ORIGINAL ARTICLE

RECORDS OF PHARMACEUTICAL AND BIOMEDICAL SCIENCES

Therapeutic efficacy of *Ganoderma Lucidum* on Thioacetamide-Induced Hepatic Fibrosis through Inhibition of TGF-β1 signaling in Male Rats

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Received on: 26. 11. 2020

Revised on: 22. 12. 2020

Accepted on: 24. 12. 2020

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Abstract

The anti-fibrosis efficacy of *Ganoderma lucidum* (GL) was investigated on thioacetamide (TAA)-induced liver fibrosis. Experimental fibrosis was induced by (200 mg/kg TAA, i.p.) twice weekly for 6 weeks in male Sprague-Dawley rats. GL was administered to TAA rats, either as (250/500 mg/kg, i.p) daily for further 3 weeks. Repeated administration of TAA caused liver fibrosis evidenced by significant elevation of hepatic TGF-β1 accompanied by an increase in the hydroxyproline content, oxidative stress levels and liver biomarkers. Contrarily, GL treatment significantly reduced ALT, AST, total bilirubin, MDA, NO concentrations and restored SOD activity. H & E and Masson trichrome staining confirmed that GL suppressed liver fibrosis, inflammation and attenuated the histopathological alterations. GL also elicited a statistical decrease in TGF-β1 level and hydroxyproline content with a concomitant decline in NF-kB and α-SMA immunoexpression in the TAA treated rats. Furthermore, GL downregulated TNF-α and IL-1β levels in TAA-treated rats compared to the control group. **Conclusion:** GL possesses a pronounced protective activity against TAA-induced fibrosis in rats; It significantly inhibits oxidative stress, inflammation and diminishes fibrosis by: inhibiting NF-kB activation, reducing TGF-β mediated by PI3K-AKT pathway thus restoring the liver function, the effect was in a dose dependent manner.

Keywords: *Ganoderma lucidum*; liver fibrosis; TGF-β1; 4-hydroxyproline.

1. Introduction

Liver fibrosis is a major global public health problem caused by several chronic liver diseases **(Li et al., 2019)**. It is defined as the remodeling and excessive deposition of extracellular matrix (ECM) proteins in liver **(Palmer et al., 2005)**. Liver fibrosis may progress into more severe stages known as cirrhosis, when liver acini are substituted by nodules and further to hepatocellular carcinoma. Although the main causes of hepatic cirrhosis may be associated with increased alcohol consumption, obesity, metabolic disorders, cholestasis, steatosis, viral infection and toxin accumulation **(Bataller and Brenner, 2005)**, poor lifestyle is also considered a risk factor **(Li et al., 2019)**. Therefore, much attention is required to prevent progression of liver fibrosis to cirrhosis.

Oxidative stress is an important primary factor which was extensively investigated in number of liver diseases as hepatic fibrosis. The latter is associated with reactive oxygen species (ROS) production, which is known to aggravate

inflammation and induce the pathogenesis of hepatic fibrosis **(Wang et al., 2013)**. ROS influence various biological processes including cell differentiation, gene expression and cytokine responses. Also, reactive nitrogen species (RNS) play a major role in the pathogenesis of various liver diseases and inducible nitric oxide synthase (iNOS)-derived NO is closely related to this process **(Iwakiri and Kim 2015)**.

Previous studies have shown that thioacetamide (TAA) causes cirrhosis in mice, the center of the injury is oxidative stress and hepatic stellate cells (HSCs) **(Kang et al., 2008)**. Lipid peroxidation of cell membranes and hepatocyte necrosis follow and result in an imbalance of the antioxidant defense system, which directly affects the HSCs and myofibroblasts comportment **(Li et al., 2015)**. In addition, some pro-fibrogenic mediators also stimulate the production of ROS in myofibroblasts and HSCs such as transforming growth factor-β1 (TGF-β1) resulting in exacerbated liver injury **(Krstic et al., 2015)**. TGF-β1 is a pleiotropic cytokine involved in extracellular matrix (ECM) production. It is known as the strongest effector in liver fibrosis, which acts as a major pro-fibrotic cytokine, promoting fibroblast recruitment, proliferation and differentiation into ECM-producing myofibroblasts **(Sheppard 2006)**. In fibrosis, hepatocyte necrosis or apoptosis occurs in hepatic tissue through Kupffer cell activation, eventually leading to cirrhosis **(Zhuo et al., 2014)**. TGF-β1 activates hepatic HSCs producing fibrosis through excessive deposition of ECM proteins, such as collagen **(Tsucada et al., 2006; Gressner et al., 2007; Schuppan, 2015)**.

Interestingly, it has been established that the progression of liver fibrosis is reversible. However, the major limitation is the lack of effective treatment strategies for liver fibrosis **(Benyon and Iredale, 2000; Issa et al., 2004)**. Therefore, novel and effective therapeutic targets are required for the treatment of liver fibrosis.

Ganoderma lucidum (GL) is a significant source of natural fungal medicines that had been used for the treatment of various diseases for many years and it has the ability to enhance body resistance **(Yu Cao et al., 2018)**. GL is widely used in China, America, Japan, Korea, and other countries. It is one of the most well-known medicinal species regarded as the "marvelous herb" **(Meng et al., 2011)**. Several studies reported that the polysaccharides isolated from GL have diverse biological activities including antitumor, anti-allergy, anti-inflammatory, antioxidant and hypoglycemic effects **(Li et al. 2015; Zhang et al. 2014)**.

Recently, **Hassan et al. (2020)** confirmed the efficacy of ganoderic acid in GL to suppress chemotherapy-induced hepatic injury experimentally in rats. According to traditional Chinese medicine (TCM) theory, the water or ethanol extracts of GL showed protective actions against acute hepatitis in rats and mice **(Lin et al., 1995)**. In addition, **Park et al. (1997)** demonstrated that the polysaccharides extracted from GL could antagonize liver fibrosis caused by biliary obstruction. Therefore, the aim of the current study is to examine the anti-fibrotic efficacy and the underlying mechanism of GL in attenuating TAA-induced liver fibrosis in rats.

2. Materials and Methods

2.1. Chemicals

Ganoderma lucidum was purchased from DXN Pharmaceutical SDN, BHD, Malaysia as a powder form. TAA and ABTS were purchased from Sigma Aldrich Chemical Co., St. Louis, MO, USA.

2.2. Animals

Sprague-Dawley rats were supplied from the animal house at the Delta University for Science and Technology. All experimental protocols were approved by The Research Ethics Committee of the Faculty of Pharmacy, Delta University for Sciences and Technology (FPDU 18/2020), which were in accordance with National Institute of Health guidelines for laboratory animal care (NIH publication No. 85-23, revised 2011). Thirty male Sprague-Dawley rats weighed 200 ± 30 g were kept under standard laboratory conditions, 12 hours light/dark cycle at temperature 23 ± 2 °C, free access to food and water. Animals were acclimated for one week to the laboratory environment before the start of the experiment.

2.3. Experimental design and treatment

For induction of fibrosis, rats were intraperitoneally injected (i.p.) with 200 mg/kg TAA in normal saline solution twice weekly for 6 weeks **(Lee et al., 2019; Li et al., 2019; El-Baz et al., 2020)** and then randomly allocated into three groups each of 6 animals. which received the following treatments:
TAA group: Received distilled water (500 µL/day,

i.p.) daily for further 3 weeks and used as fibrosis control group. **TAA+GLLD:** Received GL (250 mg/kg, i.p) dissolved in distilled water to a final volume of (500 µL/day, i.p.) for 3 weeks after TAA treatment and served as thioacetamide and GL low dose. **TAA+GLHD:** Received GL (500 mg/kg, i.p) dissolved in distilled water to a final volume of (500 µL/day, i.p.) for 3 weeks after TAA treatment and served as thioacetamide and GL high dose. **Control group:** Received normal saline solution (500 µL/day, i.p.) twice weekly for 6 weeks, then received distilled water (500 µL/day, i.p.) daily for further 3 weeks. **GL group:** Received normal saline solution (500 µL/day, i.p.) twice weekly for 6 weeks, then received (500 mg/kg, i.p) GL dissolved in distilled water to a final volume of (500 µL/day, i.p) daily for 3 weeks and served as GL control group. At the end of the experiment, blood samples were collected via puncture of the retro-orbital venous plexus under Pentobarbital (50 mg/kg i.p) anesthesia using capillary hematocrit tubes, allowed to stand, and sera were obtained via centrifugation at 4 °C, and frozen until further use. All animals were sacrificed by cervical dislocation. Liver tissues were collected and stored at−80°C for histological and other analyses.

2.4. Phytochemical analysis of GL

For the qualitative characterization of GL, a total phytochemical screening was performed to reveal the bioactive constituents of the mushroom powder-using methanol as solvent. In addition, quantitative total polyphenols and flavonoids contents were determined in our previous study **(Hassan et al., 2020)**.

2.5. Antioxidant activity screening of GL

The total antioxidant activity of GL was evaluated according to the method reported by **Lissi et al. (1999)**, it is a result of bleaching of 2,20-azino-bis(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) derived radical cation. The radical cation was derived from the reaction of ABTS (60 µL) with MnO_2 (3 mL, 25 mg/mL) in phosphate buffer solution (10 µM, pH 7.5 mL). After shaking the solution for a few minutes, it was centrifuged and filtered. The absorbance of the resulting green blue solution of the control (ABTS radical solution) was recorded at 734 nm (A (control), and the absorbance of (20 µL of 1 mg/mL) solution of GL was measured upon its addition to the ABTS solution in spectroscopic grade using MeOH/buffer (1:1 v/v). The decrease in the absorbance is expressed as percentage inhibition, which is calculated from the equation:

$$\% \text{ Inhibition} = [A(\text{control}) - A(\text{GL}) / A(\text{control})] \times 100$$

Ascorbic acid (20 µL, 2 mM) solution was used as standard antioxidant (positive control). A blank test was done using solvent without ABTS.

2.6. Assessment of biochemical parameters

Levels of alanine and aspartate transaminases (ALT and AST) and total bilirubin (T. bilirubin) were determined in serum using commercially available rat ELISA kits (MyBioSource, San Diego, USA) Cat. Nos. (MBS269614, MBS264975, MBS730053) respectively in accordance with the manufacturer's instructions.

2.7. Assessment of oxidative stress and antioxidant activity

Levels of nitric oxide (NO) in liver homogenates (10% w/v in 0.05 M phosphate buffer, pH 7.4) were determined using assay kit provided by (Biodiagnostic Company, Dokki, Giza, Egypt). Also, level of malondialdehyde (MDA) and superoxide dismutase activity (SOD) were assessed in liver homogenates using methods previously described by **(Satoh 1978; Baehner et al., 1976)** respectively.

2.8. Assessment of fibrogenic and inflammatory mediators

Liver tissue homogenates were used to determine hepatic levels of transforming growth factor-β1 (TGF-β1), interleukin-1β (IL-1β) and Tumor Necrosis Factor-α (TNF-α) proteins quantitavely using commercially available ELISA kits purchased from (MyBioSource, Cat. # MBS824788, San Diego, USA), (Elabscience Biotechnology Inc. Cat.# E-EL-R0012, USA) and (BioLegend Cat. # 438207, San Diego, USA), respectively, according to manufacturers' instructions.

2.9. Liver hydroxyproline contents

Hydroxyproline assessment was conducted as an indirect index of collagen content in the liver homogenates using a colorimetric assay kit (Elabscience Biotechnology Inc. Cat.# E-BC-K062-S) according to manufacturer guidelines. Concisely, hydroxyproline can produce oxidation

product under the action of oxidizing agent. The generated oxidation product can react with dimethylaminobenzaldehyde and the resultant burgundy measured at 550 nm using spectrophotometer. The concentration of hydroxyproline can be calculated according to **Toyoki et al. (1998).**

2.10. Histopathological and Immuno-histochemical assessments of liver tissue

Hepatic tissue samples were fixed in 4% paraformaldehyde for 72 hrs, embedded in paraffin wax, cut into 4 µm-thick sections then stained with hematoxylin and eosin (H & E) for histological assessment and Masson's trichrome stain for evaluation of the collagen deposition. Moreover, additional sections were used for immunohistochemical detection using primary antibodies for α-smooth muscle actin (α-SMA, 1:100, GeneTex, CA, USA) and nuclear factor-kappa B (NF-kB, 1:300, Santa Cruz Biotechnology Inc, CA,USA). The percentages of areas of fibrosis and positive protein immunostaining in liver tissues were assessed using ImageJ Software (National Institutes of Health, USA). All histopathological and immunohistochemical assessments were performed by two independent pathologists who were blinded to animal groups.

2.11. Statistical analysis

Data were shown as mean±SEM. Statistical analysis were carried out using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Statistical analyses and graphing were performed using GraphPad Prism 8 software (CA, USA). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effects of GL on liver functions, liver and body weights

Table 1 showed that TAA treatment (200 mg /kg) twice weekly for 6 weeks caused liver injury as indicated by elevated levels of liver enzymes. In TAA-treated rats, serum levels of ALT, AST, and T. bilirubin were significantly increased compared to the control and GL groups ($P < 0.0001$ for both groups). Contrarily, treatment of fibrotic rats with GL significantly decreased liver enzymes levels ALT, AST and T. bilirubin in a dose dependent manner. Furthermore, table 1 showed that TAA significantly increased relative ratio of liver weight/body weight compared to both normal and drug controls. Treatment of fibrotic rats with Gl remarkably attenuated such increase in a dose dependent manner.

3.2. GL ameliorated TAA-induced oxidative stress and restored anti-oxidant activity

Oxidative stress and antioxidant activity were assessed by determining the levels of hepatic MDA, NO, and SOD. As shown in Figure 1 (**a, b)**, levels of MDA and NO were significantly increased (+161%, +63.94%) in TAA-treated rats compared to those in control rats ($P < 0.0001$ for both). However, GL administration significantly reduced the levels of hepatic MDA and NO in the TAA-treated rats and these effects were more pronounced in GLHD group (-48.79%, -36.58%) at ($p<0.001$) than in GLLD (-15.07%, -30.55%) ($P < 0.05$) respectively as compared to TAA group. Moreover, the antioxidant activities were evaluated by determining SOD in liver tissues Figure 1 **(C)**.

Table 1. Effect of GL treatment on liver functions, body and liver weight in TAA treated rats.

	ALT activity (U/L)	AST activity (U/L)	T. Bilurubin (mg/dL)	Liver Wt. (g)	Body Wt. (g)	Relative liver Wt. (*100)
Control	48.05±2.41	154.62±3.32	0.60±0.04	12.28	240.5	5.11
GL	43.02±1.71	148.88±11.32	0.57±0.05	11.75	230.5	5.1
TAA	$88.33±2.25^{*@}$	$292.50±9.57^{*@}$	$1.16±0.06^{*@}$	14.85	210.5	7.05
TAA+ GLLD	$75.71±2.40^{\#}$	$241±13.01^{\#}$	$0.889±0.09^{\#}$	12.98	220	5.9
TAA+ GLHD	$70.39±3.75^{\#\#\$}$	$223.23±14.38^{\#\#}$	$0.758±0.05^{\#\#}$	12.41	225	5.52

Data are expressed as means±SEM. Results are considered significant when $P < 0.05$, indicated as $^{*}P < 0.0001$: compared to control group; $^{@}P < 0.0001$: compared to GL group; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$: compared to TAA group, $^{\$}P < 0.05$: compared to TAA+GLLD group. GL: *Ganoderma lucidum*, TAA: thioacetamide, TAA+ GLLD: thioacetamide and *Ganoderma lucidum* low dose, TAA+ GLHD: thioacetamide and *Ganoderma lucidum* high dose.

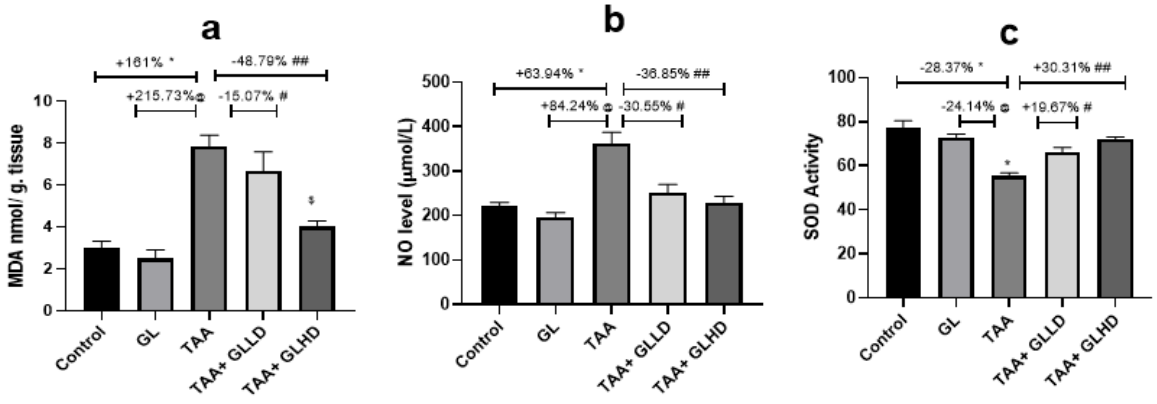

Figure 1. Antioxidant effects of GL on (**a)** MDA, (**b**) NO and (**c**) SOD levels in TAA-induced liver fibrosis in rats. Data are expressed as means±SEM. Results are considered significant when $P < 0.05$, indicated as $^{*}P < 0.0001$: compared to control group, $^{@}P < 0.0001$: compared to GL group, $^{\#}P < 0.05$, $^{\#\#}P < 0.001$: compared to TAA group, $^{\$}P < 0.05$: compared to TAA+GLLD group. MDA: malondialdehyde, NO: Nitric oxide, SOD: superoxide dismutase, GL: *Ganoderma lucidum*, TAA: thioacetamide, TAA+GLLD: thioacetamide and *Ganoderma lucidum* low dose, TAA+GLHD: thioacetamide and *Ganoderma lucidum* high dose.

TAA treatment significantly reduced SOD activities (-28.37%) and (-24.14%) compared to the control and GL groups ($P < 0.0001$ for both). However, SOD activities in liver tissue were significantly reversed by GL therapy considering that GLHD was statistically effective (+30.31) at ($P < 0.001$) more than GLLD (+19.76) at ($P < 0.05$) as compared to TAA group.

3.3. Antioxidant activity screening of GL

The data in table (2) showed that the total antioxidant content of GL elicited a progressive decrease in the concentration of the free radical, which was recorded as a reduction of the absorbance at 734 nm and expressed as percentage inhibition of the produced color.

Table 2. Antioxidant activity of GL

Compounds	% inhibition
Control of ABTS	0
Ascorbic-acid	88.3%
GL	58%

ABTS: 2,20-azino-bis(3-ethyl benzothiazoline-6-sulfonic acid).

3.4. Histopathological examination

Figure (**2**) displayed the ability of GL to protect against liver injury induced by TAA. Liver sections of the control and GL groups (**A, B**) stained with hematoxylin eosin (H&E) showed a normal histological structure of liver lobules. On the other hand, administration of TAA induced severe liver injury as reflected by hydropic to ballooning degeneration of hepatocytes, distended degeneration characterized by vacuolated cytoplasm with apoptotic nuclei (black arrowheads) and necrotic hepatocytes at the vicinity of nodules (red arrowheads) (**C**). Conversely, GL treatment significantly reduced pathological changes and preserved liver structure indicating mitigation of hepatic damage. These effects were improved by administration of GLLD which showed moderate hydropic degeneration of hepatocytes (**D**), whilst GLHD showed mild degeneration with almost normal hepatocyte architecture (**E**).

3.5. GL attenuated TAA-induced inflammatory response

The inflammatory responses were evaluated in liver tissues by determination of levels of pro-inflammatory cytokines including TNF-α, IL-1β and NF-kB. Figure 3 (**a, b**) showed that the levels of TNF-α and IL-1β in the liver were dramatically increased in the TAA-treated group compared to the control groups ($P < 0.0001$). However, GL treatment resulted in significant decrease in TNF-α and IL-1β concentration in TAA-treated rats liver tissues ($P < 0.05$) with respect to that GLHD effect was sharper than the GLLD. Moreover, immune-histochemical study showed a marked positive immune expression of NF-kB in the hepatic tissues of the TAA group compared to the normal and GL control groups ($P < 0.0001$) Figure 4 (**A-C**). Besides, hepatic tissue from TAA+GLLD group showed moderate positive NF-kB immunostaining in hepatocytes (**D**), while the TAA+GLHD group showed mild positive immunostaining against NF-

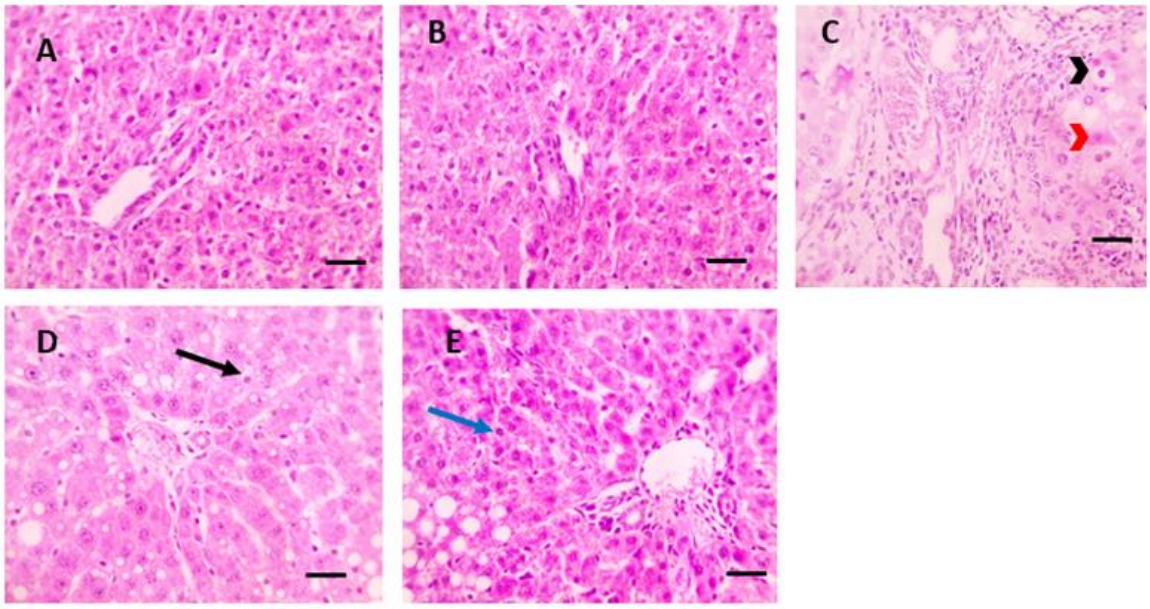

Figure 2. Effect of GL treatment in the liver tissues of TAA treated rats. Representative microscopic images of liver histology of hematoxylin-eosin (H & E) staining in: (**A**) Control group, (**B**) GL, (**C**) TAA group, (**D**) TAA+GLLD. (**E**) TAA+GLHD. Black arrowhead shows hydropic to ballooning degeneration of hepatocytes with vacuolated cytoplasm, red arrow head shows the necrotic hepatocytes on the periphery of the nodules (**C**). Treatment with GL preserved hepatic lobular architecture. Black arrow: portal infiltration with almost normal hepatocyte (**D**). Blue arrow: slight hydropic degeneration of hepatocytes (E). High magnification X: 400 bar 50. GL: *Ganoderma lucidum*, TAA: thioacetamide, TAA+GLLD: thioacetamide and *Ganoderma lucidum* low dose, TAA+GLHD: thioacetamide and *Ganoderma lucidum* high dose.

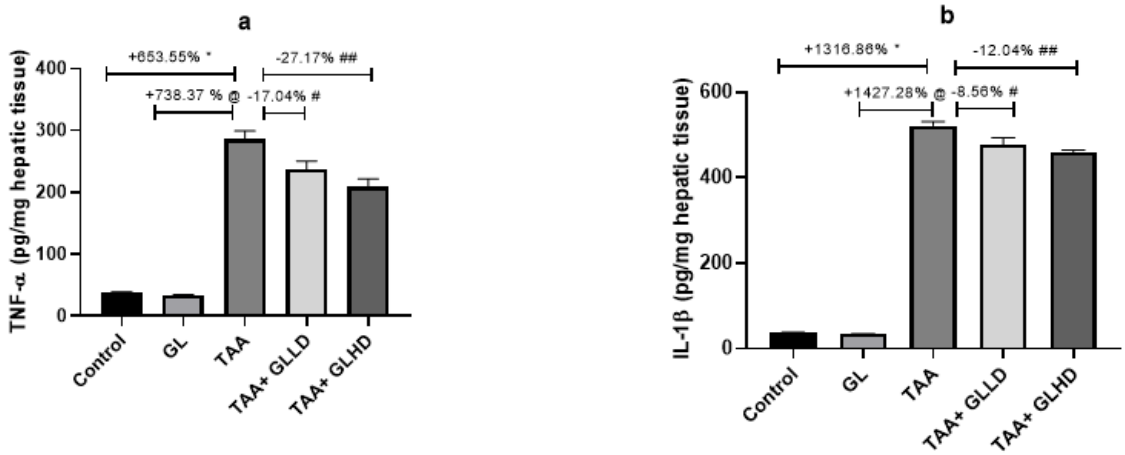

Figure 3. Anti-inflammatory effects of GL on hepatic levels of **(a)** TNF-α and (**b**) IL-1β in TAA-induced liver fibrosis in rats. Data are expressed as means±SEM. Results are considered significant when $P < 0.05$, indicated as $^{*}P < 0.0001$: compared to control group, $^{@}P < 0.0001$: compared to GL group, $^{\#}P < 0.05$, $^{\#\#}P < 0.001$: compared to TAA group. GL: *Ganoderma lucidum*, TNF-α: tumor necrosis factor alpha, IL-1β: interlukin-1 beta, TAA: thioacetamide, TAA+GLLD: thioacetamide and *Ganoderma lucidum* low dose, TAA+GLHD: thioacetamide and *Ganoderma lucidum* high dose.

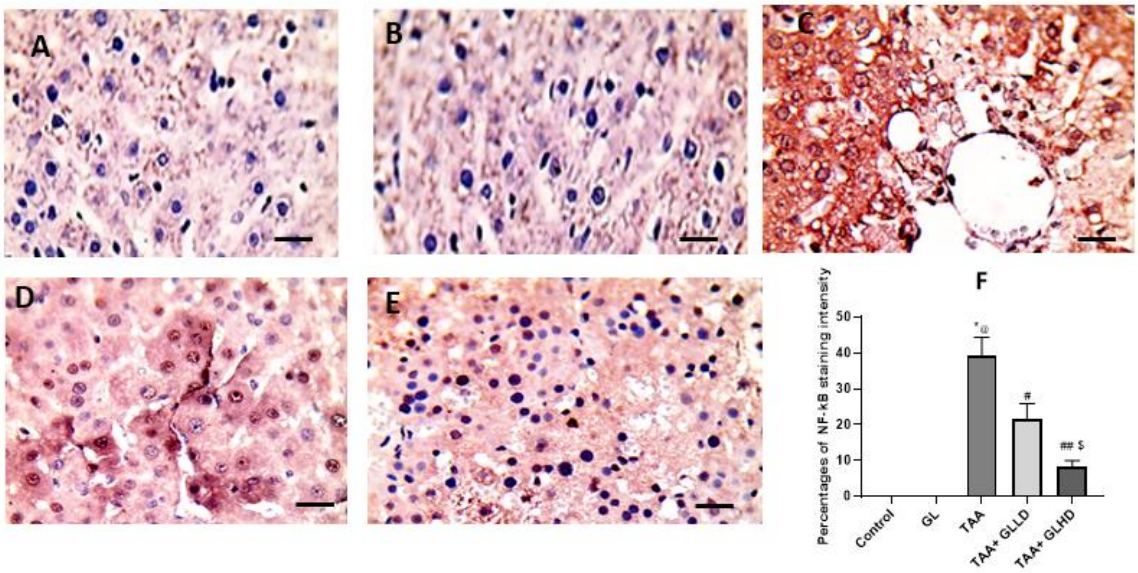

Figure 4. Effect of GL treatment on immuno expression of NF-kB in TAA-treated rats. Representative microscopic images of hepatic sections immunostained using NF-kB antibody from: (**A)** Control group, (**B**) GL group, (**C)** TAA group, (**D)** TAA+GLLD, (**E**) TAA+GLHD. Negative staining in normal and GL groups. Strong positive staining as indicated by intense brown color in TAA group, moderate positive staining TAA+GLLD, mild positive staining in TAA+GLHD. IHC counterstained with Mayer's hematoxylin. Images were observed using high magnification X: 400 bar 50. F: Statistical analysis of positive area of NF-kB protein expression. Results are considered significant when $P < 0.05$, indicated as $^{*}P < 0.0001$: compared to control group, $^{@}P < 0.0001$: compared to GL group, $^{\#}P < 0.01$, $^{\#\#}P < 0.0001$: compared to TAA group, $^{\$}P < 0.05$: compared to TAA+GLLD group. GL: *Ganoderma lucidum*, NF-kB: Nuclear factor kappa B, TAA: thioacetamide, TAA+GLLD: thioacetamide and *Ganoderma lucidum* low dose, TAA+GLHD: thioacetamide and *Ganoderma lucidum* high dose.

-kB in the portal regions (**E**) which strongly indicate anti-inflammatory activities in the hepatic rat livers extensively after GLHD administration, it is statistically presented in Figure 4 (**F**).

3.6. Effect of GL on hepatic fibrosis in TAA-treated rats GL

In this study, assessment of TGF-β1 level and 4-hydroxyproline content were used to determine the fibrosis severity and indicate the amount of collagen in the liver homogenates.

Figure 5 (**a**) showed that the hepatic level of TGF-β1 was dramatically elevated (+297.37%) after TAA-treatment as compared to the control group ($P < 0.0001$). However, GLLD treatment resulted in significant decrease (-10.49%) in TGF-β1 level in TAA-treated liver tissues ($P < 0.001$) at the same time, GLHD caused significant decrease (-24.44%) at ($P < 0.0001$) as compared to TAA group which suggest an anti-fibrotic effect of GL.

Figure 5 (**b**) showed that hydroxyproline content was significantly elevated in the TAA rats (+413.33%) as compared to control groups ($P <$ 0.0001). Nonetheless, GL treatment resulted in significant decrease in hydroxyproline concentration in TAA-treated liver tissues with the fact that GLHD effect was more distinct (-53.89%) than the GLLD (-43.04%).

3.7. Effect of GL on hepatic α-SMA expression, in TAA-administered rats

Figure 6 displayed a marked positive immune expression of α-SMA in the hepatic tissues of the TAA group compared to the normal and GL control groups (**A-C**). Further, hepatic tissue from TAA+GLLD group showed moderately positive α-SMA immunostaining in hepatocytes (**D**), while the TAA+GLHD group showed mild positive immunostaining against α-SMA in the portal regions (**E**) which powerfully indicate anti-fibrotic activities in the hepatic rat tissues widely after GLHD administration at ($P < 0.0001$) (**F**).

Our study also revealed that Masson's trichrome stained hepatic sections showed no histochemical reaction for collagen fibers deposition in control and GL groups Figure 7 (**A, B**).

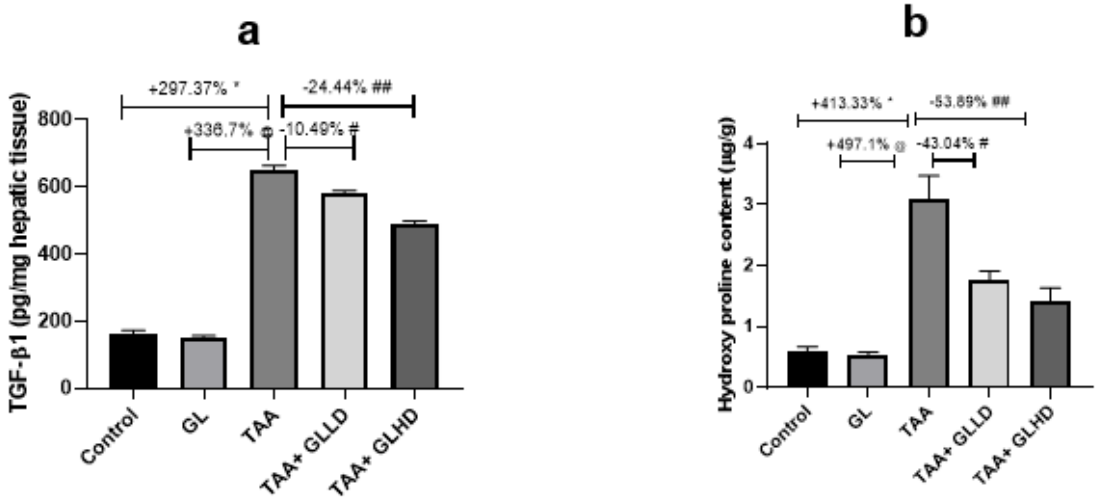

Figure 5. Effects of GL on hepatic levels of (**a**) TGF-β1, (**b)** 4-hydroxyproline content in TAA-induced liver fibrosis in rats. Data are expressed as means±SEM. Results are considered significant when $P < 0.05$, indicated as $^{*}P < 0.0001$: compared to control group, $^{@}P < 0.0001$: compared to GL group, $^{\#}P < 0.0001$, $^{\#\#}P < 0.001$: compared to TAA group. GL: *Ganoderma Lucidum*, TAA: thioacetamide, TAA+GLLD: thioacetamide and *Ganoderma lucidum* low dose, TAA+GLHD: thioacetamide and *Ganoderma lucidum* high dose.

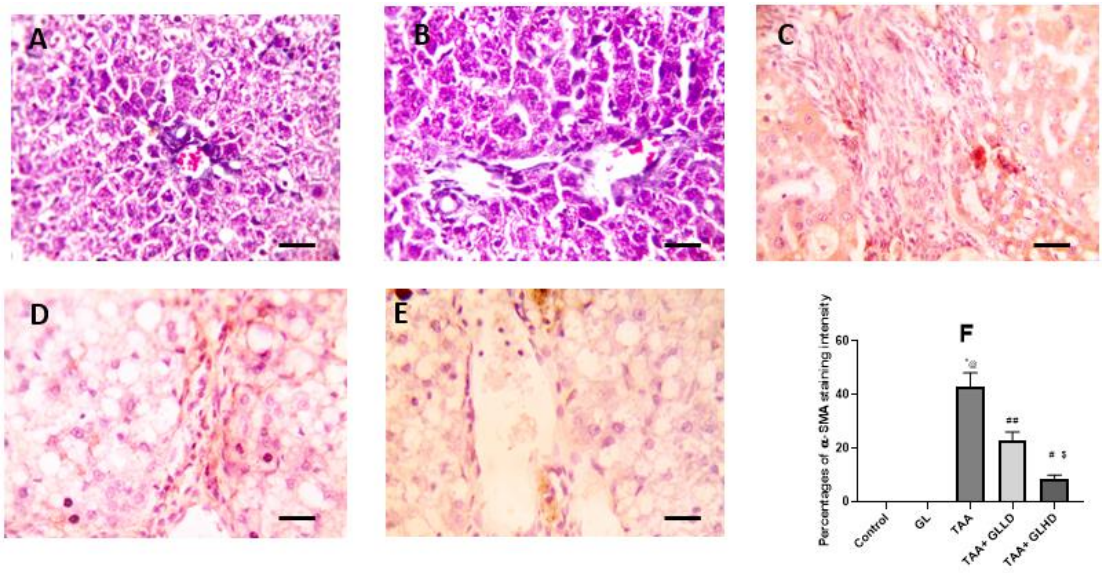

Figure 6. Effect of GL treatment on α-SMA protein expression in TAA-treated rats. Representative microscopic images of immunostained hepatic sections against α-SMA from: (**A**) Control group, (**B**) GL group, (**C**) TAA group, (**D**) TAA+GLLD, (**E**) TAA+GLHD. Negative expression in normal and GL groups. Hepatic sections from TAA group showed marked positive expression of α-SMA mainly around hepatic nodules. Moderate positive expression against α-SMA in portal areas in TAA+GLLD and weak positive expression of α-SMA in portal areas of TAA+GLHD were detected. IHC counterstained with Mayer's hematoxylin. Images were observed using high magnification X: 400 bar 50. Thin black arrows point to positive expression. Statistical analysis of positive area of α-SMA protein expression (**F**). Results are considered significant when $P < 0.05$, indicated as $^{*}P < 0.0001$: compared to control group, $^{@}P < 0.0001$: compared to GL group, $^{\#}P < 0.0001$, $^{\#\#}P < 0.001$: compared to TAA group, $^{\$}P < 0.05$, compared to TAA+GLLD. GL: *Ganoderma lucidum*, α-SMA: Alpha smooth muscle actin, TAA: thioacetamide, TAA+GLLD: thioacetamide and *Ganoderma lucidum* low dose, TAA+GLHD: thioacetamide and *Ganoderma lucidum* high dose.

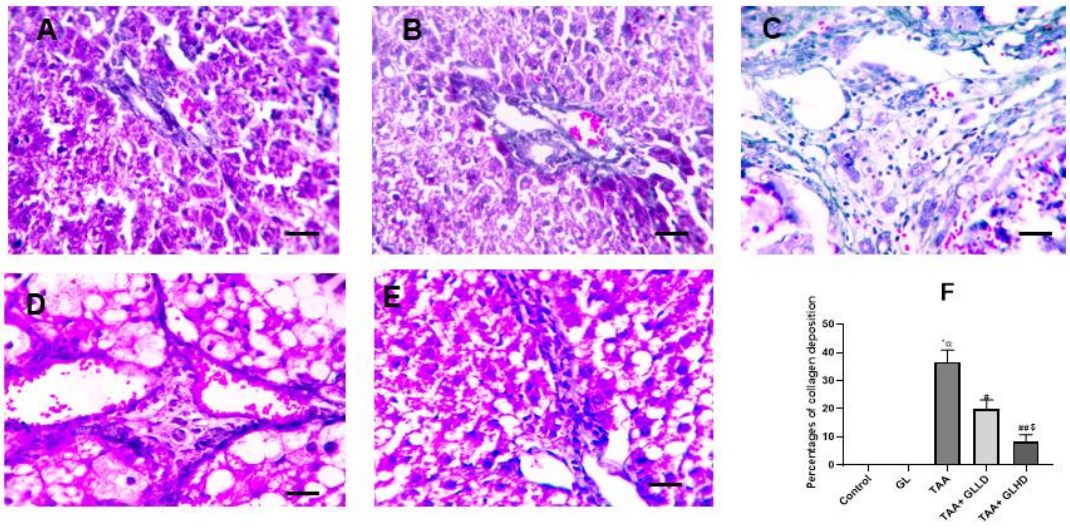

Figure 7. Effect of GL treatment on fibrosis in TAA treated rats. Representative microscopic images of Masson trichrome stained hepatic sections from: (**A**) Control group, (**B**) GL group, (**C**) TAA group, (**D**) TAA+GLLD, (**E**) TAA+GLHD. No histochemical reaction for collagen fibers deposition in control and GL groups (**A, B**). In contrast, hepatic sections from TAA group showed strong positive histochemical reaction for collagen fibers indicated by blue-stained fibrous tissues of disrupted hepatocytes. On the other hand, hepatic sections from TAA+GLLD group (**D**) showed moderate light blue stained fibrous tissue deposition in portal areas. Similarly, the TAA rats treated with GLHD showed mild to moderate light blue stained fibrous tissue deposition in hepatic portal area. Images were observed using high magnification X: 400 bar 50. Statistical analysis of percentages of area of collagen deposition in hepatic sections of the experimental groups (**F**). Results are considered significant when $P < 0.05$, indicated as $^{*}P < 0.0001$: compared to control group, $^{@}P < 0.0001$: compared to GL group, $^{\#}P < 0.01$, $^{\#\#}P < 0.0001$: compared to TAA group, $^{\$}P < 0.05$: compared to TAA+GLLD group. GL: *Ganoderma lucidum*, TAA; thioacetamide, TAA+GLLD: thioacetamide and *Ganoderma lucidum* low dose, TAA+GLHD: thioacetamide and *Ganoderma lucidum* high dose.

4. Discussion

In the current study, TAA remarkably triggered signs of liver dysfunction mimicking pathological symptoms of human liver disease which is evidenced by elevated serum levels of ALT, AST and T. Bilirubin. Our results were consistent with other studies **(El-Baz et al., 2019 and Al-Attar, 2011)**. In addition, TAA significantly raised hepatic levels of MDA and NO and suppressed SOD antioxidant activity in TAA-treated rats compared to the control group ($P < 0.0001$). These findings were in agreement with previous studies **(Lee et al., 2019; Metwaly et al., 2018).** Furthermore, **Schyman et al. (2018) and Sharma et al. (2019)** demonstrated that thioacetamide bioactivation is catalyzed by CYP450 isoenzymes, resulted in the formation of thioacetamide sulfur dioxide which may imply downregulation of enzymes involved in fatty acid β-oxidation, branched chain amino acids, methionine breakdown and upregulation of proteins related to lipid peroxidation and oxidative stress **(Low et al., 2004)**. Interestingly, treatment with GL significantly reduced hepatic MDA and NO levels and enhanced SOD antioxidant activity in a dose dependent manner. Moreover, hepatic levels of ALT, AST and T. bilirubin were significantly decreased with the fact that the state and function of bile secretion were restored in the GL-treated groups compared to the TAA group ($P < 0.001$). These results were consistent with previously reported findings **Lin and Lin (2006)** and **Hassan et al. (2020)**. Remarkably, our data revealed that the elevated antioxidant activity of GL (58%) may account for its role in attenuating hepatic antioxidant activity in rats. **Ferreira et al. (2015)** confirmed the radical scavenging abilities of polysaccharides and polysaccharide-complex isolated from different parts of the crude GL. Furthermore, in this study the histopathological examination proved that the severe liver injury as evidenced by vacuolated cytoplasm with apoptotic nuclei and necrotic liver cells induced by TAA administration had been improved upon treatment with GL, signifying mitigation of liver damage while preserving liver structure.

Hepatocyte apoptosis is known to be prominent in

liver disease and is a major step in most forms of liver injury **(Wang and Lin, 2012)**. A wide range of pro-inflammatory mediators including tumor necrosis factor and interleukin-1β activate NF-κB in HSCs, which functions as a key mediator of fibrosis. **(Hellerbrand et al., 1998; Wang et al., 2014)**. NF-κB activation is almost exclusively observed in HSCs, indicating that these cells are an important site of inflammation in a chronically injured and fibrotic liver **(Kluwe et al. 2010)**. In accordance, this study demonstrated elevated levels of TNF-α and IL-1β in TAA-treated rats compared to the control group (P <.0001). Likewise, the immune-histochemical assessment showed that NF-kB expression was dramatically increased in TAA-treated rats in comparison to the control group (P <.0001). Our data were in accordance with other studies **(Li at al., 2019; Lee et al., 2019; El-Baz et al., 2019)**.

This could be explained according to **Takehara et al. (2004)** who stated that after toxic exposure, hepatocytes undergo apoptosis and hepatic stellate cells migration to the site of injury to engulf the apoptotic bodies which promotes activation of the hepatic stellate cells to hepatic myofibroblasts promoting extracellular matrix deposition and scar formation in the liver. Therefore, progressive cirrhosis was associated with increased inflammatory cytokines. Thus inhibition of NF-κB results in decreased liver fibrosis **(Wang et al., 2014)**. Supporting this notion, our results revealed that, treatment with GL conferred a significant decrease in TNF-α and IL-1β levels with a concomitant reduction in NF-kB expression as compared to the TAA group.

Hydroxyproline was shown to be the only unique amino acid that is restricted for the synthesis of collagen fibrils in connective tissues. It was reported that during collagen degradation, the hydroxyproline content significantly correlated with fibrosis. Thus, hydroxyproline provides a characteristic biochemical marker in tissue, serum, and urine samples and could be used as a diagnostic marker for fibrotic scores especially in liver targets **(Gabr and sheriff, 2017)**. Thereafter, prolonged hepatocyte damage and HSCs were shown to play a pivotal role in liver fibrosis. Also, activated HSCs produce excessive amounts of ECM proteins such as collagen types, proteoglycans, fibronectin and laminin along with inhibitors of matrix metalloproteinase enzymes, resulting in an imbalance between fibrogenesis and fibrolysis and subsequent excessive deposition of matrix proteins. So, these cells have a significant role in progression of liver fibrosis stage to cirrhosis **(Spira et al.2002; Stalnikowitz and Weissbrod 2003; Lotersztajn et al. 2005)**. Our study revealed that TAA induced liver fibrosis as evidenced by significant increase in hepatic TGF-β1 and hydroxyproline levels which was accompanied by a strong positive histochemical reaction for collagen fibers indicated by blue-stained fibrous tissues of disrupted hepatocytes. Moreover, a marked positive immunoexpression of α-SMA was detected in TAA model as compared to control groups. On contrast, treatment with GL resulted in a statistical decrease in TGF-β1 and hydroxy proline level with a concomitant decline in α-SMA expression in the TAA treated rats which may account for the its favorable anti fibrinogenic activity. Recently, **Mi et al., (2019)** demonstrated that the downregulation of TGF-β, is mediated via the phosphoinositide 3-kinase (PI3K)-AKT signaling pathway, which leads to inhibition of inflammatory cytokine production in HSCs **(Wang et al., 2014)** that may explain the promising effect of GL in treating fibrosis.

5. Conclusion

The current study showed that the repeated administration of TAA caused serious liver fibrosis evidenced by significant elevation of hepatic TGF-β1 in parallel with an increase in the hydroxyproline content. Such effect may be related to enhanced oxidative stress and inflammation. GL possesses a pronounced protective activity against TAA-induced fibrosis in rats, it significantly inhibits oxidative stress, inflammation and diminishes fibrosis by inhibiting NF-kB activation, reduction of TGF-β mediated by PI3K-AKT pathway resulting in reduction in hydroxyproline content and α-SMA expression and restoring the liver function. The effect was in a dose dependent manner. These results indicate that GL can be used as an effective candidate in protection against liver fibrosis. Additional clinical trials to examine the therapeutic effects and to explore the detailed molecular mechanisms of GL as an anti-fibrosis dietary supplement.

Funding: No financial support was received.

Conflicts of Interest: The authors declare no conflict of interest.

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