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Antifibrotic effects of gallic acid on hepatic stellate cells: *In vitro and in vivo* mechanistic study

Naglaa M. El-Lakkany [a, *], Walaa H. El-Maadawy [a], Sayed H. Seif el-Din [a], Samira Saleh [b], Marwa M. Safar [b, f], Shahira M. Ezzat [c, g], Salwa H. Mohamed [d], Sanaa S. Botros [a], Zeinab Demerdash [d], Olfat A. Hammam [e]

[a] Department of Pharmacology, Theodor Bilharz Research Institute, Warak El-Hadar, Imbaba P.O. Box 30, Giza 12411, Egypt
[b] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt
[c] Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt
[d] Department of Immunology, Theodor Bilharz Research Institute, Warak El-Hadar, Imbaba P.O. Box 30, Giza 12411, Egypt
[e] Department of Pathology, Theodor Bilharz Research Institute, Warak El-Hadar, Imbaba P.O. Box 30, Giza 12411, Egypt
[f] Department of Pharmacology and Biochemistry, Faculty of Pharmacy, The British University in Egypt, Suez Desert Road, P.O. Box 43, ElSherouk City, Cairo 11837, Egypt
[g] Department of Pharmacognosy, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), 6th of October, Giza 12566, Egypt

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ABSTRACT

Few studies reported the antifibrotic effects of gallic acid (GA) despite its known hepatoprotective and antioxidant activities. Accordingly, this study investigated the antifibrotic effects of GA through clarifying its mechanisms on hepatic stellate cells' (HSCs) activation, proliferation and/or apoptosis. *In vitro* effects of GA on HSC-T6 activation/proliferation, morphology and safety on hepatocytes were assessed. *In vivo*, hepatic fibrosis was induced *via* chronic thioacetamide (TAA)-intoxication. TAA-intoxicated rats were treated with silyamrin or GA. At end of experiment, liver functions, hepatic MDA, GSH, PDGF-BB, TGF-β1, TIMP-1 and hydroxyproline were determined. Histological analysis and Sirius red staining of hepatic sections, expressions of alpha-smooth muscle actin (α-SMA), proliferating cellular nuclear antigen (PCNA) and caspase-3 were examined. *In vitro*, GA resulted in a concentration and time-dependent inhibition in HSCs activation, proliferation (IC_{50}= 45 and 19 μg/mL at 24 and 48 h respectively); restored the quiescent morphology of some activated HSCs plus its safety on hepatocytes. *In vivo*, GA reduced ALT, AST, MDA, PDGF-BB levels, collagen deposition and fibrosis score (S1 vs S4); increased caspase-3 expression and restored GSH stores, TGF-β1 level, α-SMA and PCNA expressions. In conclusion, GA counteracted the progression of hepatic fibrosis through reduction of HSCs proliferation/activation mutually with their apoptosis induction.
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1. Introduction

Liver fibrosis, a major cause of morbidity and mortality worldwide, is a wound-healing response to repeated liver injury, driven by different causes such as chronic hepatitis, autoimmune disease, alcoholic and nonalcoholic fatty liver diseases.[1] It results in impaired hepatic regenerative capacity that ultimately leads to cirrhosis and hepatocellular carcinoma.[2]

Liver fibrosis is characterized by excessive deposition of extracellular matrix (ECM), culminating to major changes in liver architecture where ECM acts as a reservoir for pro-inflammatory and pro-fibrogenic mediators.[3] Hepatic stellate cells (HSCs) are the key fibrogenic effector cell type in the liver and the main ECM-producing cells.[4] When hepatic injury persists, HSCs become activated and transform into myofibroblast-like cells.[5] Activation of HSCs is promoted by tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), and reactive oxygen species (ROS) produced by apoptotic hepatocytes and Kuppfer cells, which in turn, secrete pro-inflammatory cytokines and further perpetuating an inflammatory state.[6]

* Corresponding author.
E-mail address: n.ellakkany@tbri.gov.eg (N.M. El-Lakkany).
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Regardless the vast comprehension on potential mechanistic targets for anti-fibrotic drug, clinical success has not yet been attained[7] revealing an extreme need for hepatic fibrosis treatment.[8] Interest in natural products and herbal medicines for treating liver fibrosis have increased,[9] due to their wide range of biological activities, high abundance and few adverse effects.[10] Despite their popularity for the treatment of hepatic diseases, data related to their mechanisms are still lacking.[8]

Edible fruits have also attracted substantial interest for containing several antioxidants and bioactive phytocompounds that may act as possible remedial agents.[11] *Punica granatum* L. (*Punicaceae*), commonly called pomegranate, a folkloric medicinal plant, contains large amounts of polyphenols and flavonoids in the fruit, juice and peels.[12] Peels of pomegranate have reported higher antioxidant activity than the pulp,[13] where gallic acid (GA) was found to be present in highest quantities followed by ellagic acid and quercetin.[14]

GA (3, 4, 5-trihydroxybenzoic acid), possesses many pharmacological properties including antioxidant,[15] anti-inflammatory[16] and anticancer properties.[17] Furthermore, GA exhibited protective effects against CCl4-induced hepatic damage in rats,[18] selectively induced apoptosis in tumor cells[19] and in lung fibroblasts.[20] Despite the role of GA in these diseases, yet few studies have documented its therapeutic effect on liver fibrosis.[21–23] Accordingly, this study seeks to investigate and correlate the antifibrotic effects of GA *in vitro* and in thioacetamide (TAA)-intoxicated rats through focusing on its effects on HSCs proliferation, activation and/or apoptosis.

2. Materials and methods

2.1. Plant material

Pomegranates (*Punica granatum* L.) were obtained from the Agricultural Research Center, Giza, Egypt. The plants were authenticated by Dr. Reem Samir Hamdy, Lecturer of Plant Taxonomy, Botany Department, Faculty of Science, Cairo University, Giza, Egypt. Voucher samples no (2014043) of the plants were deposited at the museum of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

2.2. Extraction and isolation of GA

The peels of pomegranates were manually removed, shade-dried and powdered to yield 1 kg, which was extracted with a Soxhlet extractor using methanol for 4 h according to the method of Singh et al.[24] The extract was filtered to remove the peels particles and then was concentrated under vacuum at 40 °C. The dried extract powder (300 g) was further used for isolation of GA. The extract was fractionated over a diaion HP−20 AG column, then elution was carried out using water and methanol in different ratios, and the solvent in each fraction was removed by evaporation under reduced pressure and monitored by TLC. The fraction containing GA (75% methanol in water v/v) was purified over several sephadex LH−20 columns using methanol or water-methanol (1:1 v/v) as eluent.

2.3. Experimental animals

Male Sprague-Dawley rats (Animal house, Theodore Bilharz Research Institute, Giza, Egypt) weighing 250−300 g were used in this experiment. The animal protocol was designed to minimize pain or discomfort to the animals. Rats were housed under an environmentally controlled room at 20−22 °C, 12 h light/dark cycle and 50−60% humidity with free access to food and water *ad libitum* throughout the acclimatization and experimental periods. All animals were euthanized by a lethal intraperitoneal injection of 10% chloral hydrate for blood and tissue collections. All the animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH 1985) and its amendments and were approved by the Institutional Review Board of TBRI (2942013).

2.4. HSC-T6 cell line, hepatocytes isolation and cell culture

In vitro experiments were performed on rat hepatic stellate cell line (HSC-T6) and primary hepatocytes. The rat HSC-T6, an immortalized rat liver stellate cell line, with an activated phenotype,[25] was a generous gift from Prof. Scott L. Friedman (Division of Liver Diseases, Icahn School of Medicine at Mount Sinai University, New York). Primary hepatocytes were freshly isolated from rats by a two-step portal collagenase perfusion of the liver as previously described.[26] On 96-well plates, both hepatocytes and HSCs were seeded in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C under a humidified atmosphere of 5% CO_2 and 95% air. A stock solution of GA was dissolved in a small volume of dimethyl sulfoxide (DMSO, equivalent to < 1% of the final volume), filtered through a 0.22 μm membrane and aliquots were stored at −20 °C protected from light.

2.4.1. HSC-proliferation/morphology and hepatocytes cytotoxicity assays

Micro cultures of 5×10^3 HSCs or hepatocytes were cultured in 96-well tissue culture plates (Nunc, Roskilde, Denmark) in 200 μL DMEM supplemented with 10% FBS. After 24 h, cells were treated with different concentrations of GA (0−300 μg/mL) for 24 and 48 h where cell survival ratios corresponding to untreated cells were examined. Each test was performed in triplicate. The antiproliferative effect of GA on HSCs proliferation was assessed using sulforhodamine base (SRB) assay.[27] Briefly, cells were fixed, washed and stained with the SRB dye. Then, the unbound dye was removed and the optical density was measured at 490 nm using a microplate ELISA reader (ELX 808-Biotek, USA). Moreover, HSC morphology was observed under phase-contrast microscope (EVOS® xl core cell culture microscope (Advanced Microscopy Group, USA). The antiproliferative activity of the GA was expressed in terms of the IC_{50} value.

Moreover, viability/cytotoxicity of GA on isolated rat hepatocytes was assessed using thiazolyl blue tetrazolium bromide (MTT)[28] where MTT solution (20 μL of 0.5 mg/mL) was added to each well and incubated for another 4 h at 37 °C. The MTT-formazan crystals produced by viable cells were dissolved by DMSO and the optical density corresponding to the amount of formazan generated was measured using an ELISA reader at 570 nm.

2.4.2. HSCs activation assay

HSCs activation was assessed by alpha-smooth muscle actin (α-SMA) expression according to the manufacturer's instructions (Santa Cruz Biotechnology, CA, USA).

2.5. Liver fibrosis induction and experimental design

Thirty-two male rats were randomly divided into four groups, 8 each. TAA was injected intraperitoneally (200 mg/kg, twice a week for 12 weeks) to groups of rats II, III, and IV, while group I was injected intraperitoneally with the same amount of saline and served as normal control. Rats in groups III and IV were administered silymarin (50 mg/kg)[29] and GA (50 mg/kg)[30] via oral gavage, respectively for 8 weeks starting from the 5th week of TAA-

intoxication where an apparent stage of fibrosis (S2) was recorded via histopathological examination of hepatic tissues. A pilot study was conducted at the beginning of the experiment to determine the safety of GA administration in normal rats via immunohistopathological examinations of hepatic tissues.

At the end of the 12th week after initiation of TAA injection, all rats were euthanized with a lethal intraperitoneal injection of 10% chloral hydrate. Blood samples were collected; sera were separated and stored at −80 °C for assessment of liver functions. Livers were harvested and divided into two portions; the first was fixed with formalin for histological, Sirius red and immunohistochemical examinations and the second was rapidly washed with 0.9% ice-cold saline and stored at −80 °C for assessment of oxidative stress, fibrosis markers and hydroxyproline (HP) content.

2.6. Biochemical assays

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined spectrophotometrically[31] using the commercially available kits (Spectrum, Egypt). The level of reduced glutathione (GSH) and the extent of lipid peroxidation (MDA) were estimated in liver homogenates according to the methods described by Ellman[32] and Ohkawa et al.[33] respectively.

2.7. Quantification of liver fibrosis markers

Tissue inhibitor matrix metalloproteinases, type 1 (TIMP-1), transforming growth factor-β1 (TGF-β1) and platelet-derived growth factor-B (PDGF-B) levels were measured in liver tissue homogenates by commercial ELISA kit (R&D system, MN, USA). The HP content was determined in liver tissue samples as described by Woessner.[34] Briefly, liver tissues (300 mg) were hydrolyzed in 6 N HCl for 18 h at 110 °C and aliquots were dried and incubated with 50% isopropanol and chloramine−T solution followed by reaction with *p*-methylaminobenzaldehyde (Ehrlich's solution) at 60 °C for 20 min. The absorbance was read at 540 nm by UV spectrophotometer (Optizen 3220 double beam spectrophotometer, Korea). The amount of HP was expressed as μg/g wet tissue.

2.8. Histopathological and immunohistochemical examinations

Liver specimens were fixed in 10% formalin and embedded in paraffin blocks. Tissue sections were stained with hematoxylin-eosin (H&E) (4-μm thickness) and Sirius red (20-μm thickness) for analysis of overall liver histology and collagen distribution respectively. Collagen was quantified using imaging analysis software (Axiovision L.E. 4.8; Carl Zeiss MicroImaging, Jena, Germany). Briefly, paraffin sections were stained in 0.1% Sirius red F_3B (SR) in saturated picric acid. The red-stained area (mm^2) was measured in five consecutive fields (x50) and a numerical scoring system for morphometric analysis of hepatic fibrosis score was used.[35] Briefly, fibrosis was graded into 4 stages: S0, no fibrosis; S1, expansion of fibrosis in portal area; S2, peripheral fibrosis in portal area with retention of intralobular architecture; S3, fibrous septum accompanied by intralobular structural disorders; S4, early hepatic cirrhosis.

Additionally, hepatic sections were immunohistochemically stained for caspase-3, proliferation cell nuclear antigen (PCNA) and α-SMA with a horseradish-peroxidase complex kit (Abcam Inc, UK). The percent of positively stained brown nuclei (PCNA)[36] or brown cytoplasm (α-SMA and caspase-3)[37] were examined in 10 microscopic fields (at x400 under Zeiss light microscopy, Jena, Germany).

2.9. Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed either by Student's *t*-test for comparison between 2 means or by the one-way ANOVA test followed by Tukey's *post hoc* test for multiple comparisons (GraphPad Software, San Diego, CA, USA, version 5.03). Differences were considered significant when p values < 0.05.

3. Results

3.1. Spectroscopic data and identification of GA

^{1}HNMR spectrum of GA compound revealed the resonance of two methine protons in the aromatic region at δ 7.07 p.m. (2H) assigned to H-2, 6. In addition, ^{13}CNMR spectrum revealed five quaternary carbons at δ 121.4 (C-1), 109.8 (C-2, 6), 146.0 (C-3, 5), 140.3(C-4) and 167.3 (C = O). Based on the spectroscopic data and by comparing to the published literature,[38] this compound was identified as GA.

3.2. Effect of GA on HSCs proliferation, morphology and hepatocytes viability

Our *in vitro* results showed that GA inhibited the proliferation of cultured HSCs in a concentration and time-dependent manner ($p < .05$) compared with the untreated cells as evidenced by 50% inhibition concentration (IC_{50}) of 45 and 19 μg/mL at 24 and 48 h respectively (Fig. 1A). Moreover, our results revealed that toxicity of GA was specific to HSCs as evidenced by safety of GA to hepatocytes even after prolonged (48 h) and high concentration exposure (up to 300 μg/mL) (Fig. 1B).

Under phase contrast microscope, untreated control HCSs (Fig. 2A) appeared flattened, elongated and longitudinally spindle-like with numerous fat droplets in their cytoplasm. Interestingly, GA treatment (Fig. 2B) caused differential morphological effects on activated HSCs. Some cells exhibited cellular swelling and disintegration and others retained back their quiescent morphological features. These morphological changes were accompanied by a decrease in α-SMA stained cells due to GA treatment (Fig. 2D) versus those of untreated control (Fig. 2C).

3.3. Effect of GA on liver functions and hepatic oxidative stress

Chronic TAA-intoxication resulted in a remarkable elevation ($p < .05$) in serum ALT and AST levels when compared to normal control, this was accompanied with a significant depletion in hepatic GSH stores as well as an increase in MDA level (Table 1). Silymarin and GA treatments caused a notable decrease ($p < .05$) in ALT and MDA with normalization of AST levels when compared to TAA-intoxicated group. Additionally, GA restored the hepatic GSH content (Table 1).

3.4. Effect of GA on liver fibrosis markers and HP content

TAA-intoxication resulted in a marked elevation ($p < .05$) in hepatic pro-fibrogenic cytokines (PDGF-BB and TGF-β1) levels, TIMP-1 and HP content when compared to their corresponding normal groups (Fig. 3). Treatment of TAA-intoxicated rats with silymarin or GA showed a significant decline ($p < .05$) in PDGF-BB, TIMP-1 and HP content when compared to TAA-intoxicated rats. Moreover, silymarin reduced TGF-β1 level whereas GA normalized its level.

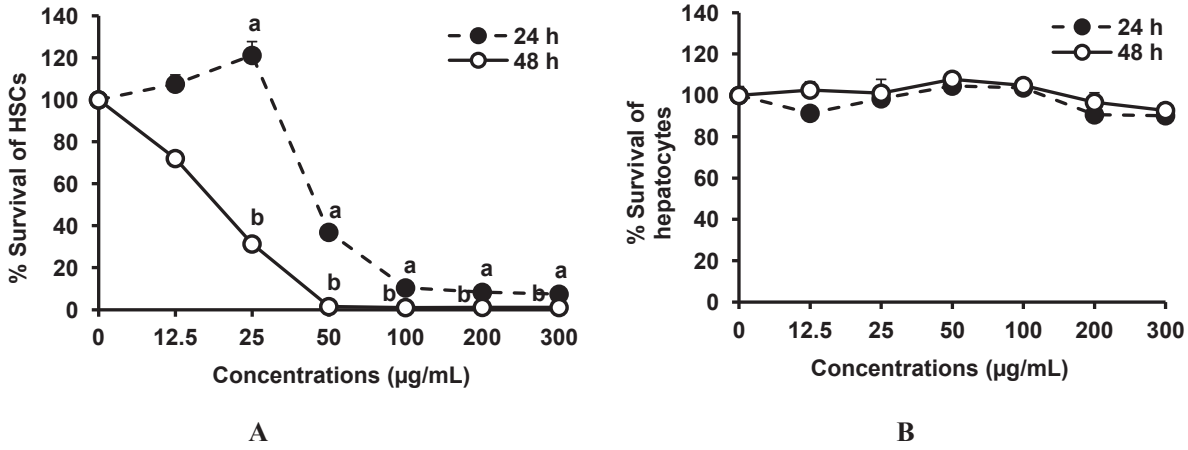

Fig. 1. Effect of various concentrations of GA (0–300 µg/mL) on HSCs (A) and hepatocytes (B). Statistical analysis was done using Student's *t*-test. [a] $p < .05$ vs control untreated cells at 24 h. [b] $p < .05$ vs control untreated cells at 48 h. GA, gallic acid; HSCs, hepatic stellate cells.

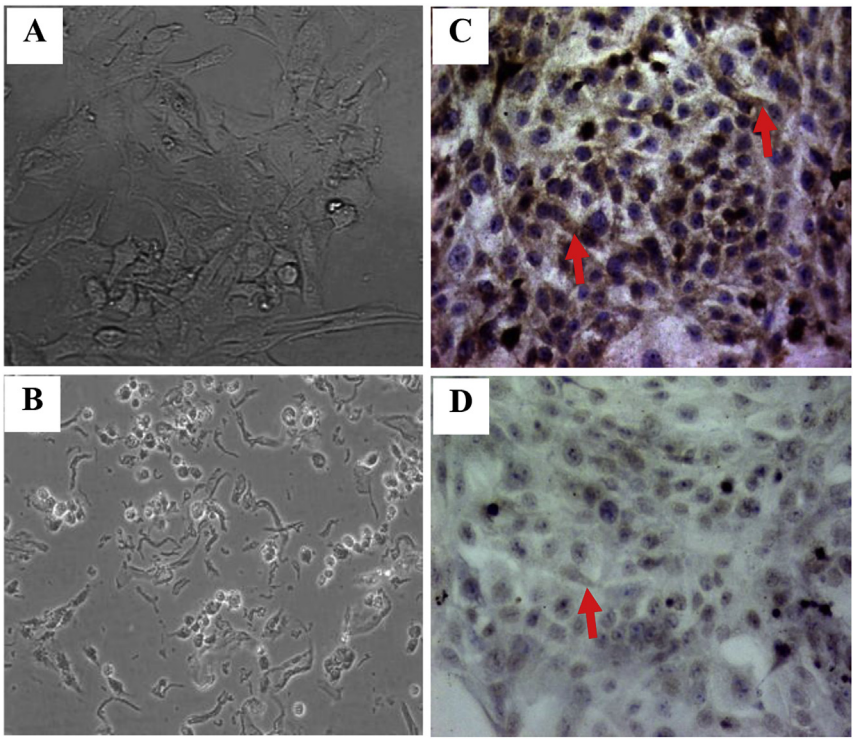

Fig. 2. Photomicrographs showing HSCs morphology of untreated control cells (A) and GA-treated cells (20 µg/mL, corresponding to approximately IC_{50}) for 48 h (B) observed under a phase-contrast microscope (x400). α-SMA immunoreactivity was observed in control activated HSCs (C) and GA-treated cells (D) (x400). The expression of α-SMA was estimated as the number of positively stained cells. HSCs, hepatic stellate cells; α-SMA: alpha-smooth muscle actin; GA, gallic acid.

Table 1
Effect of GA treatment on liver functions, oxidative stress markers and fibrosis score in TAA-induced fibrotic rats.

Animal groups	Liver Functions		Oxidative stress markers		Fibrosis score
	ALT (U/L)	AST (U/L)	GSH (mg/g liver)	MDA (µmol/g liver)	
Normal control	55.88 ± 1.99	122.38 ± 5.24	1.48 ± 0.10	18.22 ± 0.89	0.00 ± 0.00
TAA	107.63 ± 3.41[a]	161.75 ± 4.82[a]	0.47 ± 0.04[a]	63.30 ± 2.84[a]	3.50 ± 0.22[a]
TAA + Silymarin	74.63 ± 4.13[ab]	137.25 ± 4.69[b]	0.95 ± 0.06[ab]	40.89 ± 2.45[ab]	1.67 ± 0.33[ab]
TAA + GA	79.00 ± 3.51[ab]	139.00 ± 4.15[b]	1.22 ± 0.09[bc]	45.77 ± 3.25[ab]	1.17 ± 0.31[ab]

Data are presented as mean of 8 rats ± SEM. [a]$p < .05$ vs normal control group, [b]$p < .05$ vs TAA-intoxicated group, [c]$p < .05$ vs silymarin group.

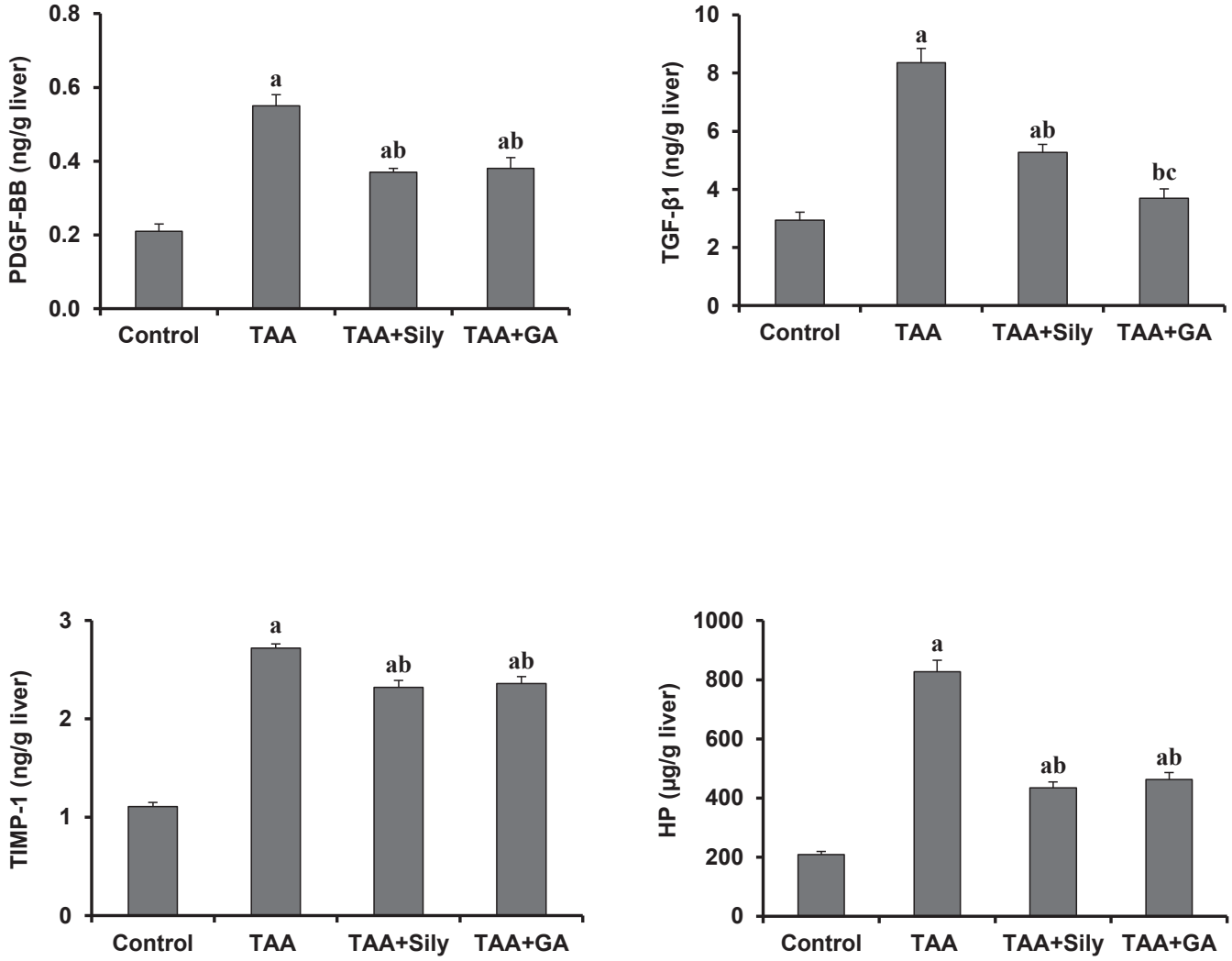

Fig. 3. Effect of GA treatment (50 mg/kg) daily via oral gavage for 8 weeks on profibrotic cytokines (PDGF-BB and TGF-β1), TIMP-1 and HP levels in liver homogenates. Data are expressed as mean (n = 6) ± SEM. [a] $p < .05$ vs normal control group, [b] $p < .05$ vs TAA-intoxicated group, [c] $p < .05$ vs silymarin group. Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparisons test. GA, gallic acid; PDGF-BB, platelet derived growth factor BB; TGF-β1, transforming growth factor-β1; TIMP-1, tissue inhibitor of matrix matalloproteinases type-1; HP, hydroxyproline; Sily, silymarin; TAA, thioacetamide; SEM, standard error of the mean.

3.5. Effect of GA on liver histology

Administration of GA to normal rats (in our pilot study) revealed that GA exhibited no toxicity signs on fibroblasts in portal tract, around bile ducts (periductal) and blood vessels as presented in Fig. 4. The Effect of GA on liver fibrosis grade and histopathological changes are shown in Fig. 5. Liver tissue of normal control rats showed normal lobular architecture with central veins, radiating hepatic cords and little collagen deposition. Meanwhile, liver tissue of TAA-group showed disrupted architecture with extensive damage of hepatocytes exemplified by centrilobular necrosis showing high fibrosis (S4) when compared to control rats. Moreover, collagen fibers were deposited around hepatic lobules mainly in the periportal regions appearing as thick fibrous septa generating micro and macronodules as confirmed by Sirius red staining. Treatment with silymarin showed better liver recovery with less damage in liver tissue (S2) as indicated by reduced levels of necrosis, hepatocyte degeneration and infiltration of inflammatory cells, with presence of mild thin fibrous bands. However, enhanced recovery was observed in GA-administered rats revealing intact hepatic architecture with almost normal hepatocytes (S1) together with notable decrease ($p < .05$) in collagen deposition when compared to silymarin-treated group.

3.6. Effect of GA on blockade of HSCs activation and induction of apoptosis

The degree of HSC activation was determined in liver tissues using IHC staining for α-SMA (Fig. 6), which was only expressed in the hepatic vascular smooth muscle cells of the blood vessels in normal liver. However, the livers of TAA-intoxicated rats displayed a substantial increase ($p < .05$) in α-SMA expression, which was concentrated in the centrilobular and periportal fibrotic areas. Silymarin treatment induced a notable decrease in α-SMA expression, whereas normalization in the expression of α-SMA in GA-treated rats was recorded.

Additionally, the number of caspase-3 positive cells (apoptotic cells) in the normal control group was extremely low (Fig. 6), meanwhile it was moderately elevated in the TAA-intoxicated group. Treatment with either silymarin or GA showed a marked increase ($p < .05$) in the number of apoptotic cells when compared to fibrosis group. However, the difference between the two treated

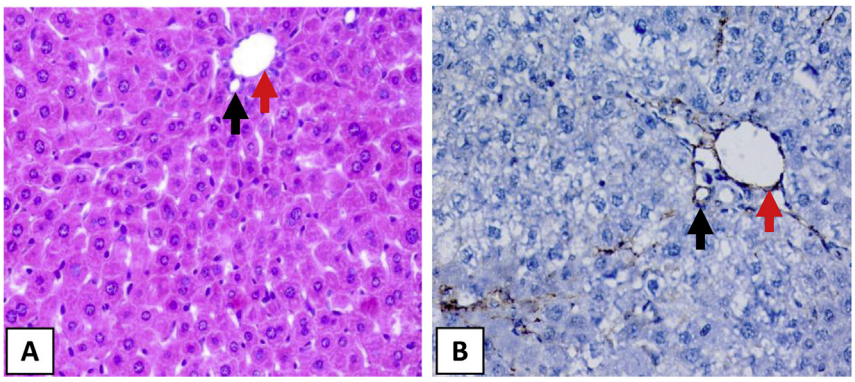

Fig. 4. Photomicrograph of hepatic sections of normal rats treated with GA (50 mg/kg) daily via oral gavage for 8 weeks (n = 6). (A) Liver sections stained with H&E (x400) showed normal hepatic architecture, almost normal portal tract, bile duct (black arrow) and blood vessels (red arrow). (B) Liver sections immunohistochemically stained α-SMA (x400) showed normal hepatic architecture, almost normal portal tract with positively stained periductal (bile ductal) fibroblasts (black arrow) and in the wall of blood vessels (red arrow). H&E, Hematoxylin and Eosin; α-SMA: alpha-smooth muscle actin; GA, gallic acid.

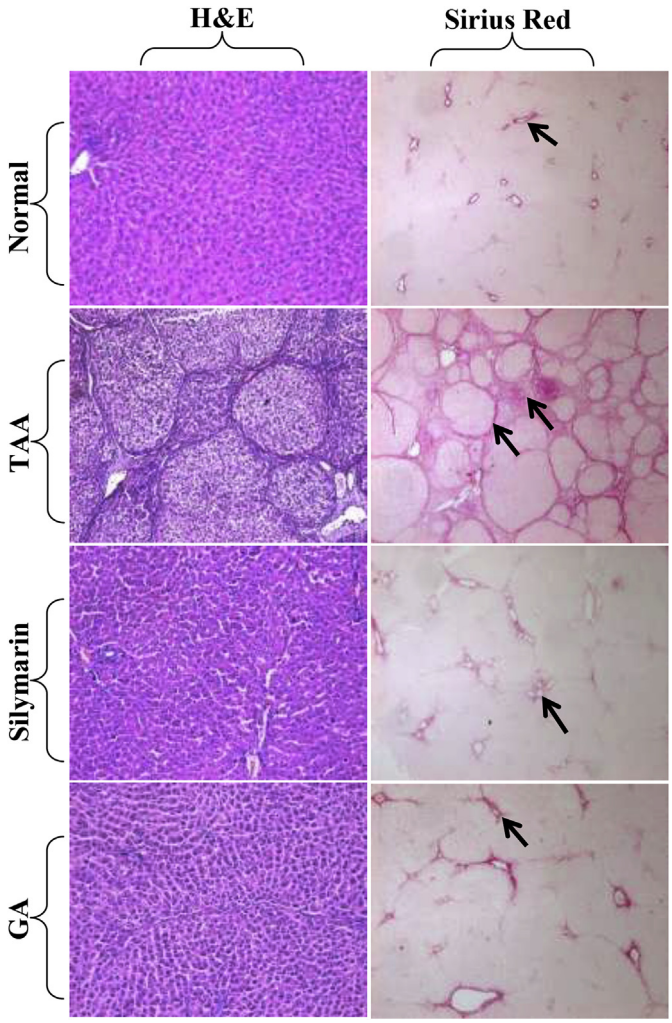

Fig. 5. Representative photomicrographs chosen from each experimental group (n = 6) showing liver histology for percent area stained with either of H&E (x200) and Sirius red (x5) in liver sections of normal; TAA-intoxicated (200 mg/kg intraperitoneally, twice a week for 12 weeks) rats; TAA-intoxicated rats treated with either silymarin (50 mg/kg) or GA (50 mg/kg) daily via oral gavage for 8 weeks starting from the 5th week post TAA intoxication (apparent stage of fibrosis S2). arrows = strands of fibrosis. H&E, Hematoxylin and Eosin; TAA, thioacetamide; GA, gallic acid.

groups was not significant. Positively caspase-3 stained cells were more apparent in portal and periportal areas around fibrotic septa (active HSCs) rather than in parenchymal cells (hepatocytes).

3.7. Effect of GA on proliferation of hepatocytes

The degree of hepatocytes proliferation was investigated using IHC staining for PCNA that was seldom positive in hepatocytes of normal control (Fig. 6). In contrast, PCNA expression was up-regulated in hepatocytes of the TAA-intoxicated animals indicating increased degree of proliferation, in an attempt to repair the damaged liver tissue. Although silymarin and GA-treated groups showed decreased PCNA expression, yet GA substantially lowered ($p < .05$) the expression of PCNA in the hepatocytes than silymarin, thus inhibiting the proliferation of damaged hepatocytes.

4. Discussion

HSCs play an important role in orchestrating hepatic fibrosis progression making them an appealing target for antifibrotic therapy, which upon injury are activated and acquire a myofibroblast-like phenotype accompanied by increased proliferation and ECM synthesis.[39] Suppression of HSC activation, proliferation and induction of their apoptosis has been proposed as therapeutic targets against hepatic fibrosis. Accordingly, this study focuses on the antifibrotic effects of GA, clarifying its possible mechanisms on HSCs through *in vitro* and *in vivo* investigations.

In this study, GA markedly inhibited the *in vitro* proliferation of activated HSCs in a time and concentration-dependent manner where almost 100% cytotoxic effects were observed at 50 μg/mL and higher concentrations (at 48 h exposure time), such cytotoxicity was specific to activated HSCs without showing any cytotoxicity signs to hepatocytes. This observed anti-proliferative activity might be related to the correlation between the dietary polyphenolic pro-oxidant activities and cytotoxicity,[40] where phenolic acids have been reported to exert antioxidant and pro-oxidant *in vitro* behavior.[41] Chang et al.[21] as well postulated that natural antioxidants such as GA can selectively induce oxidative damage and cytotoxic effects on HSCs through maneuvering its anti- and pro-oxidative capacity in hepatic diseases promoting cell death and inactivation of activated HSCs.[42] Moreover, some GA-treated HSCs recovered the quiescent stellate morphology accompanied with suppressed α-SMA expression that ultimately leading to blockade of HSCs activation. Such morphological change suggests that there might be a reorganization of the actin cytoskeleton of HSCs

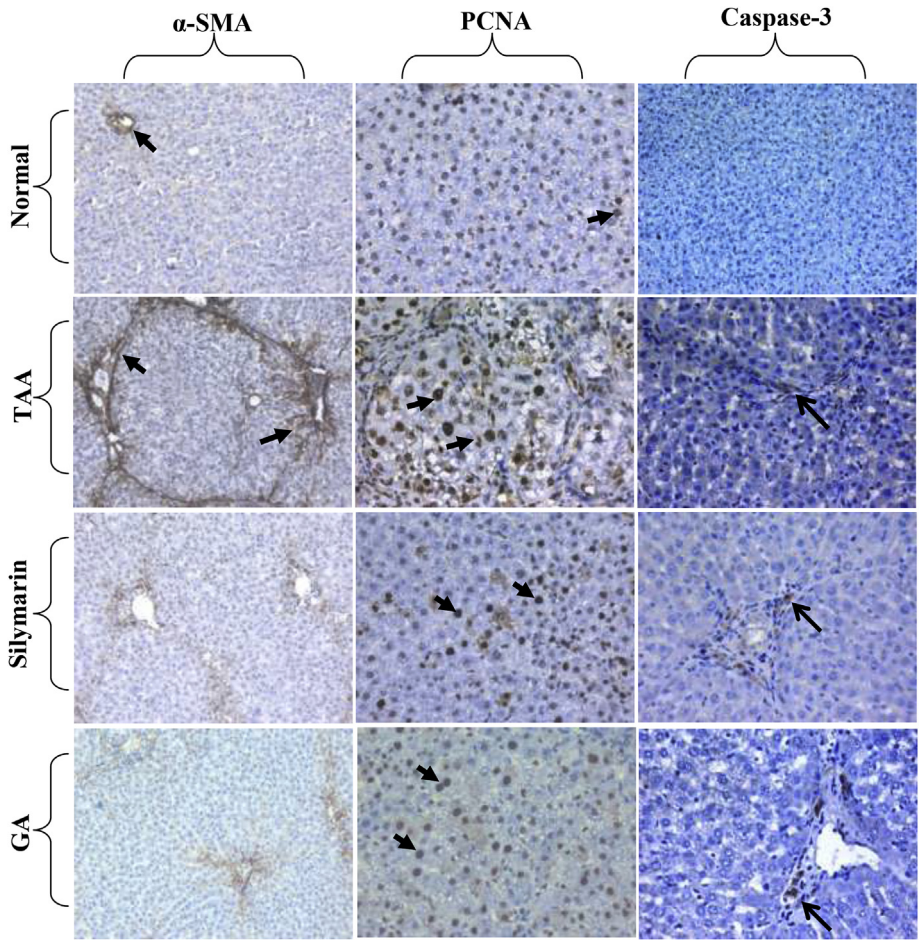

Fig. 6. Immunohistochemical examinations of expression of α-SMA (x200), PCNA (x400) and caspase-3 (x400) in liver sections of normal; TAA-intoxicated (200 mg/kg intraperitoneally, twice a week for 12 weeks) rats; TAA-intoxicated rats treated with either silymarin (50 mg/kg) or GA (50 mg/kg) daily via oral gavage for 8 weeks starting from the 5th week post TAA intoxication (apparent stage of fibrosis S2) (n = 6). The expression of α-SMA, caspase-3 and PCNA were estimated as percentage of positively stained cells (black arrows). α-SMA: alpha-smooth muscle actin, PCNA: proliferating cellular nuclear antigen, TAA: thioacetamide, GA: gallic acid.

inducing marked suppression of α-SMA expression.[43]

In the present study, the *in vivo* antifibrotic activity of GA was assessed using TAA-induced fibrosis model in rats where silymarin, a polyphenolic flavonoid, was used in this study as a reference drug, since it is one of the oldest and thoroughly researched plants in the treatment of liver diseases.[44] Although, silymarin's pharmacological and hepatoprotective profiles have been well investigated both *in vitro* and *in vivo*[45] yet, some clinical trials have indicated that the standard doses of silymarin were ineffective in many patients with chronic liver diseases, also some adverse effects as gastroenteritis have been reported.[46] Moreover, caution should be warranted with combined administration of silymarin and dietary polyphenolic compounds. Such co-administration could lead to undesirable side effects, through altering the metabolism of silymarin, which is metabolized mainly by CYP2C8 and CYP3A4.[47] It was reported previously that dietary polyphenols that interact with CYP3A4, could modify the metabolism of xenobiotics and drugs, and consequently change the active doses of prescribed medicines as well the nature of the prescribed compounds.[48] It was evidenced as well that GA have inhibitory activities on CYP3A family.[49] Therefore, the need for new and safe hepatoprotective candidates is quite apparent.[50]

Chronic TAA-intoxication, a hepatotoxin containing thionosulfur compound, is well known to induce hepatic damage and fibrosis through generation of ROS.[51] This was evidenced by disrupted hepatic architecture as exemplified by extensive damage and centrilobular necrosis with a fibrosis score of S4, typical of TAA-induced liver fibrosis. This finding was also accompanied with elevation in serum aminotransferases, exacerbation of lipid peroxidation and GSH depletion. However, GA-treatment counterbalanced TAA-induced liver damage as indicated by reduced lipid peroxidation and aminotransferases levels together with normalized GSH content. Such hepatoprotective effects could be related to the phenolic hydroxyl groups in GA, which are known to be potent in scavenging free radicals especially the OH-group at the para position to the carboxylic group.[52] These results were also supported by the histological findings, which showed intact hepatic architecture with almost normal hepatocytes.

Moreover, the free radicals resulting from TAA metabolism have been reported to initiate the activation of HSCs, which is a critical step in hepatic fibrosis.[53] HSCs activation is a complex process that requires the coordinated activation of multiple cellular signaling cytokines including TGF-β1 and PDGF.[54,55] PDGF is the most potent proliferative cytokine on HSCs[56] whereas TGF-β1 mainly affects the stimulation of ECM synthesis and deposition.[53] GA-treatment resulted in suppression of HSCs proliferation and activation, caused by TAA-intoxication, as denoted by down-regulation of PDGF-BB and attenuation of TGF-β1 levels. This was associated with remodeling of ECM deposition as represented by a decrease in TIMP-1 level and HP content, a major component of collagen,[57]

leading to a decrease in collagen deposition, which are main components of ECM.[58] These findings were complemented with reduction in α-SMA expression, a key marker of HSCs activation,[59] as elaborated through both *in vitro* and *in vivo* results denoting the ability of GA to reduce the activation of HSCs and hence controlling hepatic fibrosis progression.

Herein, GA treatment triggered the tendency of HSCs to undergo apoptosis *via* increasing caspase-3 expression, which could be linked to the recorded reduction in TIMP-1 levels. Over expression of TIMP-1 was reported to be correlated with failure of activated HSCs to undergo apoptosis leading to down-regulation of caspases.[60,61] However, GA treatment increased the susceptibility of HSCs to undergo apoptosis where activated HSCs were removed from fibrotic and cirrhotic livers, fortifying the contribution of apoptosis in the resolution of fibrotic changes.[62,63] Furthermore, GA-treatment inhibited the proliferative activity of damaged hepatocytes as manifested by reduction in PCNA staining when compared to TAA-intoxicated rats. This finding supported the idea that GA treatment did not only regulated HSCs activation but also the cell cycle progression of hepatocytes thus playing a positive role in liver regeneration.

5. Conclusion

Our findings demonstrated that the antifibrotic activities of GA could be related to the *in vitro* inhibition of the proliferation and activation of cultured HSCs together with reversion of the morphology of some of the activated HSCs to their quiescent stellate shape. GA as well ameliorated the progression of hepatic fibrosis *in vivo* through suppression of HSCs activation, proliferation and increasing their apoptotic tendency. Adding on, GA administration exerted superior effects over silymarin in terms of HSCs activation (TGF-β1 and α-SMA) and hepatocyte proliferation (PCNA). These outcomes would encourage further studies on the potential of GA as an alternative medication in treating liver fibrosis.

Conflict of interest

All authors declare that they have no conflict of interest.

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