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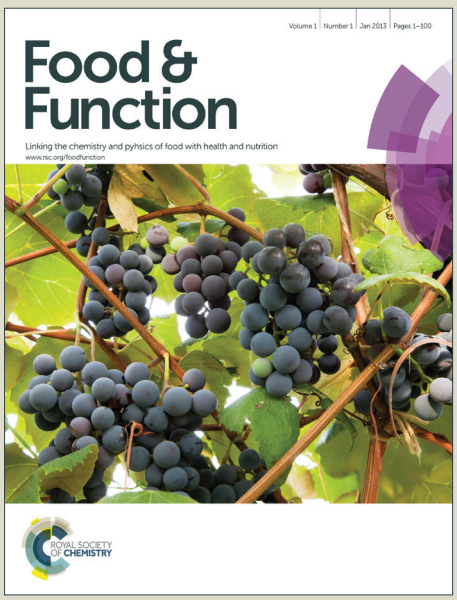

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Protective effect of theaflavin-enriched black tea extracts against dimethylnitrosamine-induced liver fibrosis in rats

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Running title: Theaflavin-enriched black tea extract inhibits DMN-induced liver fibrosis

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Abbreviations:

α-SMA, alpha-smooth muscle actin; DMN, dimethylnitrosamine; HCC, hepatocellular carcinoma; HSC, hepatic stellate cells; TF, theaflavin; TF2A, theaflavin-3-gallate; TF2B, theaflavin-3'-gallate; TFDG, theaflavin-3,3'-digallate; TF-BTE, theaflavin enriched black tea extract; TGF-β1, transforming growth factor- β1; TNF-α, tumor necrosis factor-alpha

Abstract

Liver cirrhosis is responsible to hepatic fibrosis resulting in high mortality and is also the risk factor in developing hepatocellular carcinoma (HCC), which is the fifth most common cancer in men and the seventh in women globally. Several studies have found effective anti-cancer activities of theaflavins, the major black tea polyphenols. The objective of this study was to investigate the protective effects of theaflavin-enriched black tea extracts (TF-BTE) on hepatic fibrosis induced by dimethylnitrosamine (DMN) administration in Sprague–Dawley (SD) rats. Treatment of SD rats with DMN (10 mg/ kg bw) for 4 weeks produced inflammation and remarkable liver fibrosis assessed by serum biochemistry and histopathological examination. Fibrotic status and the activation of hepatic stellate cells were improved by oral administration of 40% theaflavins in black tea extracts (40% TF-BTE) as evidenced by histopathological examination. Oral administration of 40% TF-BTE at low dose of 50 mg/kg bw/day and high dose of 100 mg/kg bw/day attenuated the DMN-induced elevation of serum GOT (glutamate oxaloacetate transaminase) and GPT (glutamic pyruvic transaminase) levels and reduced necrosis, bile duct proliferation, and inflammation. Western blot analyses revealed that TF-BTE inhibited the expression of liver alpha-smooth muscle actin (α-SMA) and transforming growth factor-β1 (TGF-β1) protein. The histochemistry examination showed the inhibitory effect of TF-BTE on the p-Smad3 expression. Overall, these data demonstrated that TF-BTE exhibited hepatoprotective effects on experimental fibrosis, potentially by inhibiting the TGF-β1/Smad signaling.

1. Introduction

Hepatic fibrosis is caused by a variety of chronic stimuli including alcohol intake, drug abuse, autoimmune and metabolic diseases, cholestasis, and hepatic viruses [1]. It further evolves into cirrhosis resulting in high mortality [2] and is a risk factor in the development of hepatocellular carcinoma (HCC) [3] that is the fifth most common cancer in men and the seventh in women [4] and worldwide ranks the third in cancer mortality behind lung and gastric cancer. Liver fibrosis associated with a number of pathological and biochemical changes leads to structural and metabolic abnormalities.[5] During the progression of liver fibrogenesis - a wound healing process, quiescent hepatic stellate cells (HSCs) proliferate and transform to myofibroblast-like cells.[2] These proliferating cells, also called activated hepatic stellate cells, secrete extracellular matrix (ECM) proteins including collagen type I and alpha- smooth muscle actin (alpha-SMA) [6]. The excessive accumulation of ECM protein is predominantly responsible for scarring following the series of inflammatory and fibrotic process which generate proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-alpha) and fibrogenic mediators such as transforming growth factor-beta 1(TGF-β1) [7;8]. TGF-β1 is a large group of growth factors that play important roles in regulating cell growth, differentiation and function. TGF-β1 initiates cellular responses by binding to and activating the specific cell surface receptors, and the activated TGF-β1 receptors (TβRI) generate the phosphorylation of the surface receptor regulated Smad proteins, which in turn form complexes with Smad 4 in the nucleus and regulate the transcription of the target genes [9]. TGF-β1 is considered as the most powerful mediator of HSC activation both in vitro and in vivo, and plays a central role in initiating fibrogenic cascade in liver [10]. Therefore, regulation of anti-inflammation and anti-fibrogenesis, perticularly inactivation of HSC and elimination of pro-fibrogenic signaling, is a promising strategy to prevent

further liver damage.

Dimetylnitrosamine (DMN) is a potent hepatotoxin, mutagen and carcinogen and widely used to induce liver damage in rats, which mimics the progression of liver fibrosis and cirrhosis in humans [11;12]. Specifically, DMN-induced liver fibrosis in rats reproduces most of the features of human liver fibrosis, such as ascites, nodular regeneration, overproduction of ECM including collagen and histopathological changes [13]. Liver histology in vivo study is the clinical gold standard for evaluating the degree of hepatic injury since it can also be used to follow morphological changes and pathophysiology of liver injury associated with liver damage.

Among tea category, black tea is the most popular tea beverage, accounting for 78% consumption worldwide. Transformed from green tea catechins through enzymatic catalyzed oxidation and chemical condensation, theaflavins are usually the main polyphenols responsible for unique color and taste of black tea infusion[14-16]. In the manufacturing process of black tea, there are four major theaflavins formed: theaflavin (TF1), theaflavin-3-gallate (TF2A), theaflavin-3'-gallate (TF2B), and theaflavin-3,3'-digallate (TFDG) [17]. Recently, theaflavins in black tea have received much attention in terms of bioactivity study on health benefits[15]. It also has been reported that theaflavins have stronger anti-oxidative properties than typical antioxidants such as glutathione, ascorbic acid, or tocopherol under certain conditions[18]. Reports in anti-inflammation [19;20] and cancer chemoprevention of theaflavins have been documented and studies on chemopreventive activity of theaflavins were based on several cell lines including liver cancer HepG2, colon cancer HT29, breast cancer MCF-7 and prostate cancer PC-3 [21-24]. Fibrosis is a dynamic process and may be reversible prior to become advanced architectural changes to liver[25]. Currently, there is no animal model based report showing the hepatoprotective potential of theaflavins, especially the anti-fibrotic activity. In

current study, we have investigated the anti-fibrotic effect of theaflavin complex on hepatic fibrosis induced by DMN administration in rats by biochemistry and histopathological examination and further elucidated the molecular mechanisms of liver fibrosis inhibition by theaflavins. Our results indicate that theaflavin enriched black tea extract (TF-BTE) attenuated HSCs activation by the inhibition of the α-SMA through the TGF-β1/Smad signaling inhibition.

2. Materials and Methods

2.1. Reagents and chemicals

The chemical structures of theaflavins are showed in Figure 1. Theaflavin (40%) enriched black tea extract (TF-BTE) was purchased from Jiangsu Dehe Biotechnology, Jiangyin, China. All reagents and chemicals were purchased from Sigma, Inc. (St. Louis, MO, USA) unless specified otherwise. *N*-Nitrosodimethylamine (dimethylnitrosamine; DMN) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). α-SMA and antibody were obtained from Epitomics, Inc. (Burlingame, CA, USA). β-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TGF-β, antibody were purchased from Transduction Laboratories (BD Biosciences, Lexington, KY, USA)

2.2. Tissue protein extraction and western blot analysis

Liver tissue from each rat was homogenized individually and total proteins of livers were extracted by using ice-cold gold lysis buffer [20 mM Tris–HCl, pH 7.4; 10 mM NaF; 137mM NaCl; 1 mM ethylene glycol tetraacetic acid (EGTA); 10% glycerol; 1% Triton X-100; 100 μM β-glycerophosphate; 1 mM $Na_4P_2O_7$; 5 mM ethylenediaminetetraacetic acid (EDTA); 1 mM Na_3VO_4 and 1 Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN)] to the cell pellets on ice for 30 min, followed by centrifugation at 10,000×g for 30 min at 4 °C. The total proteins were measured by Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany). The samples (50 μg of protein) were mixed with 5× sample buffer containing 0.3 M Tris–HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium dodecyl sulfate (SDS), 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue. The mixtures were boiled at 100 °C for 5 min and were subjected to 10% SDS–polyacrylamide minigels at a constant current of 20 mA. Electrophoresis was then carried out on SDS–polyacrylamide gels.

Proteins on the gel were electrotransferred onto an immobile membrane (PVDF; Millipore Corp., Bedford, MA) with transfer buffer composed of 25 mM Tris–HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membranes were blocked with blocking solution containing 20 mM Tris–HCl, and then immunoblotted with primary antibodies including α-SMA, TGF-β1, and β-actin at room temperature for 1 h. The blots were rinsed three times with PBST buffer (0.2% Tween 20 in 1 × PBS buffer) for 10 min each. Then blots were incubated with 1:5000 dilution of the horseradish peroxidase (HRP)-conjugated secondary antibody (Zymed Laboratories, San Francisco, CA, USA) and then washed again three times with PBST buffer. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.3. Animals and treatment

Thirty-two male Sprague–Dawley rats (4 weeks of age), weighing 200–250 g, were purchased from BioLASCO Co. (Taipei, Taiwan). All animal experimental protocols used in this study were approved by Institutional Animal Care and Use Committee of the National Kaohsiung Marine University (IACUC, NKMU, #099-AAA9-02, validity dates: 08/01/2009–07/31/2012). Procedures were realized according to Taiwan law on care and use of laboratory animals. The animals were housed in a humidity-controlled room at 25 ±°C with a 12-h dark/light cycle with free access to MFG diet (BioLASCO Co., Taipei, Taiwan) and distilled water ad libitum throughout the study. After one-week of acclimation, the animals were randomly assigned into three DMN-treated groups and one control group with eight rats in each group. The DMN-treated animals were administered DMN (10 mg/kg body weight) via *i.p.* injection on Monday, Wednesday, and Friday for four consecutive weeks. Control, untreated animals were given an equal volume of normal saline. Two DMN groups

were also administered 50 mg and 100 mg 40% TF-BTE per kg body weight, respectively, by gavage feeding daily. At the end of the study period, all animals were sacrificed under CO_2 anesthesia. Blood was collected by cardiac puncture and serum was harvested and stored at -80 °C until analysis. After rinsing with normal saline, the weights of livers, spleens, and kidneys were recorded. The liver samples were either immediately frozen in liquid nitrogen and kept at -80°C for further analysis or fixed with 10% buffered neutral formalin and embedded in paraffin for histological examination.

2.4. Histopathological examinations

Liver tissue sections (3 μm thickness) were measured for the portal inflammation and collagen distribution by hematoxyline and eosin (H&E) and Sirius satin, respectively. The right lobe of the liver was sliced, and tissue slices were fixed in 10% buffered neutral formalin for 24 h. The fixed liver tissue slices were embedded in paraffin, sectioned, deparaffinized, and rehydrated using standard techniques. Sections of 3 μm in thickness were subjected to H&E and Sirius red staining. An arbitrary scope was given to each microscopic field viewed at a magnification of 100. A minimum of 10 fields were scored per liver slice. The extent of fibrosis was graded as 0 (no increase), 1 (slight increase), 2 (moderate increase), 3 (distinct increase), or 4 (severe increase).

2.5. Immunohistochemical staining of α-SMA

Three micrometer sections of liver were deparaffinized, rehydrated and treated with hydrogen peroxide (H_2O_2) for 15 min to block endogenous peroxidase. Sections were pressure cooked (4 × 7 min) in 10 mM citrate buffer, pH 6.0 (Immuno DNA retriever with citrate, BIO SB, Santa Barbara, CA) to unmask epitopes. Sections were incubated with primary antibody to α-SMA (1:100 dilutions in phosphate-buffered

saline) for 1 h. Immunoreactivity was determined using biotin-labeled secondary antibody and streptavidin–biotin peroxidase for 30 min each. 3,3'-Diaminobenzidine tetrahydrochloride (DAB) was used as the substrate, and positive signal was detected as a brown color under a light microscope. The detailed procedures for the stained tissue analysis method were reported previously [26]. For α-SMA, the criterion for positive expression was membrane staining. For the immunoreactive score, the scores for the percentage of positive cells and the staining intensity were multiplied.

2.6. Biochemical analysis of liver function

Liver function was assessed by the serum levels of aspartate transaminase (AST), alanine transaminase (ALT), triacylglycerol (TG), and total cholesterol (T-chol). Briefly, serum was spotted onto respective Fujifilm Dri-Chem slides (Fujifilm, Kanagawa, Japan) and each biochemical indicator was determined using a blood biochemistry analyzer (Fujifilm Dri- Chem 3500s; Fujifilm, Kanagawa, Japan) according to the manufacturer's instructions.

2.7 Statistical analysis

Values are presented as means ± standard deviations for the number of experiments indicated. Significant differences were statistically detected by a one-way analysis of variance (ANOVA), followed by using Duncan's test. Results were considered statistically significant when $p < 0.05$.

3. Results

3.1 Body and organ weights of normal, DMN-treated, and combined DMN- and TF-BTE- treated rats

The body weight of each rat was monitored weekly. Food and water intake were measured 3 times per week. As shown in **Figure 2**, after 2 weeks, the body weight of rats treated with DMN was significantly decreased compared with both the control (DMN-untreated animal) and TF-BTE treated rats. This phenomenon is in agreement with the previous result [27;28] that DMN treatment may cause appetite reduction resulting in the decrease of average body weight of the animals. We also found that the body weight of the rats treated with TF-BTE alone was lower than the control which might be due to the effect of the tea polyphenol. These experimental data support that TF-BTE inhibits the growth and suppresses lipogenesis. The molecular mechanisms of fatty acid synthase gene suppression by tea theaflavins may attribute to the down-regulation of EGFR/PI3K/Akt/Sp-1 signal transduction pathways [29]. The body weights of rats treated with TF-BTE at low dose (50 mg/kg bw/day) and high dose (100 mg/kg bw/day) were not significant different and also not much different from the DMN treated rats. These results o indicated that 40% TF-BTE complex in combination with DMN treatment had no effect on the body weights of rats. We also examined liver, kidney and spleen weight to evaluate if these modulated treatments have undesirable side effects on the body. The results were shown in Table 1.The liver weight of DMN treated rats was significantly lower than that of the untreated animals. Renal toxicity may not be reflected from the treatments because the kidney weight from all treatments was not significantly different ($p<0.05$). The spleen weights of DMN treated rats were significantly higher than those in the control group, but were not significant different from those of the DMN-treated groups combined with TF-BTE at both high and low dose animals. These results suggested that the dosage of

TF-BTE had no impact on the weight of the kidney nor on the spleen of the DMN-treated animals. However, it should be reminded that in evaluating the effect of compound on splenic toxicity, the weights should always be interpreted in conjunction with histopathologic study because of the inherent variability in lymphoid organ weights [30]. Figure 3 showed the comparative liver organ sizes of animals. In animals treated with DMN, smaller liver size and deposition of collagen were observed, however TF-BTE administration caused significant reduction of collagen, and rendered the same normal liver size as the untreated animals.

3.2 Physical and biochemical characteristics

The serum biochemical indicators for liver inflammation, GOT (glutamate oxaloacetate transaminase) and GPT (glutamic pyruvic transaminase) are enzymes released into the bloodstream when the liver is injured. The hepatic serum concentrations of GOT and GPT (also known as AST and ALT) were significantly increased after 4 weeks of DMN treatment compared with untreated rats ($p < 0.05$) (Table 2). The combination treatment with DMN and TF-BTE slightly decreased serum GOT and GPT levels compared to the DMN-treated group ($p < 0.05$), but the difference was not statistically significant in terms of GOT level. TF-BTE dose dependence was observed for the significant reduction ($p < 0.05$) of GPT level induced by DMN. There were no effect was shown in the serum TG or T-chol levels. The specific pathological changes including fatty liver change, necrosis, bile duct proliferation and inflammation were detected to see the effect of TF-BTE on liver injury in terms of the liver injury score as indicated in Table 3. In comparison, the injury score of necrosis, bile duct proliferation, and inflammation of the DMN-treated rats were significantly ($p < 0.05$) higher than the control rats. TF-BTE at high dose (100 mg/kg bw/day) significantly reduced the injury score of necrosis, bile duct

proliferation and inflammation, implying that the improvement of DMN-induced hepatic fibrosis by TF-BTE treatment may result partially from attenuation of hepatocytes injury, because liver fibrosis is not only the result of derangements in the synthesis and degradation of matrix, but also the result of the liver injury.

3.3 TF-BTE complex inhibited liver fibrosis development and HSC activation

DMN-induced liver injury results in the activation of quiescent hepatic stellate cells (HSCs) and the proliferation of myofibroblast-like cells that cause liver fibrosis [31]. Activated HSCs are associated with cell proliferation and the accumulation of ECM proteins, including α-SMA and collagen type I and III. We therefore evaluated the protective effect of TF-BTE on DMN-induced histopathological changes in liver tissues. The liver sections were histopathologically and histomorphometrically examined by Sirius red (SR) and Hematoxylin/eosin (H&E) staining. In contrast to the control group of rats (Figure 4A and 5A), the DMN-treated group showed widespread destruction of liver architecture and enhancement of collagen I as indicated in the arrows (Figure 4B and 5B). Oral administration of TF-BTE significantly attenuated the deposition of collagen fibers (Figure 4 C and 5C) in a dose dependent manner. The high doses of TF-BTE groups (Figure 4D and 4D) had a stronger inhibitory effect than the low dose of TF-BTE. These results suggested the ability of TF-BTE to reduce the collagen synthesis. Immunohistochemical staining (Figure 5B) revealed that chronic DMN-treated liver exhibited the increase expression of α-SMA protein level and collagen as indicated by the arrow. The TF-BTE treated groups showed very weak α-SMA and collagen, suggesting that TF-BTE treatment effectively inhibited the activation of HSCs induced by DMN (Figure 6C and 6D). We examined the effects of TF-BTE on the levels of alpha-SMA expression in liver tissue western blotting analysis. As shown in Figure 10, the expressions of α-SMA were

315 increased in DMN-treated groups compared with the control group, consistent with
316 DMN-induced hepatotoxicity. Similarly, the levels of alpha-SMA were decreased by
317 co- administration of TF-BTE in a dose dependent manner. These data suggest that
318 TF-BTE complex from black tea can reduce DMN-induced expression of alpha-SMA
319 through inactivated HSCs.
320

321 **3.4. Inhibitory effects of TF-BTE on DMN-induced expression of TGF-β1**

322 Transforming growth factor-β1 (TGF-β1) is one of the most important cytokines play
323 a crucial role in HSCs turnover in the fibrotic process [32]. In response to activated
324 TGF-β1, the Smad-group of proteins has been shown to be specifically activated by
325 phosphorylation of receptor regulated Smads including Smad2 and Smad3 which
326 finally translocate to the nucleus, where they regulate transcription of target gene
327 expression such as collagen type I [33]. Recent studies revealed that Smad3, but not
328 Smad2, is a key signaling pathway of fibrogenesis in response to many fibrogenic
329 mediators such as TGF-β1 [34]. Immunohistochemistry analysis by IHC staining
330 showed the expression of TGF-β1 and the induction of p-Smad 3 of the rat livers
331 treated with DMN for 4 weeks due to the hepatic fibrosis development (Figure 7B and
332 8B). Oral administration of 40% TF-BTE significantly attenuated the TGF-β1
333 expression and the induction of p-Smad 3 of the rat livers. We further confirmed the
334 effects of TF-BTE on the levels of induced p-Smad 3 by TGF-β1 expression in liver
335 tissue by western blotting analysis. As shown in Figure 9, the expression of TGF-β1
336 was increased in DMN-treated group compared with untreated group, compatible with
337 DMN-induced hepatotoxicity. Levels of TGF-β1 decreased compared with those of
338 the DMN-treated group when TF-BTE was co-administrated (Figure 7C and 7D). The
339 data reported herein strongly suggested the suppressive activity of TF-BTE on the
340 development of liver fibrosis as well as the activation of hepatic stellate cells,

potentially by inhibiting the TGF-β1/Smad signaling pathway.

4. Discussion

Theaflavin enriched black tea complex (TF-BTE) is mainly consisted of the following two groups of compounds, namely, (1) green tea catechins: epigallocatechin gallate (EGCG) and epicatechin gallate (ECG); (2) theaflavins: theaflavin (TF1), theaflavin-3-gallate (TF2A), theaflavin-3'-gallate (TF2B), and theaflavin-3,3'-digallate (TFDG). A recent interesting study revealed that tea flavanols could efficiently inhibit the formation of dehydroascorbic acid-induced advanced glycation end products[35]. Several reports demonstrated antioxidant [36], anti-viral, anti-inflammatory [37;38] and cancer chemopreventive [39;40] activities of theaflavins. Among them, anti-cancer and anti-inflammatory have been the topic of considerable interests. Although several animal models (e.g., skin, lung, esophagus, stomach, liver, pancreas, small intestine, colon, bladder, prostate and mammary gland) were used to examine the efficacy of tea constituents as anticancer compounds, there is no report of the cancer-preventive activity of theaflavin enriched black tea extract on hepatocellular carcinoma (HCC) associated liver fibrosis in rat model. Hepatic fibrosis involves the formation or development of excess fibrous connective tissue as a result of liver injury. Chronic liver disease can progress to liver cirrhosis, leading to human suffering, hospital costs and even death. The inhibition and prevention of fibrosis development by theaflavin enriched black tea and a understanding of molecular mechanisms might be a potential therapeutic and sustainable strategy for combating hepatic fibrosis and cirrhosis. The present study is the first time to demonstrate that theaflavin enriched black tea extract has efficacious inhibitory property on DMN-induced hepatic fibrosis in Sprague–Dawley rats. We find that the DMN damaged the animal livers by reducing in liver size and caused dark discolorations. The decreased liver size was caused by congestion, which is consistent with clinical liver disease patients.

Herein, our experimental data revealed that the oral administration of 40% TF-BTE with DMN had no adverse effect on the body weight, kidney, and spleen. It also led to a recovery of reduced liver weight (Table1) after 4 weeks of treatment. In addition, the 40% TF-BTE complex reduced the hepatic inflammation by the reduction of DMN-induced serum GOT and GPT level (Table. 2) with no effect on the lipid serum (TG and T-Cho). Attenuation of liver injury dose dependently by TF-BTE was concluded by the significant decrease of liver injury such as necrosis and bile duct proliferation caused by inflammation (Table 3). The histological examination of the liver sections show that TF-BTE decreased DMN-induced liver fibrosis due to the reduction of the hemorrhagic necrosis, disruption of tissue architecture (Figure 4C and 4D) and the remarkable reduction of collagen deposition as indicated by the percentage of Sirius Red (Figure 6C and 6D). The recent evidence implicated that the improvement of DMN-induced hepatic fibrosis by TF-BTE may result partially from the attenuation of hepatocytes injury. Furthermore, our present data in immunohistochemical staining showed that DMN increased the number of alpha-SMA cells in the liver (Figure 6B) and these proliferations are suppressed by oral administration with TF-BTE (Figure 6C and 6D). The expression of α-SMA is used as a biomarker of activated HSCs which play a critical role during hepatic fibrogenesis[7;41]. Taken together, these findings suggest the anti-fibrotic effect of TF-BTE may be due to the suppression of HSC activation.

During hepatic fibrosis, activated HSCs cause ECM protein accumulation leading to the induction of fibrogenic cytokines including TGF-β1[41]. In response to activated TGF-β1, the Smad-group of proteins has been shown to be specifically activated by phosphorylation of receptor- regulated Smads, which further form heteromeric complexes, then the Smad complexes translocate to the nucleus, where they regulate transcription of target gene expression such as collagen type I [42]. TF-BTE reduced

biomarkers in the fibrosis process including the inhibition of TGF-β1 expression in the liver (Figure 7A and 7B). This finding was in the gene level and it correlated well with that in the protein level of TGF-β1 in liver tissue (Figure 9). Hence, the anti-fibrotic effect of TF-BTE is partially due to inhibition of HSC proliferation through down-regulation of TGF-β1 gene. At the same time, TF-BTE suppressed the induction of p-Smad 3 protein (Figure 8), indicating a suppressive activity of TF-BTE on the development of liver fibrosis and the activation of hepatic stellate cells, potentially by inhibiting the TGF-β1/Smad signaling pathway.

In conclusion, TF-BTE exhibited efficacious anti-fibrotic effects against liver injuries induced by DMN by improving the liver function and histopathological appearance of hepatic morphology. The molecular mechanism appeared to be mediated by inactivation of HSCs and inhibition of α-SMA, resulting in inhibiting the TGF-β1 induction.

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Figure legends

Figure 1. Chemical structure of theaflavins

Figure 2. Effect of 40%TF-BTE on the change of Body Weight of experimental rats.

DMN was given intraperitoneally at a dose of 10 mg/ kg body weight three times a week for four consecutive weeks to each group except the control group. DMN, DMN alone; 40%TF(50mg/kg)+DMN(10mg/kg), DMN with 50mg/kg/d 40%TF by oral gavage; 40%TF(100mg/kg)+DMN(10mg/kg),DMN with 100mg/kg/d 40%TF by oral gavage; 40%TF(100mg/kg), 40%TF(100mg/kg) alone.

Figure 3. View of organs from Spargue-Dawley rats.

DMN was intraperitoneally given at a dose of 10 mg/kg on three days per week for 4 weeks to each group except control group. (A)normal group ; (B) animals treated with DMN ; (C) animals treated with 40% TF-BTE 50mg/kg+DMN10mg/kg ; (D) animals treated with 40% TF-BTE 100 mg/kg+DMN10mg/kg.

Figure 4. Representative photomicrograph of rat liver section from the DMN study with hematoxylin/eosin staining.

(A) normal group ; (B) animals treated with DMN ; (C) animals treated with 40%TF-BTE 50 mg/kg + DMN 10mg/kg;(D) animals treated with 40%TF-BTE 100 mg/kg + DMN 10mg/kg. The arrows indicate the areas of DMN-induced collagen deposition.

Figure 5. Representative photomicropraph of rat liver section from the DMN study with sirius red staining.

519 (A) normal group ; (B) animals treated with DMN ; (C) animals treated with
520 40%TF-BTE 50 mg/kg + DMN 10mg/kg ; (D) animals treated with 40%TF-BTE 100
521 mg/kg + DMN 10mg/kg. The arrows indicate the areas of DMN-induced collagen
522 deposition.
523
524 **Figure 6. Representative photomicrograph of rat liver section from the**
525 **DMN-induced α-SMA expression study. IHC staining (40X)**
526 (A) normal group ; (B) animals treated with DMN ; (C) animals treated with
527 40%TF-BTE 50 mg/kg + DMN 10mg/kg ; (D) animals treated with 40%TF-BTE 100
528 mg/kg + DMN 10mg/kg. The arrows indicate the areas of DMN-induced α-SMA
529 expression.
530
531 **Figure 7. Representative photomicrograph of rat liver section from the DMN**
532 **-induced TGF-β1 expression study. IHC staining (40X)**
533 (A) normal group ; (B) animals treated with DMN ; (C) animals treated with
534 40%TF-BTE 50 mg/kg + DMN 10 mg/kg ; (D) animals treated with 40%TF-BTE 100
535 mg/kg + DMN 10 mg/kg. The arrows indicate the areas of DMN-induced TGF-β1
536 expression.
537
538 **Figure 8. Representative photomicrograph of rat liver section from the DMN**
539 **-induced p-smad3 expression. IHC staining (40X)**
540 (A) normal group ; (B) animals treated with DMN ; (C) animals treated with
541 40%TF-BTE 50 mg/kg + DMN 10mg/kg ; (D) animals treated with 40%TF-BTE 100
542 mg/kg + DMN 10mg/kg. The arrows indicate the areas of DMN-induced p-smad 3
543 activation.
544 **Figure 9. Effects of 40% TF-BTE on the DMN-induced hepatic protein**

accumulation of TGF-β1 in rats.

Total liver cell lysates were analyzed for the hepatic protein accumulation of TGF-β1 by Western blot analysis. The western blot is a representative of at least three independent experiments. Quantification of TGF-β1expression was normalized to β-actin using a densitometer.

Figure 10. Effects of 40% TF-BTE on the DMN-induced hepatic protein accumulation of α-SMA in rats.

Total liver cell lysates were analyzed for the hepatic protein accumulation of α-SMA by Western blot analysis. The western blot is a representative of at least three independent experiments. Quantification of α-SMA expression was normalized to β-actin using a densitometer.

559 **Table 1. Relative organ weight of DMN-treated rats with or without 40%TF**

Groups	Relative organ weight (g/bw)		
	Liver	Kidney	Spleen
Control	5.46±0.35[a]	1.14±0.08[a]	0.25±0.03[cb]
DMN	2.52±0.84[b]	1.08±0.14[a]	0.36±0.08[a]
LTF (50 mg/kg)+DMN	2.85±0.45[b]	1.08±0.14[a]	0.38±0.08[a]
HTF (100 mg/kg))+DMN	2.67±0.76[a]	1.02±0.14[a]	0.32±0.09[ab]

560 The average body weight of each group is expressed as the mean ± SE (n = 6 per group), and
561 statistical analysis was done by one-way ANOVA and Duncan's Multiple Range Test and
562 results were indicated by different letters a, b, c. LTF, (40%TF-BTE 50 mg/kg)+DMN; HTF,
563 (40%TF-BTE 100 mg/kg)+DMN.
564

565

566 **Table 2. Effect of 40%TF on activities of serum GOT, GPT and in rats treated with**

567 **DMN**

Groups	Activity			
	GOT (U/L)	GPT (U/L)	TG (mg/dl)	T-cho (mg/dl)
Control	106.67±21.18[b]	31.33±4.46[c]	50.83±12.84[b]	80.00±19.28[ab]
DMN	252.75±122.19[a]	144.50±46.18[a]	92.00±27.91[a]	64.50±12.13[cb]
LTF (50 mg/kg)+DMN	200.20±50.47[ab]	113.20±41.47[ab]	105.80±47.93[a]	58.00±16.29[c]
HTF (100 mg/kg))+DMN	194.83±47.72[ab]	100.33±26.04[b]	85.33±20.19[a]	50.50±9.71[c]

568

569 The average body weight of each group is expressed as the mean ± SE (n = 6 per group), and
570 statistical analysis was done by one-way ANOVA and Duncan's Multiple Range Test and
571 results were indicated by different letters a, b, c. LTF, (40%TF-BTE 50 mg/kg)+DMN; HTF,
572 (40%TF-BTE 100 mg/kg)+DMN.

573 **Table 3. Injury of score of fatty change necrosis, bile duct proliferation and**

574 **inflammation in rats treated with or without DMN**

Groups	Activity			
	Fatty change	Necrosis	Bile duct proliferation	Inflammation
Control	1.0±0.0[a]	0.0±0.0[d]	0.0±0.0[b]	1.0±0.0[b]
DMN	1.0±0.0[a]	4.0±0.0[a]	1.7±0.6[a]	2.7±0.6[a]
LTF (50 mg/kg)+DMN	1.0±0.0[a]	3.0±0.0[b]	1.0±0.0[a]	2.0±0.0[a]
HTF (100 mg/kg))+DMN	1.0±0.0[a]	1.0±0.0[c]	0.0±0.0[b]	1.0±0.0[b]

575 Rats were fed diet for 4 weeks as described under Materials and Methods, and the body

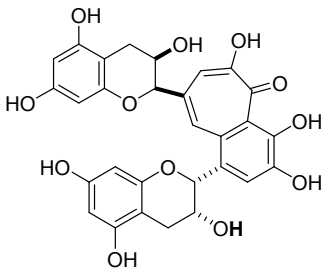
576 weights were monitored twice weekly. The average body weight of each group is expressed as

577 the mean ± SE (n = 6 per group), and statistical analysis was done by one-way ANOVA and

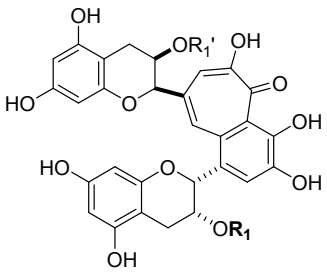
578 Duncan's Multiple Range Test and results were indicated by different letters a, b, c. LTF,

579 (40%TF-BTE 50 mg/kg)+DMN; HTF, (40%TF-BTE 100 mg/kg)+DMN.

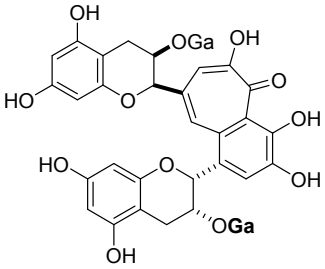
580



TF1 (Theaflavin)



TF2 (Theaflavin monogallate)
(TF2a: R_1=galloyl, R_1'=H; or
TF2b: R_1=H, R_1'=galloyl)



TF3 (Theaflavin digallate)

(Ga = galloyl)

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582

583 **Figure 1.**

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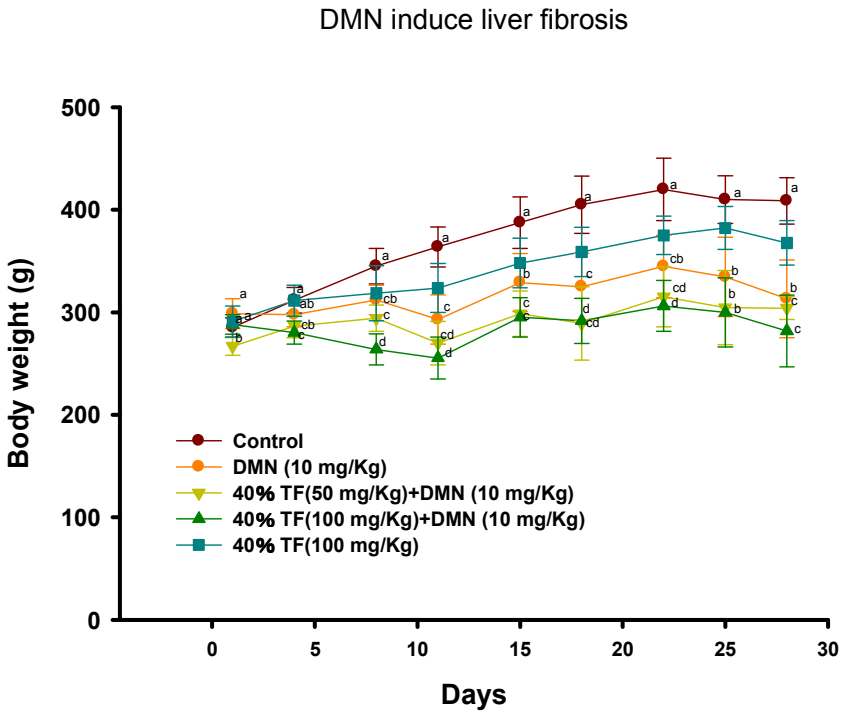

588

589 **Figure 2.**

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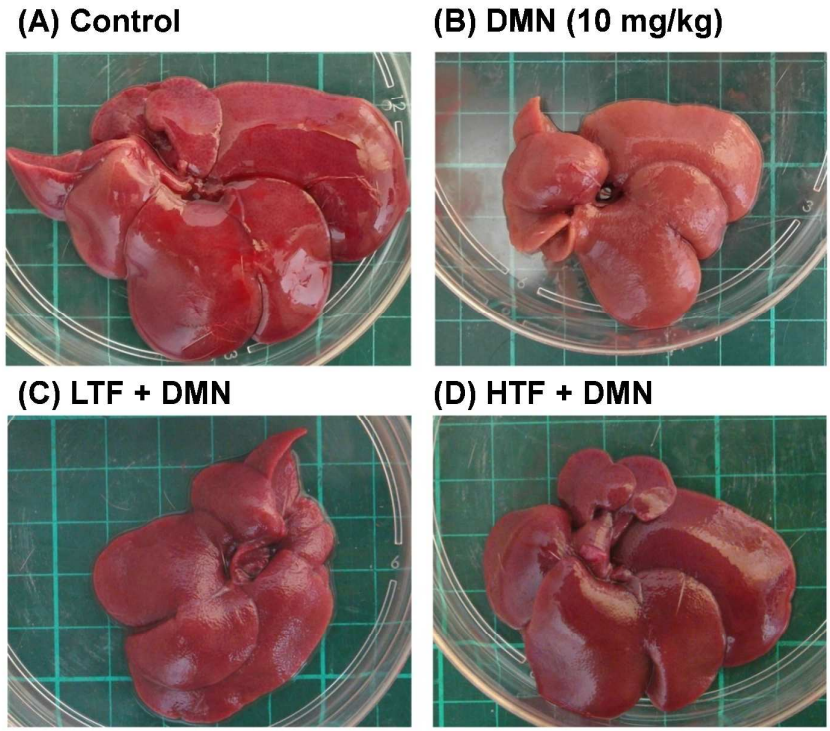

592

593

594 **Figure 3.**

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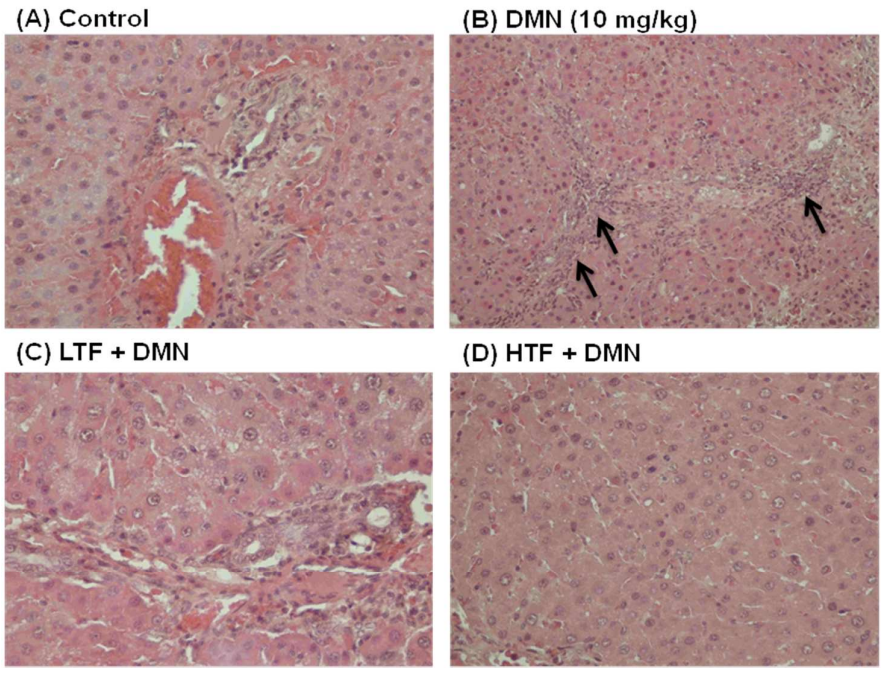

596

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598 **Figure 4.**

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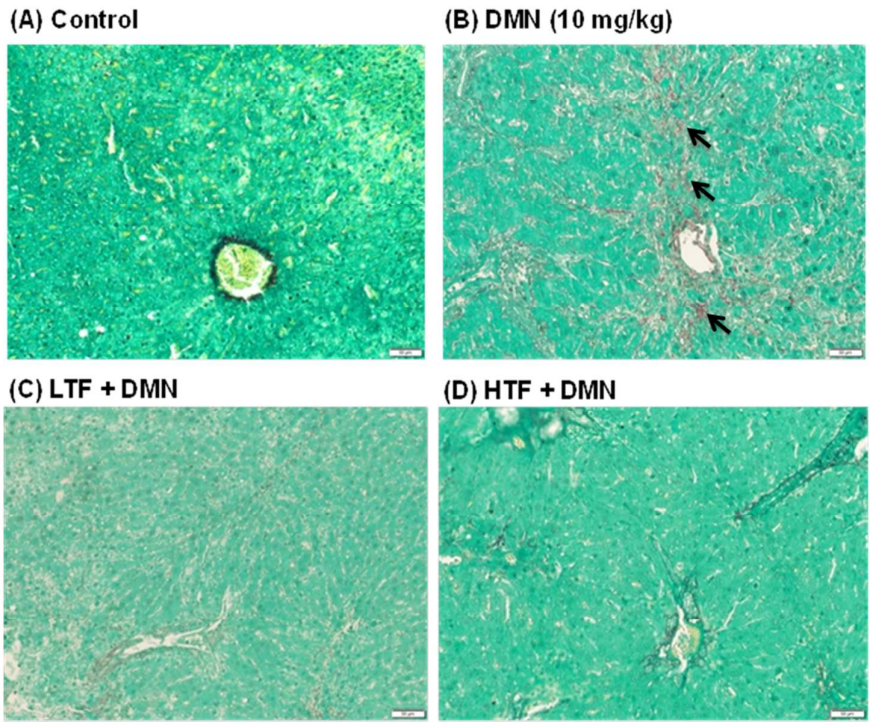

600

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602 **Figure 5.**

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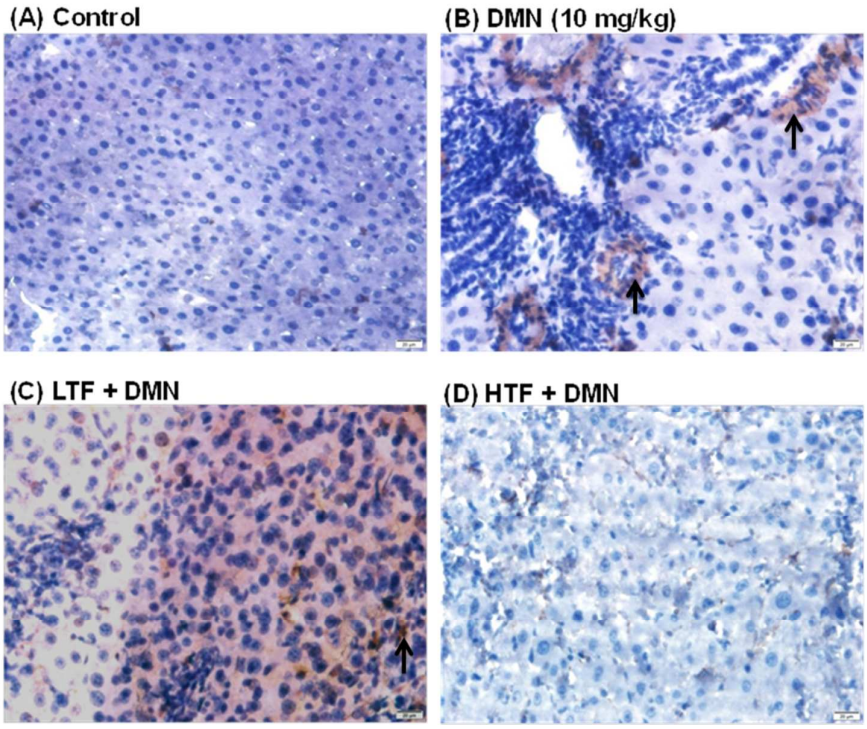

605

606 **Figure 6.**

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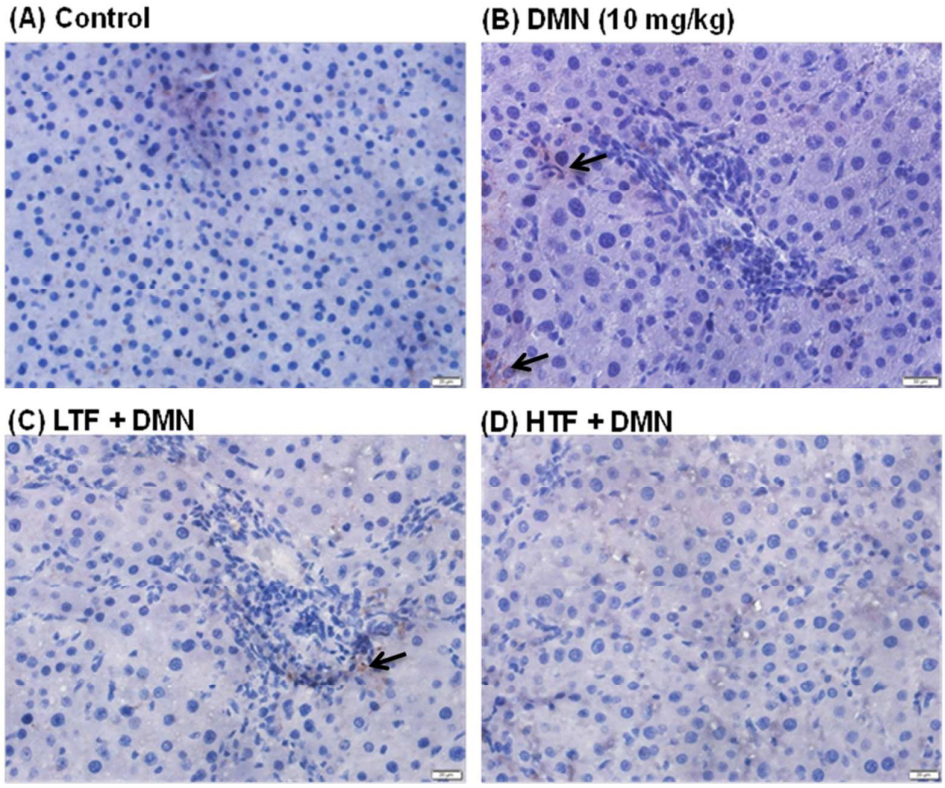

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610 **Figure 7.**

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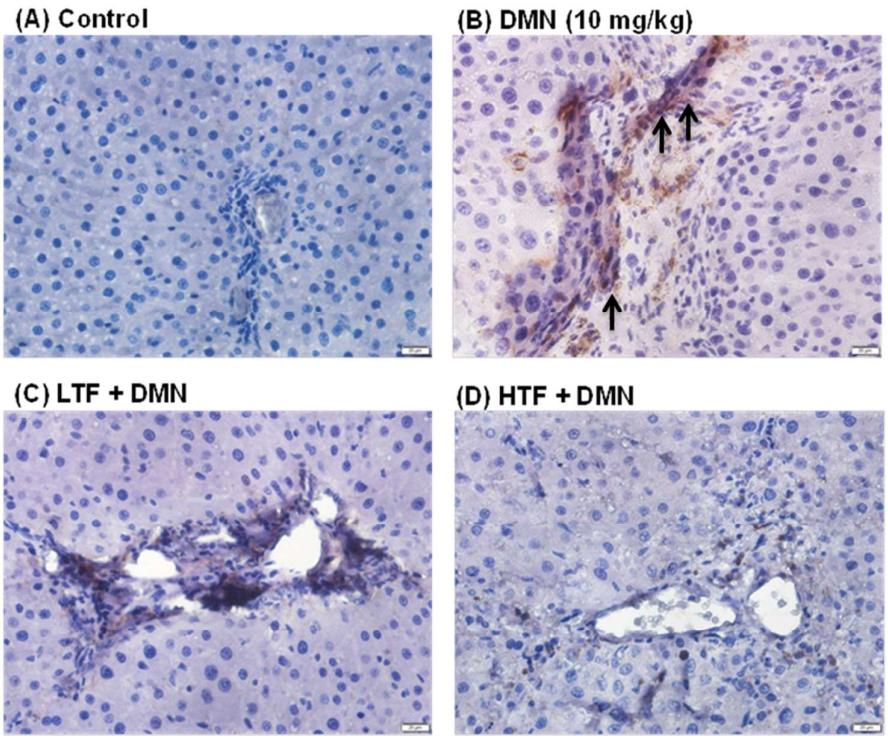

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614 **Figure 8.**

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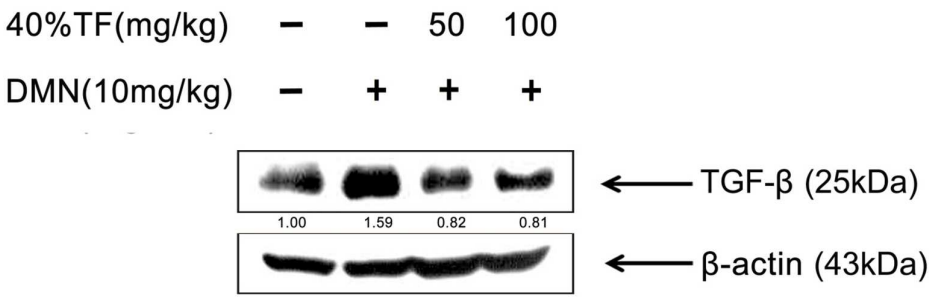

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618

619 **Figure 9.**

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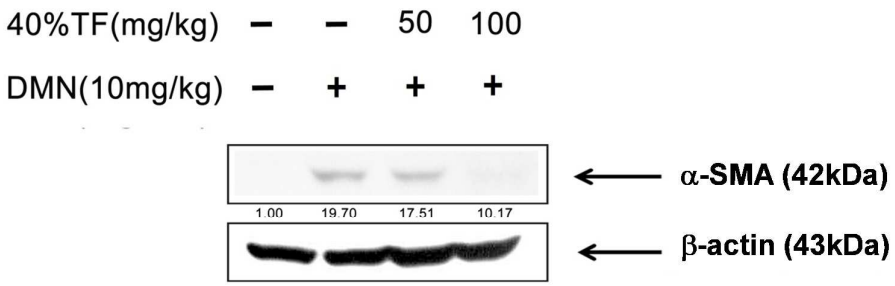

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623

624 **Figure 10.**

625

626