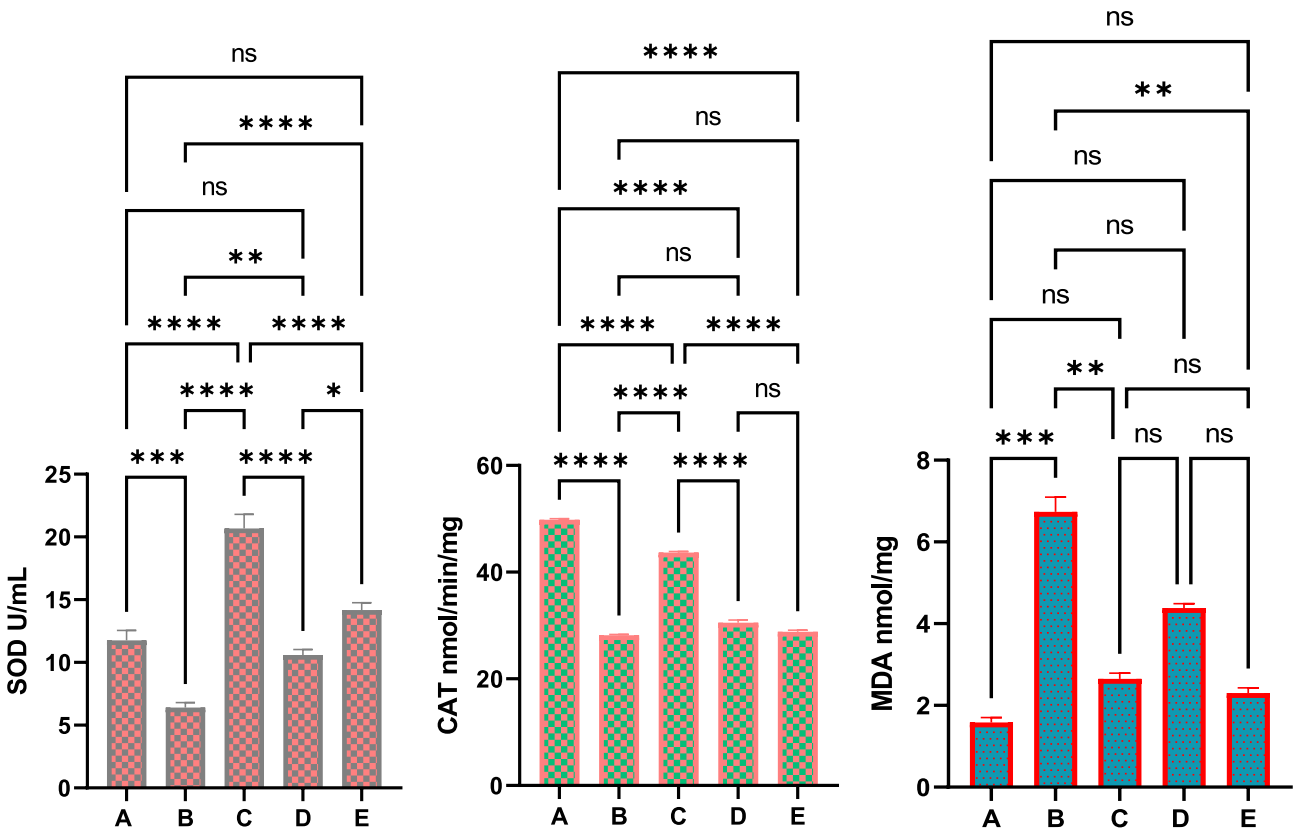

Fig. 8 Effect of apocynin on oxidative stress markers in rats exposed to TAA hepatotoxicity. Group A rats had distilled water; group B rats received TAA+10% tween 20; group C rats received TAA + Silymarin; groups D and E rats received TAA+50 or 100 mg/kg apocynin, respectively. TAA control rats showed increased oxidative stress in their liver tissues, denoted by reduced concentration of SOD, CAT, and increased MDA contents. Apocynin-treatment resisted TAA-mediated oxidative stress, shown by higher SOD, CAT, and lower MDA levels, strengthening cytoprotective mechanisms against aggressive factors such as TAA

denoted by higher serum protein/albumin and significantly lower ALT, bilirubin, AST, and ALP levels compared to those of TAA control rats (Table 2). The overall maintained liver functionality in Apocynin-treated rats justifies its hepatoprotective effects against TAA intoxication to a margin near normalization of hepatic excretory and synthetic functions (Table 2).

Discussion

The acute toxicity evaluation was conducted to complement the evidence on the safety of Apocynin in long-term use as a supplement and health-promoting food. Animals are sensitive to toxic substances found in herbal products and their isolates; thus, administration of relevant doses of natural products provides a piece of scientific evidence for their safety margins [35]. In the present toxicity trial, supplemented rats received oral doses of 250 or 500 mg/kg of Apocynin and were kept under observation for the following two weeks. The results denoted the absence of any mortality or acquired toxic signs/symptoms in rats even after the trial. Moreover, food consumption and weight gain were found to be comparable between supplemented rats and normal controls. The literature search showed few data on the toxic effects of apocynin, but those available are reassuring about the safety of apocynin consumption. An experimental trial has estimated the LD50 of apocynin as 9 g/kg in an oral gavage model using mice. Moreover, intraperitoneal injection of apocynin at 120 mg/kg was found to be completely safe based on relevant estimations of urine samples collected 20 hours later. An intravenous delivery of 420 mg/kg apocynin in mice showed minimal toxicity signs (reviewed in [36]. Numerous clinical/preclinical investigations approved the therapeutic potential of apocynin on numerous inflammatory diseases, reassuring its safe ingestion even for long-term use [20].

TAA intraperitoneal delivery is an effective experimental protocol applied by researchers when studying any herbal products with potential hepatoprotective actions [37]. Although the TAA-induced liver damage is rare in humans, comparable chemical substances have been associated with the elevated incidence rate of liver disease, such as carbon tetrachloride (dry cleaner), vinyl chloride (plastic industry), paraquat (the herbicide), and polychlorinated biphenyls (industrial chemicals) [38, 39]. In the present trial, liver macroscopical and

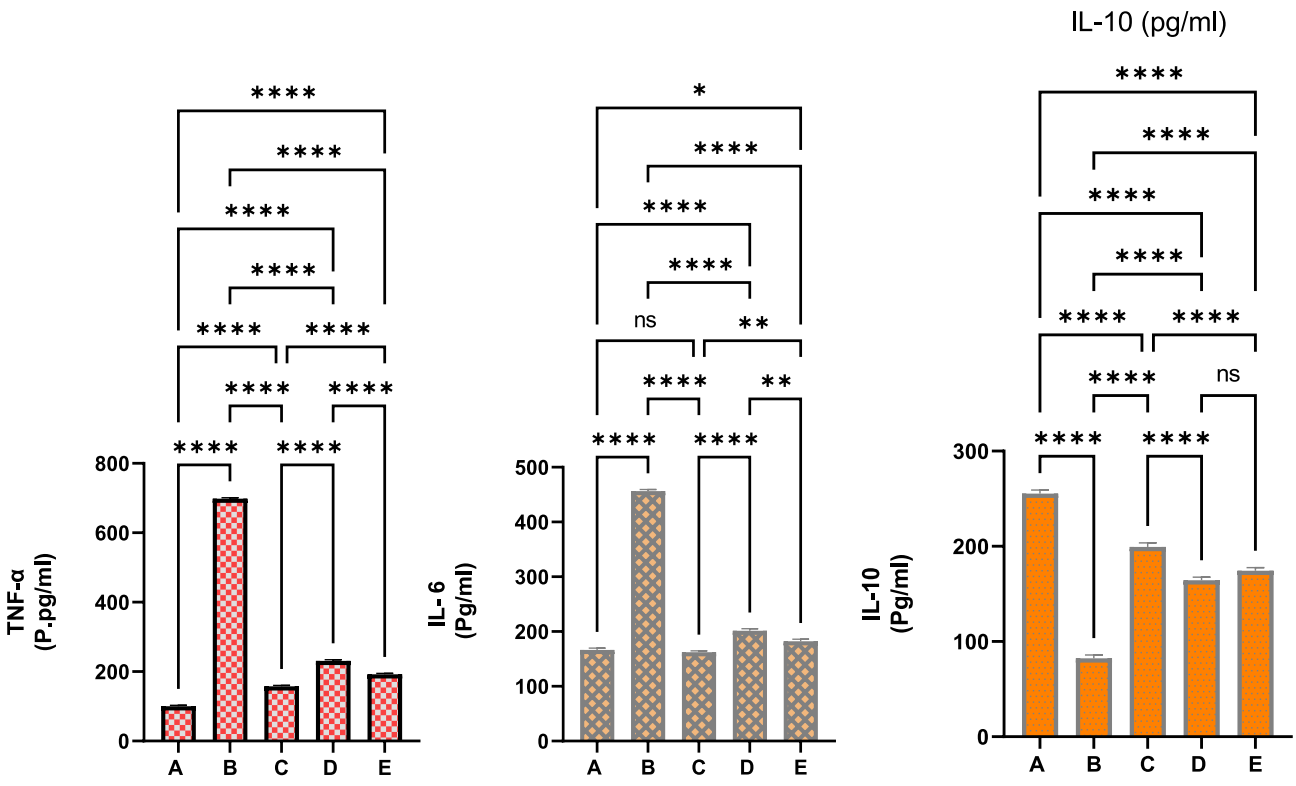

Fig. 9 Effect of Apocynin on inflammatory markers in TAA-intoxicated rats. Group A rats had distilled water; group B rats received TAA+10% tween 20; group C rats received TAAT + Silymarin; groups D and E rats received TAA +50 or 100 mg/kg Apocynin, respectively. Three TAA injections per week provoked a significant inflammatory response in TAA control rats, denoted by elevated TNF-α and IL-6, which further deteriorated liver tissues. In contrast, silymarin or Apocynin administration resisted TAA-induced inflammation, indicated by lower pro-inflammatory mediators and higher IL-10 cytokines compared to those of TAA control rats

Table 2 Effect of apocynin administration on liver-relevant serum biochemicals of experimental rats

Groups	ALP (IU/L)	ALT(IU/L)	AST(IU/L)	Total bilirubin uM	Protein(g/L)	Albumin (g/L)
A	86.24±5.3[a]	64.4±2.3[a]	165.2±3.8[a]	2.19±0.8[a]	66.5±3.1[a]	11.6±2.3[a]
B	254.9±4.1[d]	215.4±4.1[d]	312.5±3.3[c]	7.8±1.3 [d]	56.1±2.6[d]	8.1±1.4[c]
C	134.6±3.5[b]	77.9±3.7[b]	175.3±3.7[b]	5.5 ±0.6[b]	68.4±3.8[b]	12.6±1.2[b]
D	179.2±4.9 c	88.5±3.2[c]	218.2±3.5[b]	7.1±0.8[c]	61.3±2.6[c]	10.2±1.8[b]
E	164.2±3.8[c]	82.1±3.1[c]	212.5±4.5[b]	5.8±0.5[b]	66.1±3.8[b]	9.8±2.3[b]

Group A rats had distilled water; group B rats received TAA+10% Tween 20; group C rats received TAA + Silymarin; groups D and E rats received TAA + 50 or 100 mg/kg Apocynin, respectively

microscopical/histopathological evaluations evidenced clear initiation of liver fibrosis in a time-based progression, all of which was confirmed by previous researchers [40]. Microscopical observation detailed intense centrilobular necrosis, congestion of sinusoids, increased collagen deposition, lobular congestion, ultimately thick fibrotic septa, disrupted cellular architecture, dilatated central veins, and degenerated hepatic vacuoles, which were found parallel with outcomes [41]. In contrast, Apocynin daily supplementation limited TAA-hepatotoxicity and prevented further histological deteriorations. Accordingly, previous data show significant hepatoprotective effects in Cholesterol-induced liver injury model, which was backed up by restoration of liver function parameters, reduced microvascular leakage, and lowered inflammatory infiltrations, as well as down-regulation of gp91 phox, GSH/NADPH, and decreased TBARS [42]. Moreover, daily supplementation of apocynin (100 mg/kg) for two weeks mimicked CCl_4-induced liver fibrosis based on restored liver function parameters and preserved hepatic tissue architecture [27]. The current study is found in alignment with previous data declaring apocynin's preventive action against liver ischemia/reperfusion injury [43].

Cellular senescence is a cellular defence mechanism preventing the conversion of potentially damaged cells into mutated ones; however, chronic senescence causes the tissue to be more vulnerable to aggressive factors and facilitates chronic tissue injury. Conversion of cholangiocytes and hepatic stellate cells (HSCs) into senescent cells

that release numerous inflammatory mediators and anti-apoptotic proteins, and other cellular proteins (smooth muscle α-actin) that provoke HSCs and ECM proliferation [44]. Immunohistochemical proteins are key players in liver fibrosis initiation by enhancing the proliferation of liver progenitor cells in the ductular reaction (liver fibrogenesis). β-catenin has been linked with tumorigenesis due to its positive modulations of angiogenesis and the apoptotic process through stimulation of VEGF, Bcl-2, and survivin [45]. Scientists declared that increased expression of hepatic β-catenin facilitates senescence and senescent cell formation, provoking immune/inflammatory response, and liver tissue injury [46]. β-catenin silencing in HSCs resulted in the reduction of collagen type I and III mRNA appearance, indicating that the reduction of β-catenin directly downregulates the HSC activation and collagen synthesis. [47]. Indeed, researchers found that suppressing the Wnt/β-catenin mechanism by β-catenin siRNA also attenuated bleomycin-induced pulmonary fibrosis [48].

P53 is a tumour suppressor gene that activates pro-apoptotic proteins (Bax and transforming growth factor β) once cells are exposed to stress factors, and its stability in hepatic stellate cells is crucial to prevent liver fibrosis initiation [49]. In the present hepatoprotective trial, TAA inoculation caused significant expression of β-catenin and P53 appearance in hepatic tissues, enhancing continuous apoptosis and extracellular matrix formation (fibrosis). Apocynin supplementation improved expression of apoptotic proteins, shown by lower β-catenin and P53 proteins, reducing the apoptotic process in damaged hepatocytes, thereby limiting TAA hepatic damage. Similarly, daily supplementation with apocynin (16 mg/kg) led to noticeable up-regulation of anti-apoptotic Bcl-2 and down-regulation of the expression of Bax, p47phox/p67phox at protein/mRNA levels, which were considered as the main mechanism underlying its ameliorating testicular dysfunction in streptozocin-induced diabetic rats [50]. Accordingly, researchers have shown significant modulatory potentials of apocynin (50 mg/kg) on immunohistochemical (caspase 3, Bax, and Bcl-2) proteins, which were proposed as major mechanisms supporting its restoration of acute spinal cord damage [51].

Oxidative stress, an important mediator of liver fibrosis, occurs as a result of increased ROS formation in liver tissues exposed to aggressive factors such as TAA. Moreover, ROS molecules can attack macromolecules, the nucleus, and membrane lipids, up-regulating lipid pro-oxidant by-products (MDA) that enhance collagen production and subsequent liver necrosis/fibrosis [52]. In the present trial, TAA delivery enhanced oxidative stress in liver tissues, indicated by lower antioxidants (SOD and CAT) and up-regulated pro-oxidant MDA molecules in the hepatic tissue, subsequently facilitating inflammatory pathways and liver tissue injury. Apocynin supplementation showed significant protection against TAA-mediated oxidative tissue injury, shown by lower MDA and higher endogenous antioxidants (SOD and CAT) in hepatocytes. Similarly, the antioxidant potentials of apocynin have been repeatedly confirmed by different in vitro and in vivo trials [53–55]. Apocynin supplementation (100 mg/kg) led to a significant reduction of the oxidative stress markers (MDA, MPO, NO, and APOP level) and a noticeable up-regulation of CAT and SOD, all of which attenuated CCl4-mediated hepatotoxicity in rats [27]

Inflammation is a crucial pathological event associated with liver fibrosis that may be expressed by "liver-spleen-axis". As the largest peripheral lymphatic organ, the spleen has an anatomical/functional link with the liver through the splenic vein that forms the hepatic portal vein once it confluences with the superior mesenteric vein [56]. Therefore, in the case of liver diseases (cirrhosis), spleen volume increases (splenomegaly) in parallel with the portal pressure gradient/partial hypertension and fatty infiltration. As a strict innate/acquired immune regulator, the spleen is normally responsible for declaring circulatory apoptotic cells, production of antibodies (through T-cells and B-cells), as well as storing circulatory monocytes for immediate recruitment at injury/inflammation sites. The microvasculature of the spleen is involved in the generation of inflammatory/anti-inflammatory chemicals (TNF-α, IL-6, and IL-10), which are released into circulation to initiate a strong immune-inflammatory response. Indeed, pro-inflammatory mediators generated from the spleen pass through the splenic/portal veins to the liver, facilitating the liver's natural killer cell toxicity [57]. In the intoxicated injured liver tissues, necrotic hepatocytes release ligands associated with damage-associated molecular patterns (DAMPs), activating the immune/inflammatory response and subsequent sterile inflammation. Pro-inflammatory mediators are associated with the progression of fibrosis by regulating several cellular actions, including lipid metabolism, biliary obstruction, and the formation of positive/negative acute phase proteins. Moreover, scientists declared that inflammatory cytokines (chemokines) stimulate the release of local nitric oxide and several other vasodilators, initiating a hyperdynamic circulatory condition as seen in liver fibrosis, hypovolemia, and ascites formation. Cellular pathways such as NF-$_k$B participate in cytokine/chemokine regulation, leukocyte aggregation, and cell survival, all of which are considered key players of the inflammatory response [58]. In the present TAA hepatotoxic model, systemic inflammation was significantly provoked, indicated by increased TNF-α, IL-6, and lower IL-10 cytokines, further enhancing liver tissue deterioration. Apocynin oral supplementation (50 and 100 mg/kg) ameliorated TAA-mediated inflammation

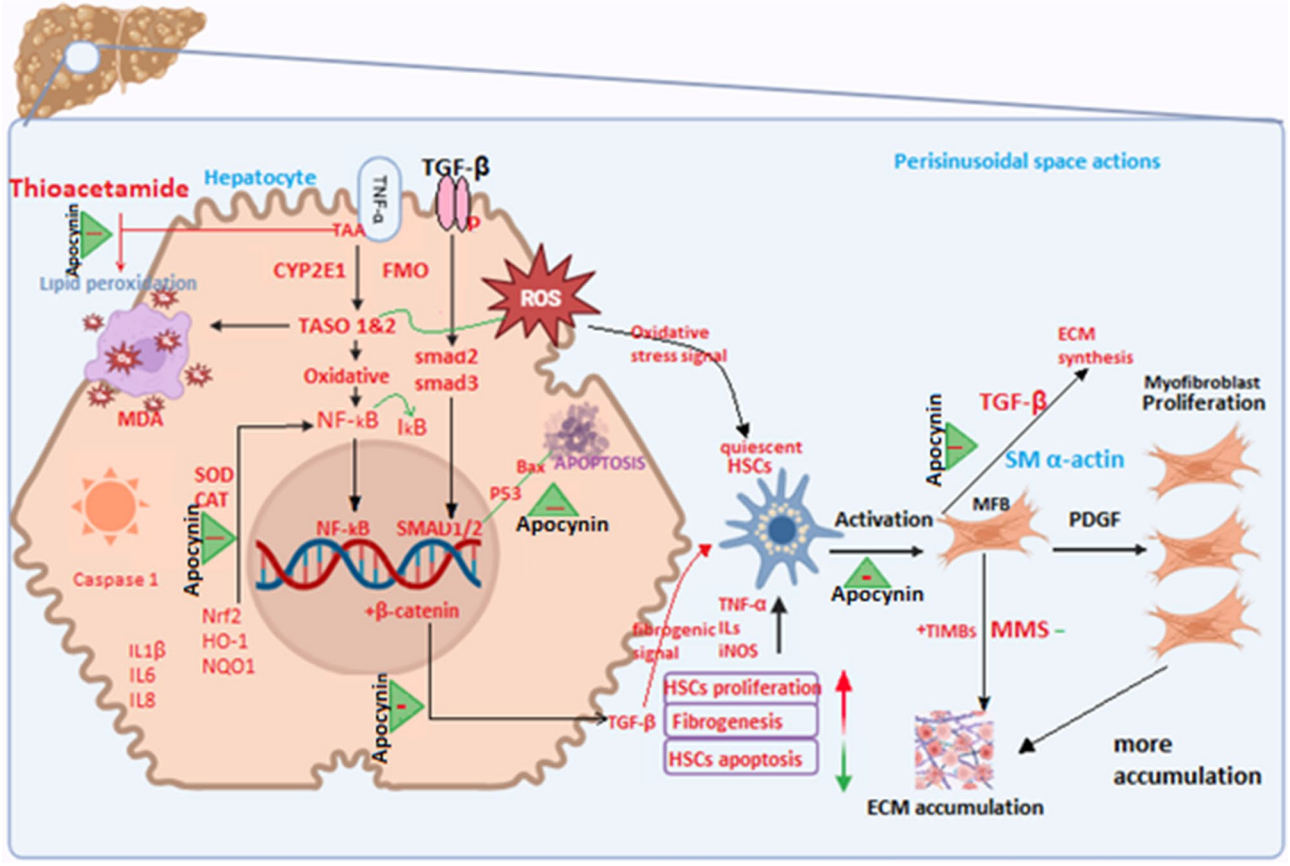

Fig. 10 Summarize molecular mechanisms associated with the present hepatoprotective effect of apocynin in the TAA-intoxicated rat model

and pro-inflammatory cytokine production. Accordingly, apocynin treatment (5 mg·kg^{-1}·day^{-1}, intraperitoneally) for 24 weeks led to a significant reduction of airway inflammation (down-regulating infiltration of inflammatory cells such as neutrophil, macrophage, and lymphocyte), lung function decline (hyperinflation), and suppressed airway collagen deposition all of which ameliorated chronic obstructive pulmonary progression and vascular damages provoked by cigarette smoke exposure (9 cigarettes/day) [59]. Similarly, intraperitoneal injection (10 mg/kg/*i.p*) apocynin attenuated inflammation by provoking IκBα and reducing IKKα, IKKβ, NF-κBp65, and p-NF-κBp65 as well as IL-6 and TNF-α cytokines, which were considered as the main pathway underlying its alleviation of TAA-mediated liver injury [60]

Serum biochemicals serve as an important reflector of the physiological and pathological processes of organs, particularly the liver. Liver enzymes (ALP, ALT, AST, GGT), waste products (bilirubin, creatinine), and liver proteins (total protein, albumin) have been monitored to understand liver functionality or possible toxicity [61]. In the present trial, TAA control rats exhibited increased leakage of liver metabolic enzymes (due to nuclear and cytoplasmic alterations), while total proteins and albumin levels were down-regulated because of suppressed transcriptional pathways (reduced mRNA) and membrane permeability disruptions. Researchers confirmed that aggressive factors can provoke liver enzyme leakage from hepatocytes, subsequently causing diminished essential proteins in the intracellular/extracellular fluids, as well as translocating nucleic acid from the nucleus into the cytoplasm [25]. In contrast, Apocynin oral administration resisted TAA-mediated biochemical alterations and restored liver functionality close to normal values in a dose-related manner. Accordingly, Apocynin (100 mg/kg) showed noticeable dose-dependent hepatoprotection against CCl4-intoxicated rodents, evidenced by a significant improvement in the serum biochemicals, including liver functional parameters [27]. Similarly, apocynin supplementation (20–100 mg/kg) led to significant restoration of liver functional parameters in different hepatotoxicity animal models [27, 60, 62]. The possible underlying mechanism of the hepatoprotective effects of Apocynin in TAA-intoxicated rats is summarized in Fig. 10.

Conclusions

The present study was geared to evaluate the acute toxicity and hepatoprotective potential of Apocynin. The toxicity trial unveiled an increased safety margin of Apocynin up to 500 mg/kg in rats based on histopathological and biochemical estimations. In the hepatoprotective experiment, Apocynin supplementation resisted TAA intoxication and restored liver function close to normal values, indicated by restoration of hepatocyte alteration, including reduced endothelial penetrations, less necrosis/fibrosis, less collagen depositions, and fewer inflammatory cell infiltration than TAA control rats. Apocynin supplementation improved immunohistochemical markers, lowering β-catenin and P53 expression, all of which suppressed autophagy and reduced ECM generation in damaged hepatocytes. Additionally, the prooxidant marker (MDA) and pro-inflammatory mediators (TNF-α and IL-6) were found to be significantly lower in Apocynin-treated rats than those of TAA control rats. The outcomes present significant hepatoprotective potentials of Apocynin, which could be a potent therapeutic source for biopharmaceutical products for liver fibrosis.

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Author contributions
K.A.A., A. A.j., conceptualization; A.A.J., K.M.A., method and investigation; T.S.A., N.A.I., G.N.S., A.I.A., formal analysis, and software; M.A.A., D.K.A., resources, and validation; S.S.A., M.A.M., H.I.A., design and review; A.A.J., writing manuscript.

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Data availability
All relevant data are contained within the article.

Declarations

Ethical approval
The animal handling procedures comply with the **ARRIVE** and EU Directive 2010/63/EU standards [23, 24]. The study protocol was confirmed by the ethical committee of Erbil Polytechnic University (No. 30, 1/2/2025).

Informed consent
N/A.

Competing interests
The authors declare no competing interests.

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