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Protective effects of garcinol on dimethylnitrosamine-induced liver fibrosis in rats

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ABSTRACT

Garcinol, a polyisoprenylated benzophenone derivative, mainly isolated from *Garcinia indica* fruit rind, has been suggested to exhibit many biological benefits including antioxidative, anti-inflammatory, and anti-tumor activities. The aim of this study is to evaluate the protective effects of garcinol on dimethylnitrosamine (DMN)-induced liver fibrosis in rats. The administration of DMN for six consecutive weeks resulted in the decrease of body weights, the elevation of serum aminotransferases, as well as histological lesions in livers. However, oral administration of garcinol remarkably inhibited the elevation of aspartate transaminase (AST) and relieved liver damage induced by DMN. Furthermore, our results revealed that garcinol not only effectively reduced the accumulation of extracellular matrix (ECM) components but also inhibited the expression of α-smooth muscle actin (α-SMA) in livers. The expression of transforming growth factor-β1 (TGF-β1) and the phosphorylation of Smad 2 and Smad 3 were also suppressed by garcinol supplementation. In conclusion, our current study suggested that garcinol exerted hepatoprotective and anti-fibrotic effects against DMN-induced liver injury in rats.

Keywords: Garcinol, liver fibrosis, dimethylnitrosamine, hepatic stellate cells, transforming growth factor-β1, α-smooth muscle actin
1. INTRODUCTION

Hepatic fibrosis is the crucial characteristic in the development of chronic liver diseases to cirrhosis and hepatocellular carcinoma (HCC), which is one of the most reported cancer incidences worldwide. A variety of pathological factors attribute to chronic hepatic fibrosis, such as chronic hepatitis B or C infections, alcohol abuse, chemical intoxication, metabolic syndrome and autoimmune disease. Hepatic fibrosis is a wound-healing response of the liver to repeated injury in association with the excessive accumulation of extracellular matrix (ECM) components including collagens, proteoglycans and carbohydrates, leading to architectural disorder and functional impairments. In the pathogenesis of chronic liver disease, reactive oxygen species (ROS) produced from damaged hepatocytes and inflammatory cytokines activate Kupffer cells (the resident macrophages in the liver) and stimulate the recruitment of activated T cells. Kupffer cells are the principal cells in the liver for antigen presentation, phagocytosis, and cytokine production. Injured hepatocytes and Kupffer cells release ROS and pro-inflammatory cytokines, such as transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), as well as interleukins (IL) to further stimulate the activation of hepatic stellate cells (HSCs; also known as perisinusoidal cells, lipocytes, or Ito cells). The activation of HSCs has consistently been shown to play a crucial role in hepatic fibrogenesis. In the normal liver, quiescent HSCs typically locate in the perisinusoidal space of Disse and serve as the major storage site of lipid-soluble vitamin A. During liver injury, HSCs undergo an activation or transdifferentiation process in which the quiescent cells transform into the proliferative, contractible, and alpha-smooth muscle actin (α-SMA)-expressing myofibroblast-like cells that synthesize ECM proteins.
Many studies have identified that TGF-β is the most important cytokine contributing to the activation of inflammatory cells and stimulating fibrogenesis of HSCs. For TGF-β signaling, binding of the ligand to TGF-β type II receptor leads to recruitment and phosphorylation the Type I receptor into the complex. The activation of the Type 1 receptor further recruits and phosphorylates Smad 2 and Smad 3, so-called receptor-activated Smads (R-Smads), which follows binding to Smad 4 to form the complex and translocate into the nucleus where it regulates the target genes such as collagen type I. Therefore, the interruption of the pro-fibrotic signaling for reducing hepatic fibrogenesis and elaboration of antioxidant activities for scavenging free radicals such as ROS are both the potential strategies to inhibit the development of hepatic fibrosis.

Dimethylnitrosamine (DMN) is a potent carcinogen, mutagen as well as a hepatotoxin, and its hepatotoxicity has been first reported in 1954. DMN is mainly metabolized by a specific cytochrome P450 isozyme called CYP2E1 and its metabolites induce hepatotoxicity. George et al reported that the administration of DMN by intraperitoneal injection for three weeks led to liver injury with hepatocyte necrosis, apoptosis and dysplasia, as well as collagen fiber deposition suggesting that the pathological features and abnormalities of DMN-induced liver injury in rats reflect human hepatic fibrosis. Therefore, the DMN-induced liver injury in rats is a relevant model to study progression from fibrosis to cirrhosis and HCC.

Garcinol, a polyisoprenylated benzophenone derivative, is one of the major active compounds isolated from fruit rind of Garcinia indica (also called Kokum) (Figure 1). The rind of Garcinia indica contains 2-3% garcinol (w/w) and has been used as a food coloring and traditional medicine in India. Many studies reported that garcinol possesses many biological benefits, such as antioxidant activity, anti-
inflammatory activity and anti-cancer activity\textsuperscript{12-17}. The structural features that confer
garcinol with potent antioxidant activity are a β-diketone moiety and phenolic
hydroxyl groups. Yamaguchi \textit{et al}\textsuperscript{12} demonstrated that garcinol suppressed the
formation of superoxide anion as well as hydroxyl radical in different chemical
systems indicating that garcinol is a potent free radical scavenger. Moreover, garcinol
also exhibits anti-inflammatory effects through inhibiting the expression of inducible
nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in lipopolysaccharide
(LPS)-stimulated macrophages and astrocytes\textsuperscript{17, 18}. Our previous study reported that
garcinol could effectively inhibit the growth of Hep 3B cells, a human hepatocellar
cancer cell without functional p53, through the elevation of DNA damage-inducible
gene 153 (GADD153) expression and Bax/Bcl-2 ratio, as well as the reduction of
mitochondrial membrane potential\textsuperscript{19}. In addition, Sethi \textit{et al}\textsuperscript{20} also found that garcinol
exerted inhibitory effects not only on the growth of HCC cells by suppression of the
nuclear translocation, phosphorylation and acetylation of signal transducer and
activator of transcription 3 (STAT3) but also on the size of human HCC xenograft
tumors in athymic nu/nu mice. A recent study also documented that the administration
of garcinol markedly alleviated LPS/D-galactosamine (D-Gal)-mediated liver injury
in rats through suppressing hepatocyte apoptosis\textsuperscript{21}.

Since liver fibrosis and cirrhosis are the risk factors in the development of HCC,
here, we tested the hypothesis that garcinol has potent anti-fibrotic effects in a DMN-
induced fibrosis model in rats. To our knowledge, it is the first time to utilize DMN-
induced liver injury as an animal model to evaluate the protective effects of garcinol
against liver fibrosis. The possible mechanisms of the anti-fibrotic effects involved in
garcinol-mediated regulation of TGF-β/Smad signaling pathway were also
investigated.
2. METHODS AND MATERIALS

2.1 Reagents and chemicals

Garcinol was isolated from *G. indica* dried fruit rind\(^6\). All reagents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). \(N\)-Nitrosodimethylamine (dimethyl \(N\)-nitrosamine; DMN) was purchased from Wako Pure Chemical industries Ltd. (Osaka, Japan). Beta-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TGF-\(\beta\)1, p-Smad 2 and p-Smad 3 antibodies were purchased from Transduction Laboratories (BD, Biosciences, Lexington, KY, USA). Alpha-SMA antibody was obtained from Epitomics, Inc. (Burlingame, CA, USA).

2.2 Animals and treatment

Healthy male Sprague-Dawley rats (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). All animals were housed under a controlled environment (25 ± 1 °C, 65 ± 5% relative humidity, 12-h lighting period, 0700-1900) and provided with a commercial rodent diet (laboratory rodent diet 5001, LabDiet Co, St. Louis, MO, USA) and distilled water *ad libitum* throughout the study.

After a one-week acclimation, animals were randomly divided into four groups (n=8) including: (i) control, (ii) DMN treatment, (iii) DMN + garcinol supplementation (10 mg/kg bw), and (iv) DMN + garcinol supplementation (25 mg/kg bw). The DMN-treated animals were administered DMN (10 mg/kg bw) via intraperitoneal injection three times a week (Mon, Wed, and Fri) for six consecutive weeks. The control group was given normal saline. After administration of DMN for
three weeks, the animals of the DMN + garcinol supplementation groups were fed
with either 10 or 25 mg/kg bw/day of garcinol for three consecutive weeks by oral
gavage (Figure 2). The control group was fed distilled water.

At termination of this study, all animals were fasted overnight and euthanized by
CO$_2$ anesthesia. The blood samples were collected by cardiac puncture and
centrifuged at 1200 $\times$ g for 10 min to obtain serum. The vital organs including livers,
spleens and kidneys were collected, blotted and weighed. A part (1 cm $\times$ 1 cm) of the
liver tissue from the right lobe was fixed in 10% buffered formalin solution and
embedded in paraffin. The paraffin-embedded tissues were stained with either
hematoxylin & eosin (H&E) or Sirius red. A pathologist reviewed the histology of
tissue samples. Quantification of Sirius red-positive areas within hepatic parenchyma
was performed by computer-assisted color image analysis (Image J). The remaining
liver tissues were frozen in liquid nitrogen and then stored at -80 °C.

2.3 Biochemical analysis of liver function
The serum activities of aspartate transaminase (AST), alanine transaminase (ALT),
the levels of total triglyceride (TG) and cholesterol (T-chol) were analyzed by
enzymatic methods using an automatic blood biochemistry analyzer (Fujifilm Dri-
Chem 3500s; Fujifilm, Kanagawa, Japan).

2.4 Tissue protein extraction and western blot analysis
The protein extraction and western blot analysis were carried out as previously
described$^{22, 23}$. Briefly, liver tissues from each mouse were homogenized individually
and total proteins of livers were extracted by using ice-cold lysis buffer (20 mM Tris-
HCl, pH 7.4; 1 mM NaF; 150 mM NaCl; 1 mM ethylene glycol tetraacetic acid; 1
mM phenylmethanesulphonyl fluoride; 1% NP-40; and 10 µg/mL leupeptin). The concentrations of total protein were determined by Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). The protein sample (50 µg) from each mouse was mixed with 5 × sample buffer, following subjected to SDS-polyacrylamide gel and electrotransferred onto immobile polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Bedford, MA). The membranes were blocked by blocking solution and then immunoblotted with primary antibodies against TGF-β1, α-SMA, phospho-Smad 2 (p-Smad 2), phospho-Smad 3 (p-Smad 3) and β-actin for 12 h. After washing with TBST buffer solution, the membranes were applied with horseradish peroxidase-conjugated secondary antibody for 1 h and then visualized by enhanced chemiluminescence agent (ECL; Amersham Corp., Arlington Heights, IL, USA). The densities of the bands were quantified by densitometric scanning (Alliance 4.7, UVItec, Cambridge, UK). The values are presented as the relative levels of TGF-β1, p-Smad 2, p-Smad 3 and α-SMA over the control group.

2.5 RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Liver tissues from each mouse were homogenized individually and total RNA was extracted using Trizol reagent (Invitrogen, Carsbad, CA, USA). The RNA samples extracted from liver tissues were transcribed into cDNA by SuperScript II Reverse Transcriptase (Invitrogen, Renfrewshire, UK). The reverse transcription reaction was achieved at 42 °C for 60 min and then 72 °C for 15 min in Gene Cycler thermal cycler (Bio-rad Laboratories, Munich, Germany). The thermal cycle conditions were initiated at 95 °C for 1 min, and then 25 cycles of amplification (94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s), followed by extension at 72 °C for 3 min. The coding
sequences of primers are: α-SMA, forward primer 5’-CGCTGAAGTATCCGATAGAACAC-3’, reverse primer 5’-CAGTTGTACGTCCAGAGGCATA-3’; β-actin, forward primer 5’-AAGAGAGGCATCCTCACCCT-3’, reverse primer 5’-TACATGGCTGGGGTGTTGAA-3’. The amplified products were resolved by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized under ultraviolet light. The values are presented as the relative levels of TGF-β1, p-Smad2, p-Smad3 and α-SMA over the control group.

2.6 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 Effects of garcinol on the body weights and relative organ weights of the DMN-treated rats.

The body weights of rats during this study are shown in Figure 3. The body weights of DMN-treated rats were significantly lower than the control rats at the end of the study, whereas no significant differences were found among the DMN-treated animals regardless of garcinol supplementation. The relative organ weights are given in Table 1. The relative weights of livers and kidneys in the DMN-alone group showed no significant differences when compared with the control group, whereas the relative weight of spleens was significantly higher than the control group. The supplementation of garcinol did not alter the relative weights of livers, kidneys and spleens when compared with DMN-alone group.

3.2 Effects of garcinol on the serum biochemical parameters in the DMN-treated rats.

ALT, AST, TG and T-cho in serum of rats are shown in Table 2. AST and ALT are the well-known indicators of liver injury. The activities of ALT and AST in DMN-alone group were significantly higher than the control group, whereas the levels of TG and T-cho were significantly lower than those of the control rats. However, the administration of garcinol to DMN-treated rats did not affect the activities of ALT, the levels of TG and T-cho when compared with DMN-alone group, whereas the activity of AST was significantly reduced.

3.3 Effects of garcinol on the development of liver fibrosis and the regulation of fibrotic-related signaling pathway in the DMN-treated rats.
Histological examinations of livers in the control and DMN-treated rats by H&E staining were shown in Figure 4. No histological abnormalities were observed in the control group, whereas the DMN-alone group exhibited the congestion and destruction of hepatic architecture, massive and severe hepatocyte necrosis, as well as marked mononuclear cell infiltrates. In contrast, these abnormalities and alterations in the livers were reduced by supplementation with garcinol. The extent of liver fibrosis was also documented by Sirius red staining (Figure 5). A marked increase in Sirius red staining (stained in red) was found in the livers of DMN-alone group when compared with the control group. In contrast, the increase in Sirius red staining was markedly reduced after supplementation with garcinol.

The hepatic content of TGF-β1, phospho-Smad 2, phospho-Smad 3 as well as α-SMA were also measured to evaluate the effect of garcinol against DMN-induced liver fibrosis (Figure 6). TGF-β1 is a crucial fibrotic mediator and our results showed that the expression of TGF-β1 in DMN-alone group was significantly higher than the control group. However, the supplementation of garcinol suppressed the expression of TGF-β1. Similarly, the phosphorylation of Smad 2 and Smad 3 significantly increased by DMN treatment, whereas their phosphorylation was suppressed by garcinol supplementation.

Activated HSCs are associated with the accumulation of collagens and the expression of α-SMA. Our results indicated that the expression of α-SMA significantly increased in the DMN-alone group, whereas garcinol supplementation reduced its expression. These results were also consistent with the results of RT-PCR analysis of α-SMA (Figure 7).

4. DISCUSSION
Liver fibrosis involves the excessive deposition of ECM proteins, which is a common characteristic of most types of chronic liver diseases; the excessive accumulation of ECM proteins leads to disorganization of the normal lobular architecture by forming a fibrous scar, hepatic functional impairment, and development of nodules of regenerating hepatocytes, consequently resulting in cirrhosis. Hepatic fibrosis and cirrhosis are common risk factors in the development of HCC. It is estimated that HCC is not only the fifth most common cancer but also the third most common cause of cancer-related deaths. As a result, the prevention of hepatic fibrosis and cirrhosis might be an effective strategy to improve the prognosis of chronic liver disease. DMN-induced liver injury is considered as an appropriate preclinical model to investigate the therapeutic effects of a drug against liver fibrosis because the histopathological features and biochemical alterations induced by DMN resemble the development of liver fibrosis in humans. Thus, in our current study, we utilized the DMN-induced liver fibrosis model to mimic human liver fibrosis and investigate the protective effects of garcinol against liver fibrosis.

In this study, we found that the administration of DMN to rats for six consecutive weeks resulted in the decrease of body weights, the increases of serum activities of AST and ALT, as well as the accumulation of ECM components in livers (Tables 1, 2 and Figure 5). George et al. discovered that the decrease in body weights of rats and hepatic accumulation of collagen in livers are found in rats after the administration of DMN for three consecutive weeks by intraperitoneal injection. However, garcinol supplementation reduced the elevation of AST activity and the accumulation of ECM components when compared with the DMN-alone group. These results suggested that garcinol exhibited protective effects against DMN-induced hepatotoxicity as well as liver fibrosis. Similarly, histopathological lesions
including the destruction of hepatic architecture, hepatocyte necrosis, mononuclear cell infiltrates induced by DMN also greatly attenuated by supplementation of garcinol (Figure 4). Most importantly, previous studies indicated that supplementation with garcinol did not have negative effects on the livers of rats. After being fed a diet containing 0.05 % garcinol for 5 consecutive weeks, the body weights and liver weights of F344 rats showed no significant differences when compared with the control group\textsuperscript{25}. Similarly, dietary administration of the diet containing 500 ppm garcinol (8.3 mg garcinol/rat/day) for 32 weeks did not cause the decreases in body weights, liver weights, kidney weights and survival rate of F344 rats\textsuperscript{26}. It is noteworthy that intraperitoneal injection with garcinol (10 mg/ kg bw) did not cause elevation of AST and ALT activities in BALB/c mice\textsuperscript{27}.

Garcinol is a polyisoprenylated benzophenone derivative that is mainly derived from \textit{Garcinia indica}. Many studies demonstrated that both curcumin and tetrahydrocurcumin exhibit potent antioxidant activities against various diseases due to their β-diketone moieties and hydroxyl groups on the aromatic rings\textsuperscript{28}. Like curcumin and tetrahydrocurcumin, garcinol is also a potent antioxidant because it contains both a phenolic hydroxyl group and a β-diketone moiety. In many studies, the potent antioxidant activity of garcinol has been used to explain its observed biological benefits\textsuperscript{29}. DMN is a well-known carcinogen and previous studies have indicated that the metabolic activation of DMN resulted in oxidative stress, which may be one of key factors to induce the pathological conditions such as hepatocellular necrosis, carcinogenicity and tumor formation. For example, Vendemiale \textit{et al} indicated that the elevation of oxidative stress has also been implicated in DMN-induced fibrosis in rats\textsuperscript{30}. As a result, liver damage including the elevation of serum activities of AST and ALT, the disruption of hepatic architecture, as well as
hepatocyte necrosis found in DMN-treated rats are partially associated with the generation of ROS and RNS. Since many studies have demonstrated that garcinol is a potent antioxidant, the hepatoprotective effects of garcinol against DMN-induced liver damage including decreasing AST and ALT levels, as well as reducing hepatocyte necrosis found in our current study may be, at least in part, due to its potent free radical scavenging ability.

Kupffer cells, the resident macrophages of liver, are responsible for protecting hepatocytes by removing foreign particles, mainly microorganisms and bacterial endotoxins, from the portal circulation. They play a key role in innate immune responses and host defense through secretion of inflammatory mediators and ROS. Many pieces of evidence from different studies suggest that Kupffer cells may be implicated in the pathogenesis of various liver diseases such as viral hepatitis, HCC, alcohol-related liver disease, and liver fibrosis\(^3\). An animal study indicated that HSC activation and the accumulation of collagens induced by DMN were effectively inhibited by suppression of macrophage infiltration\(^31\). In response to liver injury, Kupffer cells produce the pro-inflammatory cytokines and growth factors that induce HSC myofibroblastic transformation. Among the various mediators produced from Kupffer cells or apoptotic hepatocytes, TGF-\(\beta1\) is recognized as a key cytokine to drive HSC transdifferentiation resulting in increased accumulation of collagen. Many studies have documented that TGF-\(\beta1\) is the main cytokine that drives fibrosis in different animal models, including alcohol-induced liver fibrosis, carbon tetrachloride (CCL\(_4\))-induced fibrosis, as well as DMN-induced liver fibrosis\(^22, 23, 32, 33\). Some studies reveal that natural occurring antioxidants exhibit their protective activities against liver fibrosis by inhibition of TGF-\(\beta1\) expression\(^34-36\). Similarly, our current study also demonstrated that the expression of TGF-\(\beta1\) and the phosphorylation of the
receptor-activated Smads (R-Smads) including Smad 2 and Smad 3 were significantly
reduced by supplementation of garcinol. These results suggested that the anti-fibrotic
effect of garcinol is partially due to its inhibitory effects on HSC activation through
suppressing TGF-β1 expression and the TGF-β-mediated signaling pathway. Moreira
has also suggested that inactivation of HSCs have been identified as a potential
therapeutic target in liver fibrosis such as inhibition of TGF-β and induction of HSC
apoptosis^4.

Liver fibrosis is a complicated pathological process in which multiple cells are
involved including HSCs, Kupffer cells and hepatocytes. After acute or chronic liver
injury, HSCs undergo an activation or transdifferentiation process and become
myofibroblast-like cells with several phenotypes, such as increased proliferation,
expression of α-SMA and synthesis of collagens. α-SMA is not only a commonly
used marker for the early stage of hepatic fibrosis, but also widely recognized as a
reliable indicator of HSC activation^4. According to the results of histopathological
examinations by H&E, Sirius red staining and western blotting analysis, our data
revealed that DMN-induced liver injury resulted in hepatic accumulation of ECM and
the elevation of α-SMA expression through the activation of HSCs (Figures 5 and 6).
Consistent with a role for garcinol in the inactivation of HSC, our results showed that
the expression of α-SMA induced by DMN was also suppressed (Figure 6). The
mRNA expression of α-SMA suppressed by garcinol was also confirmed by RT-PCR
analysis (Figure 7). Thus, our results suggested that the suppression of α-SMA gene
expression induced by DMN was at least in part due to the inactivation of HSCs by
garcinol through inhibiting TGF-β1 expression.
5. CONCLUSION

Our current study demonstrated that garcinol exhibited both hepatoprotective and anti-fibrotic effects against DMN-induced liver injury. The mechanism of anti-fibrotic effects by garcinol was associated with inhibiting the expression of TGF-β1 and α-SMA, as well as the phosphorylation of Smad 2 and Smad 3. In addition, garcinol might also exert beneficial effects on reducing the oxidative stress induced by DMN as a potent antioxidant, which exhibited the protective effects against liver damage. Thus, our findings suggest that garcinol supplementation may serve as a potential therapeutic strategy against liver fibrosis.
ABBREVIATIONS

ALT Alanine transaminase
AST Aspartate transaminase
COX-2 Cyclooxygenase-2
DMN Dimethylnitrosamine
ECM Extracellular matrix
HCC Hepatocellular carcinoma
HSCs Hepatic stellate cells
iNOS Inducible nitric oxide synthase
RNS Reactive nitrogen species
ROS Reactive oxygen species
TGF-β Transforming growth factor-β
TNF-α Tumor necrosis factor-α
LPS Lipopolysaccharide
α-SMA α-Smooth muscle actin
STAT3 Signal transducer and activator of transcription 3
ACKNOWLEDGMENT

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Figure 1. Chemical structure of garcinol

Figure 2. Schematic diagram of the experimental procedure. Rats were divided into four groups including (a) control, (b) DMN-alone (10 mg/kg bw), (c) DMN + low dose of garcinol (10 mg/kg bw) and (d) DMN + high dose of garcinol (25 mg/kg bw). The DMN-treated animals were administered DMN (10 mg/kg bw) via intraperitoneal injection three times a week (Mon, Wed, and Fri) for six consecutive weeks. The control group was given normal saline. Rats of DMN + garcinol supplementation groups were fed with either 10 or 25 mg/kg bw/day of garcinol for three consecutive weeks by oral gavage. The control group was fed with distilled water. The animals were sacrificed on day 43. Each group consisted of eight rats.

Figure 3. Effects of garcinol on the DMN-induced body weight loss in rats. DMN was intraperitoneally given at a dose of 10 mg/kg body weight three days a week for six consecutive weeks to each group except the control group (n=8). DMN (10 mg/kg): DMN-treated rats; DMN (10 mg/kg) + LG (10 mg/kg): DMN-treated rats with supplementation of 10 mg/kg garcinol; DMN (10 mg/kg) + HG (25 mg/kg): DMN-treated rats with supplementation of 25 mg/kg garcinol.

Figure 4. Effects of garcinol on the DMN-induced histopathological alterations in rats. Representative hematoxylin and eosin-stained sections of livers are shown from control and DMN-treated rats. (A) Control group; (B) DMN (10 mg/kg): DMN-treated rats; (C) DMN + Garcinol (10 mg/kg): DMN-treated rats with supplementation of 10 mg/kg garcinol; (D) DMN + Garcinol (25 mg/kg): DMN-treated rats with supplementation of 25 mg/kg garcinol. Non-overlapping liver
sections of the animal from each group are shown. The arrows indicate inflammatory foci.

**Figure 5.** Effects of garcinol on the DMN-induced deposition of hepatic ECM components in rats. Representative Sirius red-stained sections of livers from control and DMN-treated rats. (A) Control group; (B) DMN (10 mg/kg): DMN-treated rats; (C) DMN + Garcinol (10 mg/kg): DMN-treated rats with supplementation of 10 mg/kg garcinol; (D) DMN + Garcinol (25 mg/kg): DMN-treated rats with supplementation of 25 mg/kg garcinol. Representative liver sections of the animal from each group are shown. The arrows indicate the area of DMN-induced deposition of hepatic ECM components. Different liver sections of the animal from each group are shown. Quantification of Sirius red-positive areas within hepatic parenchyma was performed by computer-assisted color image analysis (Image J). Values are expressed as mean ± standard deviation (n=8) and analyzed using one-way ANOVA and Duncan’s test. *p < 0.01 versus control. *p < 0.01 versus DMN.

**Figure 6.** Effects of garcinol on the DMN-induced hepatic protein accumulation of TGF-β1, p-Smad2, p-Smad3 and α-SMA in rats. Total liver cell lysates were analyzed for the hepatic protein accumulation of TGF-β1, p-Smad2, p-Smad3 and α-SMA by Western blot analysis. The western blot is a representative of at least three independent experiments. Quantification of TGF-β1, p-Smad2, p-Smad3 and α-SMA expression was normalized to β-actin using a densitometer.

**Figure 7.** Effects of garcinol on the DMN-induced mRNA expression of α-SMA in rats. Total liver cell lysates were analyzed for mRNA expression of α-SMA by RT-PCR analysis. Quantification of α-SMA expression was normalized to β-actin using a densitometer.
Table 1. Effects of garcinol on the DMN-induced relative organ weight alterations in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Relative organ weight (g/body weight)</th>
<th>Liver</th>
<th>Kidney</th>
<th>Spleen</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td>3.44±0.41\textsuperscript{a}</td>
<td>0.79±0.08\textsuperscript{b}</td>
<td>0.17±0.01\textsuperscript{b}</td>
</tr>
<tr>
<td>DMN (10 mg/kg)</td>
<td></td>
<td>2.71±0.91\textsuperscript{a}</td>
<td>0.96±0.13\textsuperscript{ab}</td>
<td>0.36±0.02\textsuperscript{a}</td>
</tr>
<tr>
<td>DMN+LG (10 mg/kg)</td>
<td></td>
<td>2.80±0.58\textsuperscript{a}</td>
<td>0.98±0.14\textsuperscript{a}</td>
<td>0.37±0.10\textsuperscript{a}</td>
</tr>
<tr>
<td>DMN+ HG (25 mg/kg)</td>
<td></td>
<td>2.72±0.93\textsuperscript{a}</td>
<td>1.01±0.25\textsuperscript{a}</td>
<td>0.34±0.10\textsuperscript{a}</td>
</tr>
</tbody>
</table>

DMN was intraperitoneally given at a dose of 10 mg/kg body weight three days a week for six consecutive weeks to each group except the control group. Data are expressed as mean ± standard deviation (n=8) and analyzed using one-way ANOVA and Duncan’s test. Different letters represent statistically significant differences among treatments (p<0.05).
Table 2. Effects of garcinol on the DMN-induced serum parameter alterations in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameter</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>TG (mg/dL)</th>
<th>T-cho (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>68.4±10.7b</td>
<td>23.4±4.4b</td>
<td>177.9±54.9a</td>
<td>82.0±9.9a</td>
</tr>
<tr>
<td>DMN (10 mg/kg)</td>
<td></td>
<td>206.0±69.3a</td>
<td>115.0±50.5a</td>
<td>89.3±26.2b</td>
<td>52.0±12.9b</td>
</tr>
<tr>
<td>DMN+LG (10 mg/kg)</td>
<td></td>
<td>105.3±22.0b</td>
<td>75.3±22.9ab</td>
<td>87.0±17.0b</td>
<td>52.2±8.1b</td>
</tr>
<tr>
<td>DMN+ HG (25 mg/kg)</td>
<td></td>
<td>92.6±43.3b</td>
<td>64.0±31.8ab</td>
<td>77.0±52.9b</td>
<td>45.2±25.0b</td>
</tr>
</tbody>
</table>

DMN was intraperitoneally given at a dose of 10 mg/kg body weight three days a week for six consecutive weeks to each group except the control group. Data are expressed as mean ± standard deviation (n=8) and analyzed using one-way ANOVA and Duncan’s test. Different letters represent statistically significant differences among treatments (p<0.05).
Figure 1
Figure 2

- Saline (1 ml/kg bw, i.p)
- DMN (10 mg/kg bw, i.p)
- Water (10 ml/kg bw/day, oral)
- Garcinol (10 mg/kg bw/day, oral)
- Garcinol (25 mg/kg bw/day, oral)
Figure 3
Figure 4.
Figure 5.

Sirius red-positive parenchyma (%)
Figure 6.
Figure 7.