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Original Article

Mulberry fruit extract prevents liver fibrosis via inhibition of inflammation in DEN-treated rats

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Liver disease, which involves an extensive range of liver pathologies from fatty liver to hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma, is a serious health problem around the world. Mulberry fruits are rich in polyphenols and have been reported to remove free radicals and mitigate inflammation in the presence of gastric cancer, melanoma, leukemia, or liver injury induced by alcohol or carbon tetrachloride (CCl_4). Our previous results have demonstrated the beneficial effects of mulberry water extract (MWE) on hepatocarcinogenesis via phosphoinositide 3-kinase (PI3K)/phosphorylated protein kinase B (Akt)/mechanistic target of rapamycin (mTOR) inhibition and cell death-related proteins. The purpose of this study was to assess the anti-inflammatory effect of MWE on liver cancer-related fibrosis in diethylnitrosamine (DEN)-induced rats. Oral treatment with different concentrations of MWE reduced α-smooth muscle actin (α-SMA, fibrosis marker) in DEN-induced rats.

Compared with the DEN-induced group, MWE significantly increased anti-oxidative enzyme activities. Rats receiving MWE supplementation showed reduced interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α levels compared to untreated DEN-induced rats. Inflammatory and fibrotic proteins such as collagen IV, fibronectin, phosphorylated signal transducer and activator of transcription (STAT)3, PI3K, Akt, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) were also inhibited by MWE treatment in DEN-induced rats. These results indicated that MWE is a potential health supplement for preventing liver fibrosis and hepatocarcinogenesis.

Keywords: Mulberry water extract (MWE), fibrosis, oxidative stress, inflammation

Introduction

Hepatocellular carcinoma (HCC) is one of the most aggressive and fastest growing malignancies. It is triggered by perpetual hepatocellular damage, hepatocyte regeneration, and inflammation [1]. HCC has clearly defined etiological factors, including viral hepatitis, nonalcoholic steatohepatitis (NASH), alcoholic liver disease, and cirrhosis [2]. Recently, oxidative stress has been shown to be an important factor in hepatocarcinogenesis and a common and major driving force of HCC in chronic liver disease through generation of reactive oxygen

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species (ROS) and reactive nitrogen species [3]. Modulation of ROS formation and inflammation are believed to be important in protecting against hepatocarcinogenesis.

HCC is strongly associated with liver fibrosis and cirrhosis, with about 80-90% of HCC cases having underlying fibrosis, suggesting that the environment in which HCC rises may influence tumorigenesis. This differs from most other tumors and organs, where fibrosis develops as a reaction to tumor formation [4]. While inflammation may be beneficial in the short term, chronic inflammation and its associated regenerative wound-healing response are strongly linked to the development of fibrosis, cirrhosis, and HCC [5]. Following continuous liver injury, hepatic stellate cells (HSCs) are activated, changing their morphology to become myofibroblasts and migrating to the site of tissue repair via expression of alpha-smooth muscle actin (α-SMA). Proinflammatory mediators that have a role in HCC development, including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, are secreted [6]. Although IL-6 has been reported to protect against liver fibrosis, it has also been shown to contribute to HCC development [7].

Many functional foods have been identified as effective therapeutic agents in liver disease [8]. Mulberry belongs to the genus Morus and the family Moraceae. Based on analyses of mulberry water extract (MWE) in our laboratory, it possesses high concentrations of polyphenols (MPEs) containing phenolic acids (5.12%), flavonoids (8.23%), and anthocyanins (5.61%) [9]. In recent years, we have demonstrated that MWE regulates 5' adenosine monophosphate-activated protein kinase (AMPK) signaling to reduce lipid accumulation, suppress fatty acid synthesis, and stimulate fatty acid oxidation [10]. MWE has also been shown to inhibit carbon tetrachloride (CCl_4)-induced liver damage and fibrosis by decreasing lipid peroxidation and inhibiting proinflammatory gene expression [11] and to have hepatoprotective effects on acute liver failure induced by lipopolysaccharides [12].

We found that mulberry leaf extract targets the proliferation signal pathway of the inflammatory response of adipocytes in HCC and prevents obesity-mediated liver disease [13]. Recently, we clarified the effects of MWE on inhibition of diethylnitrosamine (DEN)-induced HCC formation. Using p53-positive HepG2 cells and p53-negative Hep3B cells, we demonstrated that MPE, the major compound in the mulberry phytochemical, induces HCC cell death through different pathways, indicating that p53 status plays a critical role in HCC [14]. Here, we aimed to determine if MWE offers protection against fibrosis in liver cancer through inhibition of oxidative stress and inflammatory response.

Materials and Methods

MWE preparation

A mixture of dried *Morus alba* L. (mulberry) fruit (100 g) and water (1000 mL) was stirred. After 10 min centrifugation (8000 g), the supernatant was collected and lyophilized (-80 °C for 12 h) to obtain MWE powder. MWE powder was dissolved in distilled water, stored at -80 °C overnight, and lyophilized for future use.

Animals and treatment

The rat license for this laboratory (No. 1204) was issued by Chung Shan Medical University (CSMU). All procedures involving animals were approved according to the guidelines of the IACUC (Institutional Animal Care and Use Committee) by the Animal Center of CSMU. Male Wistar rats aged 4 to 5 weeks were purchased from BioLasco Taiwan Co., Ltd. Thirty rats were allowed to acclimate for at least 7 days on a standard laboratory diet under environmentally controlled conditions (22 ± 2 °C, 55% ± 5% relative humidity, and 12 h light/dark cycle) with free access to food and water. The animals were randomly divided into 5 groups of 6 rats each, as follows:

- Control (normal saline-treated)
- 2% MWE-treated
- DEN-treated (40 mg/kg)
- DEN-treated +1% MWE
- DEN-treated +2% MWE

DEN was dissolved in normal saline and then intraperitoneally (i.p.) administered to rats at doses

of 40 mg/mL/kg per week. The rats in the treatment groups were administered 1% or 2% MWE orally in food daily for 4 months.

Immunohistochemical analysis of hepatic tissues

Immunohistochemical staining for α-SMA antibody (Ab-5694, Abcam Company, Cambridge, UK) was performed according to the manufacturer's instructions. First, the slides were deparaffinized in xylene, rehydrated in graded alcohol series, and blocked with 3% H2O2 for 10 min and 1% bovine serum albumin in phosphate buffered saline (PBS) for 30 min. Afterwards, the slides were washed with PBS and incubated overnight at 4°C with α-SMA antibody. After removal of antibody, complement was placed, and the horseradish peroxidase (HRP) conjugate (Advance HRP Polymer) was applied for 60 min.

After washing, the sections were counterstained with Meyer's hematoxylin and washed with tap water. Images were viewed using Nikon Eclipse E600 microscopy system (100X).

RNA isolation and Real-time Quantitative Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from liver tissues using an RNA isolation kit (Ultraspec™, Biotecx Laboratories, Houston, TX, USA) according to the manufacturer's protocol and quantified spectrophotometrically by measuring the absorbance of an aliquot at 260 nm. The RNA samples were reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (PE Applied Biosystems, Foster City, CA). The primers for RT-PCR are shown in Table 1. RT-PCR was carried out in triplicate using the GeneAmp PCR System 2700 (Applied Biosystems; Foster City, CA, USA). The amount of mRNA was calculated by the comparative CT method, which depends on the ratio of the amount of target genes to reference gene β-actin..

Determination of hepatic oxidative stress markers

Livers were excised, cleaned, washed with ice-cold saline (pH 7.4), and homogenized in Tris–HCl (0.1 M)-EDTA buffer (pH 7.4, 0.001 M). After 12,000 g centrifugation at 4°C for 30 min, the supernatant was collected to detect oxidative stress. Activities of antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-PX) were measured in the liver tissue homogenates using commercial colorimetric assay kits (Zell Bio GmbH, Ulm, Germany).

Western blotting

Collagen IV, fibronectin, phosphoinositide 3-kinase (PI3K), and p-mechanistic target of rapamycin (mTOR) proteins were purchased from Santa Cruz Biotechnology (Dallas, USA). p-protein kinase B (Akt) protein was purchased from Cell Signaling Tech (Beverly, MA). IL-1b, IL-6, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and TNFα were purchased from Abcam Company (Cambridge, UK). Liver tissues were homogenized in lysis buffer (1% NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, pH 7.5) with fresh protease inhibitor. Proteins were collected by centrifugation at 12,000

Table 1. Sequences of reverse transcription-polymerase chain reaction primers.

Gene Name	Sequence
TNF-α	forward, 5′-CTACCTTGTTGCCTCCTCTTT-3′ reverse, 5′-GAGCAGAGGTTCAGTGATGTAG-3′
IL-1β	forward, 5′-AACCTGCTGGTGTGTGACGTTC-3′ reverse, 5′-CAGCACGAGGCTTTTTTGTTGT-3′
IL-6	forward, 5′-ACAACCACGGCCTTCCCTACTT-3′ reverse, 5′-CACGATTTCCCAGAGAACATGTG-3′
β-actin	forward, 5′-AGGTATCCTGA CCCTGAAGTA-3′ reverse, 5′-CACACGCAGCTCATTGTAGA-3′

g and 4°C. Total protein samples underwent 8-12% SDS-polyacrylamide gel electrophoresis and were transferred onto nitrocellulose membranes and incubated in blocking buffer (0.5% bovine serum albumin) at room temperature for 1 h and then at 4 °C overnight with the primary antibodies. After three washes with Tris-buffered saline containing 0.05% Tween 20 (TBST), membranes were incubated with secondary HRP-conjugated antibody for 1 h at room temperature.

Antigen-antibody complexes were then developed with an electrochemiluminescence (ECL) kit (Millipore) and analyzed using AlphaImager Series 2200 software (Alpha Innotech, San Leandro, CA, USA).

Statistical analysis

Data were collected from 6 rats in each group. The results are presented as means ± SD. The differences among three groups (control, DEN-induced, and MWE) were analyzed by one-way ANOVA. The differences between two groups were analyzed by Student's *t*-test with Sigmaplot software (version 12). Statistical significance was set at 0.05.

Results

MWE treatment reduces DEN-induced liver fibrosis

As an indicator of HSC activation, α-SMA is a predictor of fibrosis in liver disease [15]. Our previous research demonstrated that MWE treatment reduces DEN-induced serum alanine aminotransferase, aspartate aminotransferase, and γ-glutamyl transferase activities [14]. In Fig. 1, the expression of α-SMA was elevated in comparison with control rats following DEN treatment. Different concentrations of MWE reduced the levels of fibrosis marker of liver injury in DEN-induced rats. Also in Fig. 1, the oral administration of 2% MWE did not cause changes in the level of α-SMA in control rats.

MWE prevents liver oxidative stress in DEN-treated rats

Oxidative stress is important in the pathogenesis of fibrosis and inflammation associated with HCC [16]. To demonstrate the antioxidant effects of MWE, antioxidant enzymes CAT, SOD, GSH-PX were analyzed [17, 18]. In DEN-induced rats there was obvious depletion of CAT, SOD, and GSH-PX in liver tissues (Table 2). Treatment with 2% MWE significantly reduced oxidative damage in the livers of DEN-induced rats. This indicated that MWE has prophylactic potential against oxidative stress-associated liver damage.

MWE reduces inflammatory gene expressions in the livers of DEN-induced rats

Following continuous liver injury, HSCs are activated and change their morphology to become myofibroblasts that express α-SMA. They then migrate to the site of tissue repair where they secrete extracellular matrix (ECM), chemokines, and cytokines. As shown in Fig. 2, up-regulated

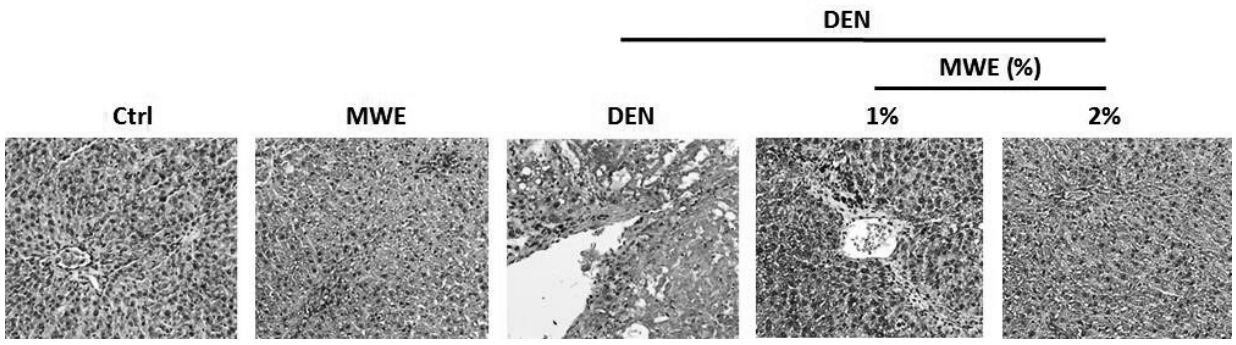

Figure 1. MWE decreases the expression of fibrosis marker induced by DEN in liver tissues. Immunohistochemical staining of paraffin-embedded sections of liver from control, 2% MWE, DEN, DEN + 1% MWE, and DEN + 2% MWE groups probed with α-SMA antibody. Representative photomicrographs are magnified 100X.

Table 2. The activities of antioxidant enzymes were recovered by MWE.

Enzyme activity	Control	MWE	DEN	DEN+ 1%MWE	DEN+ 2%MWE
Catalase (U/mg protein)	871.66±203.72	1090.11±83.86	366.03±26.02†	417.13±225.63	583.54±78.13*
SOD (U/mg protein)	36.41±2.06	31.62±2.84	21.05±2.68†	23.41±2.99	29.75±1.69*
GSH-PX (U/mg protein)	0.623±0.0960	0.630±0.0730	0.227±0.0294†	0.297±0.0403	0.344±0.0343*

Control: normal rats; MWE: rats were fed with 2% MWE; DEN: rats were injected with DEN; DEN+1% MWE: rats were injected with DEN and fed with 1% MWE; DEN+2% MWE: rats were injected with DEN and fed with 2% MWE. The data are expressed as means ± SD from ten samples for each group. † P<0.05, significant difference compared with the control. *P<0.05, significant difference compared with the DEN group.

TNF-α (*Tnf-α*), IL-1β (*Il-1β*) and IL-6 (*Il-6*) expressions were found in DEN-induced rats with significant increases in the mRNA levels of these factors. MWE dose-dependently reduced liver inflammatory cytokines in DEN-treated rats. The oral administration of 2% MWE did not affect inflammatory gene expressions in control rats.

MWE treatment reduces inflammatory fibrotic factor expressions and affects related signaling pathway in the livers of DEN-treated rats

Analyzed proteins of inflammatory factors are shown in Fig. 3. Increased TNF-α, IL-1β, and IL-6 protein levels were found in DEN-induced rats.

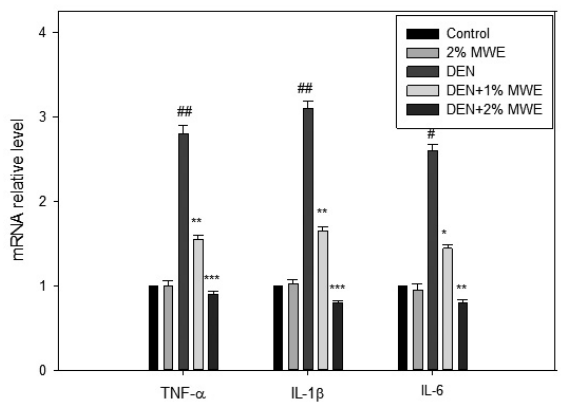

Figure 2. MWE prevents activation of pro-inflammatory factors in the livers of DEN-induced rats. The mRNA expressions of TNFα, IL-1β, and IL-6 were analyzed by RTPCR. ##P<0.001 versus control group; *P<0.05, **P<0.001 versus DEN group.

MWE decreased liver inflammatory cytokines in DEN-treated rats but did not affect inflammatory protein expressions in control rats. Oncogenic and hepatic activation of signal transducer and activator of transcription (STAT)3 occurs through various cytokines, such as IL-6 [19, 20]. Subsequently, we investigated whether IL-6/STAT3 pathway is persistently activated in fibrosis in DEN-induced rats. Fig. 3 revealed that phosphorylated STAT3 is

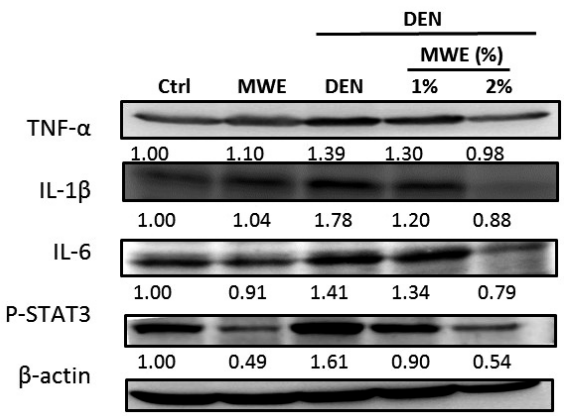

Figure 3. MWE decreases the expressions of inflammatory proteins induced by DEN. Rats were injected with DEN and fed MWE (1%, 2%). After four months, these animals were sacrificed and liver homogenates were prepared. Total homogenates were subjected to SDS-PAGE followed by Western blot. The membranes were probed with TNF-α, IL-1β, IL-6, and p-STAT3 antibodies. The results were obtained using ECL system. β-actin served as the loading control.

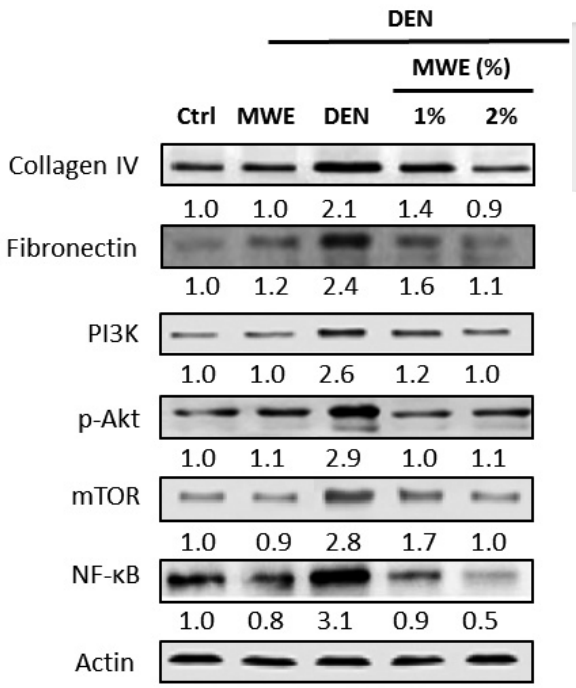

Figure 4. MWE decreases the expressions of fibrotic and inflammatory proteins in DEN-induced rats. Rats were injected with DEN and fed MWE (1%, 2%). After they were sacrificed, collagen IV, fibronectin, PI3K, p-Akt, and mTOR NF-κB expressions were detected on Western blot. The results were obtained using ECL system. β-actin served as the loading control.

attenuated by MWE in DEN-induced rats. The oral administration of 2% MWE also affected STAT3 phosphorylation in control rats.

NF-κB plays an essential role in the regulation of various inflammatory and immune responses that trigger liver fibrosis [21]. We next used Western blotting to analyze the levels of NF-κB, fibrotic markers, such as collagen IV and fibronectin, and related signaling proteins. In Fig. 4, under MWE treatment, collagen IV and fibronectin were reduced in DEN-induced rats. Earlier studies have shown that PI3K/Akt mediates liver disease by activating the expression of NF-κB under oxidative stress [22]. Here, our results demonstrated that MWE improves PI3K/NF-κB related inflammation in DEN-induced rats.

Discussion

We have reported on the chemopreventive effects of MWE and MPE in DEN-induced HCC in rats via apoptosis and autophagy of liver cancer cells [14]. In this study, the treatment of DEN-induced rats with MWE caused a remarkable reduction in liver damage including recovery of the activities of antioxidant enzymes CAT, SOD, and GSH-PX. Furthermore, MWE reduced TNF-α, IL-1β, and IL-6 expressions, inhibited liver fibrosis, and prevented hepatocarcinogenesis (Fig. 5).

Chronic inflammation and fibrosis associated with HCC and various inducing factors such as viruses and ethanol increase production of ROS, resulting in immune-mediated cell death. This leads to increased hepatocellular oxidative stress which promotes HCC development by inducing DNA mutations. Increased ROS concentrations in hepatocytes cause hepatic DNA damage [32]. Chronic inflammation leads to increased proliferation of hepatocytes, telomere dysfunction, and chromosomal instability following liver injury and gene mutations if there is predisposition to malignant transformation [23]. Telomere maintenance and Wnt signaling, receptor tyrosine kinase, angiogenesis, transforming growth factor (TGF)-β, Janus kinase (JAK)/STAT, ubiquitin proteasome pathways, inactivation of p53, chromatin remodeling, Ras signaling, mTOR signaling, and ROS pathway initiation are all involved in HCC progression [24]. Fortunately, plenty of polyphenols protect the liver from fibrosis via suppression of the activation of HSCs such as apigenin, epigallocatechin gallate (EGCG), quercetin, icaritin, curcumin, and resveratrol [25]. Polyphenols have been reported to have anti-fibrotic effects via apoptosis of activated HSCs, primarily associated with NF-κB and TNF-α signaling [26, 27]. In addition, chlorogenic acid suppresses the activation of NF-κB and Akt, reducing expression of related profibrogenic genes [28]. EGCG and wogonoside regulate profibrogenic/anti- fibrogenic balance via inhibition of PI3K/Akt/Smad pathway and PI3K/Akt/mTOR signaling, respectively [29, 30]. The results of the present study revealed that MWE improves liver fibrosis by regulating STAT3 and PI3K/Akt pathways. STAT3 is a critical element in inflammation-related tumorigenesis as it promotes the proliferation, survival, invasion,

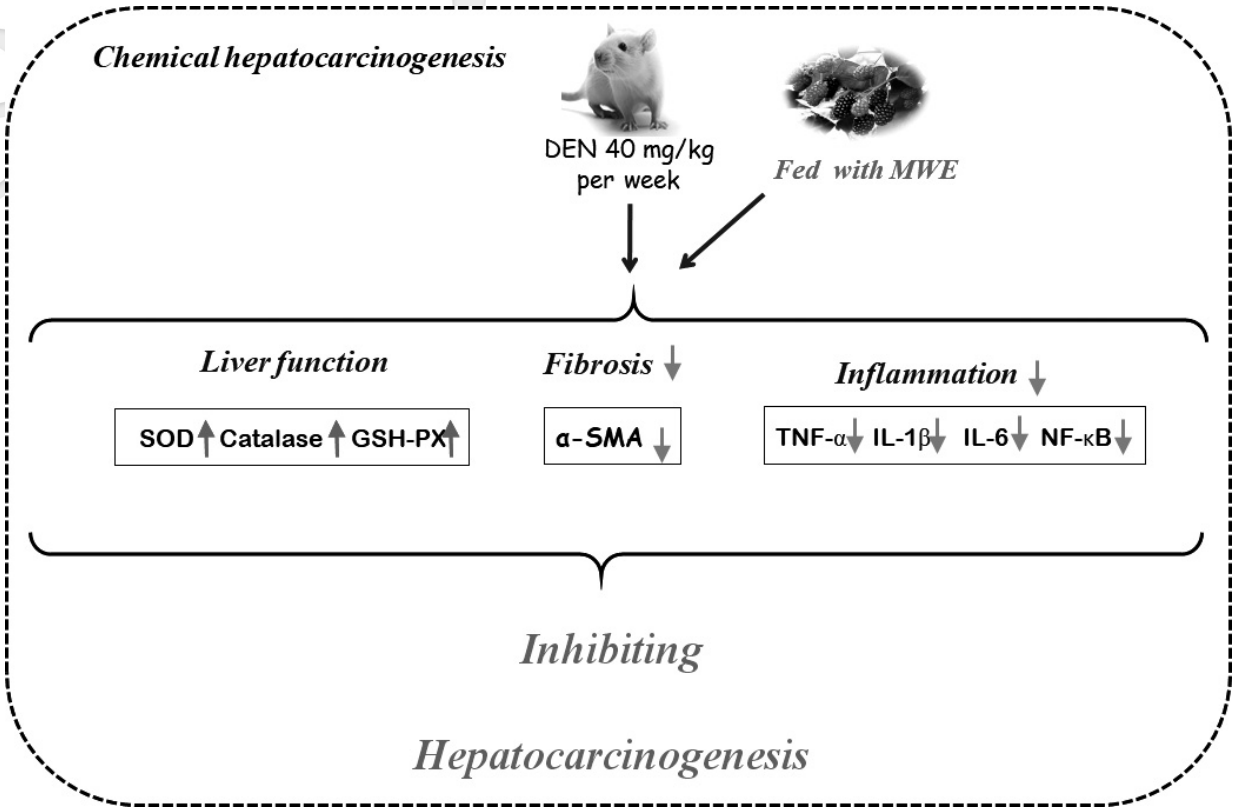

Figure 5. The effects of MWE on inhibition of fibrosis-related hepatocarcinogenesis induced by DEN. In an animal model of hepatocarcinoma induced by DEN, MWE recovered the activities of antioxidant enzymes. Moreover, MWE not only decreased TNF-α, IL-1β, and IL-6 expressions to mitigate inflammatory status, but also inhibited liver fibrosis. MWE is a potential health supplement for preventing liver fibrosis and hepatocarcinogenesis.

angiogenesis, and metastasis of tumor cells [31]. Our previous study has demonstrated that MWE and MPE induce apoptosis and autophagy of HCC by regulating PI3K/Akt/mTOR signaling. It is possible to predict both anti-initiation and anti-promotion response of MWE. MWE combated oxidative stress, suppressed inflammatory cascade, and prevented liver fibrosis by promoting cell apoptosis and autophagy, thereby inhibiting hepatocarcinogenesis.

We showed that MWE regulates two transcriptional factors, STAT3 and NF-κB. In Fig. 4, MWE markedly reduced NF-κB expression and slightly decreased the levels of collagen IV and fibronectin. STAT3 and NF-κB are activated via distinct pathways and move to the nucleus to affect transcriptional activity.

STAT3 and NF-κB, which are constitutively activated by acetylation and phosphorylation in tumor cells, have been closely linked to both cancer development and progression [32, 33]. NF-κB has been suggested to regulate the activation, survival, and inflammatory responses of HSCs and hepatic myofibroblasts. However, this may be both a profibrogenic and an antifibrogenic signaling pathway [21]. NF-κB exerts antifibrogenic effects by suppressing transcription of the Col1a1 gene [34]. In Crel deficient mice, decreased fibrosis and activation of HSCs in vitro and in vivo have been demonstrated [35]. Therefore, the role of NF-κB in regulating liver fibrosis needs to be clarified in future studies.

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