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Phyto-power dietary supplement potently inhibits dimethylnitrosamine-induced liver fibrosis in rats

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Curcumin has been extensively studied for its therapeutic effects in a variety of disorders. Fermented soy consumption is associated with a low incidence rate of chronic diseases in many Asian countries. The aim of this study was to investigate the potential underlying mechanisms of the effect of a phyto-power dietary supplement on liver fibrosis. Sprague-Dawley rats were intraperitoneally injected with dimethylnitrosamine (DMN; 10 mg kg^{-1}) three times a week for four consecutive weeks. A phyto-power dietary supplement (50 or 100 mg kg^{-1}) was administered by oral gavage daily for four weeks. Liver morphology, function, and fibrotic status were examined in DMN induced hepatic fibrogenesis. However, a phyto-power dietary supplement alleviated liver damage as indicated by histopathological examination of the α-smooth muscle actin (α-SMA) and collagen I, accompanied by the concomitant reduction of transforming growth factor-β1 (TGF-β1) and matrix metalloproteinase 2 (MMP2). These data indicate that the phyto-power dietary supplement may inhibit the TGF-β1/Smad signaling and relieve liver damage in experimental fibrosis.

1 Introduction

Hepatocellular carcinoma (HCC), one of the most common cancers,[1] currently ranks fifth in the most reported cancer incidences worldwide.[2] Numerous etiologies, including alcohol abuse, chemical intoxication, viral hepatitis infection, and autoimmune disorders, attribute to chronic liver fibrosis, which often advances further to cirrhosis.[3,4] Liver cirrhosis, a risk factor of developing HCC,[5] is associated with a poor prognosis and high mortality among the disorders of gastroenterology and hepatology.[3] Previously, liver fibrosis and cirrhosis were presumed to be irreversible responses, whereas current thoughts believe otherwise, if the underlying etiology is eradicated.[6] Therefore, establishing effective anti-fibrotic approaches could manoeuvre the course of liver fibrosis and cirrhosis and manage the treatment of chronic liver diseases.

An imbalance between oxidative stress and antioxidant defense is associated with various forms of chronic liver disease (CLD).[7,8] Reactive oxygen species (ROS) may result from damaged hepatocytes, activated inflammatory Kupffer cells, and injured mitochondria.[7] When the liver suffers from injury, wound-healing processes commence, extracellular matrix (ECM) proteins amass, and fibrosis or scarring may ensue subsequently. Hepatic stellate cells (HSC; also known as ito cells or lipocytes), one of the major components in fibrogenesis, switch from a quiescent, epithelial appearance to an activated, α-smooth muscle actin (α-SMA)-expressing myofibroblastic phenotype.[9,10] Excessive accumulation of ECM proteins followed by collagen type I is predominantly responsible for scarring.[11] The pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and fibrogenic mediators such as transforming growth factor-beta (TGF-β) are involved in a series of inflammatory and fibrotic processes.[8] TGF-β1, one of the major cytokines involved in liver fibrosis, initiates its fibrogenic cascade through binding to the TGF-β1 receptors.[12] Activated TGF-β1 receptors transmit the fibrogenic signal through Smad transcription factors, specifically by phosphorylating Smad2 and Smad3 which further form active complexes with Smad4 and translocate into the nucleus to regulate gene expression of the downstream targets such as collagen type I.[12] Therefore, anti-oxidative, anti-inflammatory, and anti-fibrotic measures, such as inactivation of HSC and elimination of pro-fibrogenic signaling, become promising strategies to prevent further liver damage.[4]

Curcumin (diferuloylmethane) is a hydrophobic polyphenol derived from the ground rhizome of the herb turmeric (*Curcuma longa*), which has been widely used as a spice and coloring agent in cooking and a constituent in folk medicine such as Ayurveda

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practice.[13] Curcumin exhibits antioxidant, anti-inflammatory, and anti-cancer properties, and has also been extensively studied for its therapeutic effects in various settings.[14] Moreover, it has been reported that curcumin may prevent cardiovascular, pulmonary, liver, and neoplastic diseases.[15]

Fermented soy consumption is associated with a lower incidence of chronic diseases in epidemiological and clinical studies in many Asian countries. Soy protein and isoflavones have been mostly studied for their multi-faceted beneficial effects.[16,17] Other biologically active soy components such as saponins and plant sterols have been suggested to have health benefits.[16] Further, many fermented soybean products, such as miso, natto, soy sauce, and tempeh, have shown a good quality of plant protein, enhanced metabolic effects, and improved immunity.[18]

A wide spectrum of studies have addressed the biological benefits and pharmacological actions of both fermented soy and curcumin in various aspects of chronic diseases; however, the effect of a fermented soy and curcumin mix on chronic liver disease has not been tackled. The objective of the current study is to investigate the effect of a phyto-power dietary supplement (a fermented soy and curcumin nutritional beverage mix) in an *in vivo* rat model of liver injury. Our data indicate that the phyto-power dietary supplement can prevent the progression of liver damage, possibly by inhibiting the TGF-β1/Smad signaling pathway, which further suggests a promising translational application of the phyto-power dietary supplement in liver disease.

2 Materials and methods

2.1 Reagents and chemicals

All reagents and chemicals were from Sigma, Inc. (St. Louis, MO) unless indicated otherwise. *N*-Nitrosodimethylamine (dimethylnitrosamine; DMN) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Jiva fermented soy and curcumin nutritional beverage mix was purchased from Essence of Life, LLC (Mount Kisco, NY) and used for the supplementation of the phyto-power dietary supplement in this study. The composition of the Jiva beverage mix was revealed in a US patent application 11/824,213, filed on June 29, 2009. 100 mg of Jiva fermented soy and curcumin nutritional beverage mix containing 50 mg of fermented whole soy bean and 2.5 mg of Curcumin C3 Complex® (Sabinsa Corporation, Piscataway, NJ), among other nutrients and minor ingredients. α-Smooth muscle actin (α-SMA) and matrix metalloproteinase 2 (MMP2) antibodies were obtained from Epitomics, Inc. (Burlingame, CA). TGF-β1, p-Smad2, and p-Smad3 antibodies were purchased from Transduction Laboratories (BD Biosciences, Lexington, KY). Beta-actin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2 Animals and phyto-power dietary supplement treatment

Adult male Sprague-Dawley rats (National Laboratory Animal Center, Taipei, Taiwan), weighing 200 to 250 g, were used in this study. All experimental procedures followed the guidelines established by the Institutional Animal Care and Use Committee of the National Kaohsiung Marine University (IACUC, NKMU, policy agreement #099-AAA9-02). All experiments with animals were performed in compliance with the relevant laws and institutional guidelines of the IACUC, NKMU. Laboratory Rodent Diet 5001 (PMI Nutritional International, Brentwood, MO) and water were available *ad libitum*. The rats were acclimated to a humidity-controlled facility with a 12 h dark/light cycle at 25 ± 1 °C for 2 weeks before experimentation. After the acclimation period, the animals were divided into 4 groups ($n = 6$): (i) control, (ii) DMN alone, (iii) DMN + phyto-power dietary supplement (50 mg kg^{-1}), and (iv) DMN + phyto-power dietary supplement (100 mg kg^{-1}). The control group was administered normal saline 1 mL. The DMN-treated animals were administered DMN (10 mg kg^{-1} body weight) *via* intraperitoneal (i.p.) injection three times a week (Mon, Wed, and Fri) for four consecutive weeks. The control group and the DMN alone group were given distilled water, whereas the DMN + phyto-power dietary supplement groups were given a phyto-power dietary supplement of 50 or 100 mg kg^{-1} body weight by dissolving 50 mg or 100 mg of phyto-power dietary supplement in distilled water, respectively, and administration by oral gavage daily for four consecutive weeks.

The animals were sacrificed under CO_2 anaesthesia after the study period of four weeks. Blood specimens were drawn by cardiac puncture and serum samples were collected and stored at −80 °C for further analysis. Livers, spleens, and kidneys were duly removed and their weights recorded. A 1 cm × 1 cm tissue fragment from the right lobe of the liver of each rat was fixed in 10% formalin and prepared for paraffin blocks. The paraffin-embedded sections were stained with Sirius red for collagen distribution or underwent immunohistochemistry for α-SMA. The remaining liver samples were instantly frozen in liquid nitrogen and stored at −80 °C.

2.3 Assessment of liver function

Serum levels of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), triacylglycerol (TG), and total cholesterol (T-chol) were measured to assess the liver function. For each biochemical parameter, serum was spotted onto the respective Fujifilm Dri-Chem slide (Fujifilm, Kanagawa, Japan) and analyzed using a blood biochemistry analyzer (Fujifilm Dri-Chem 3500s; Fujifilm, Kanagawa, Japan).

2.4 Protein extraction and Western blot analysis

Liver tissues were homogenized and total proteins were extracted using a gold lysis buffer [50 mM Tris–HCl, pH 7.4; 1 mM NaF; 150 mM NaCl; 1 mM ethylene glycol tetraacetic acid (EGTA); 1 mM phenylmethanesulfonyl fluoride; 1% NP-40; and 10 μg mL^{-1} leupeptin] and incubated on ice for 30 min, followed by centrifugation at 10 000 × *g* for 30 min at 4 °C. The protein concentration was measured by a Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). The samples (50 μg of protein) were mixed with 5 equivalents of sample buffer containing 0.3 M Tris–HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium dodecyl sulphate (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. Subsequently, electrophoresis was ordinarily carried out on SDS-polyacrylamide gels. For electrophoresis, 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, MMP2, TGF-β1, p-Smad2, p-Smad3, and β-actin at room temperature for 1 h. Detection was achieved by chemiluminescence measurements (ECL, Amersham Corp., Arlington Heights, IL) and densitometric scanning (Alliance 4.7, UVItec, Cambridge, UK).

2.5 Statistical analysis

All data were expressed as mean ± S.D. All statistical analyses were performed with the Student's *t*-test, using Sigma Plot 10.0. A value of $p < 0.05$ was considered statistically significant.

3 Results

3.1 Effect of phyto-power dietary supplement on body weights and relative organ weights of the DMN-treated rats

The body weights of the DMN-treated rats were significantly lower than those of the control animals at the conclusion of the study (Fig. 1). No difference was observed among the DMN-treated animals regardless of the administration status of the phyto-power dietary supplement (Fig. 1). It appears that the DMN treatment reduced the appetite and, consequently, the body weight of the animal. In the DMN-alone group, the weight of liver was significantly lower than that of the control animals, whereas the weights of the kidney and spleen were significantly higher than those of the control group (Table 1). Rats supplemented with the phyto-power dietary supplement, either at 50 or 100 mg kg^{-1} body weight, exhibited a significantly increase in the relative weight of the liver compared with the DMN-alone group (Table 1). The dose of the phyto-power dietary supplement changes neither the weight of the kidney nor the spleen in the DMN-treated animals (Table 1).

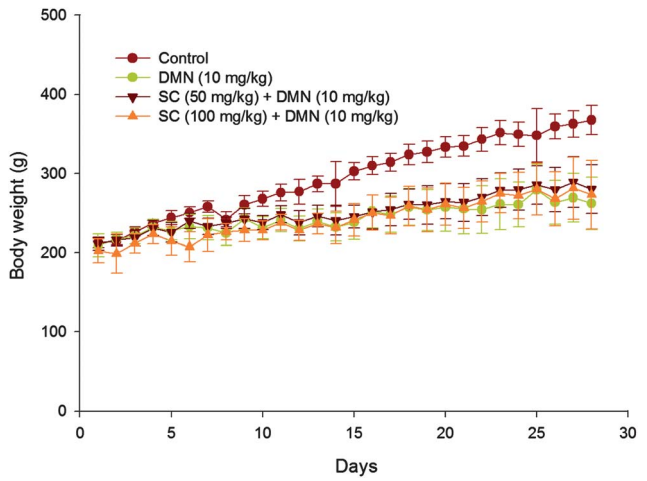

Fig. 1 Effect of the phyto-power dietary supplement (SC) on the body weight of the experimental rats. Dimethylnitrosamine (DMN) was given intraperitoneally at a dose of 10 mg kg^{-1} body weight three times a week (Mon, Wed, and Fri) for four consecutive weeks to each group except the control group. DMN (10 mg kg^{-1}), DMN alone; SC (50 mg kg^{-1}) + DMN (10 mg kg^{-1}), DMN with 50 mg kg^{-1} per day SC supplementation by oral gavage daily; SC (100 mg kg^{-1}) + DMN (10 mg kg^{-1}), DMN with 100 mg kg^{-1} per day SC supplementation by oral gavage daily.

Table 1 Effect of the phyto-power dietary supplement (SC) on the relative organ weights of the DMN-treated rats[ab]

	Relative organ weight (g/of body weight)		
Groups	Liver	Kidney	Spleen
Control	3.91 ± 0.35	0.96 ± 0.13	0.20 ± 0.04
DMN	2.49 ± 0.57[c]	1.20 ± 0.11[c]	0.51 ± 0.11[c]
SC (50 mg kg^{-1})	3.18 ± 0.41[d]	1.08 ± 0.11	0.45 ± 0.11
SC (100 mg kg^{-1})	3.20 ± 0.31[d]	1.10 ± 0.05	0.45 ± 0.09

[a] DMN was intraperitoneally given at a dose of 10 mg kg^{-1} three days a week for 4 weeks to each group except the control group. [b] The data represent the mean ± S.D. of eight rats. [c] Significantly different from the control group, $p < 0.05$. [d] Significantly different from the group treated with DMN alone, $p < 0.05$.

3.2 Effect of phyto-power dietary supplement administration on the liver function of the DMN-treated animals

GOT and GPT are biochemical indices of liver function; these two enzymes are released into the blood when the liver is injured. The DMN-alone rats exhibited significantly higher serum GOT, GPT, and TG levels than those of the control animals, whereas no difference was observed in the serum total cholesterol (T-chol) concentration (Table 2). The DMN-treated rats supplemented with the phyto-power dietary supplement, at 50 or 100 mg kg^{-1} body weight, exhibited significantly lower serum GOT and GPT levels compared with the DMN-alone animals; no effect was shown in the serum TG or T-chol levels (Table 2). These data suggest an amelioration effect of the phyto-power dietary supplement on DMN-induced liver damage.

3.3 The phyto-power dietary supplement inhibited the development of fibrosis and the activation of HSC

The morphological changes exhibited DMN-induced hepatic damage, due to the significant enhancement of collagen I (Fig. 2) and α-SMA (Fig. 3), as shown in the histopathological examinations, whereas the phyto-power dietary supplement inhibited the expression of both collagen I and α-SMA (Fig. 2 and 3). Furthermore, DMN increased the hepatic expression of both α-SMA and MMP2 proteins, whereas the phyto-power dietary supplement reduced their expression (Fig. 4A). Moreover, DMN appeared to activate TGF-β1/Smad signaling, but the phyto-power dietary supplement suppressed the expression of TGF-β1, p-Smad2, and p-Smad3 (Fig. 4B). All these data indicate a suppressive effect of the phyto-power dietary supplement on

Table 2 Effect of the phyto-power dietary supplement (SC) on the serum parameters in the DMN-treated rats[ab]

	Activity			
Groups	GOT (U L^{-1})	GPT (U L^{-1})	TG (mg dL^{-1})	T-chol (mg dL^{-1})
Control	91.17 ± 6.91	30.83 ± 23.72	58.67 ± 25.55	87.67 ± 17.40
DMN	458.00 ± 113.89[c]	241.50 ± 82.74[c]	137.17 ± 28.14[c]	77.33 ± 21.87
SC (50 mg kg^{-1})	139.17 ± 67.45[d]	102.67 ± 12.60[d]	50.83 ± 8.38	55.50 ± 6.53
SC (100 mg kg^{-1})	174.67 ± 40.05[d]	136.83 ± 49.89[d]	97.67 ± 26.88	67.67 ± 13.25

[a] DMN was intraperitoneally given at a dose of 10 mg kg^{-1} three days a week for 4 weeks to each group except control group. [b] The data represent the mean ± S.D. of eight rats. [c] Significantly different from the control group, $p < 0.05$. [d] Significantly different from the group treated with DMN alone, $p < 0.05$.

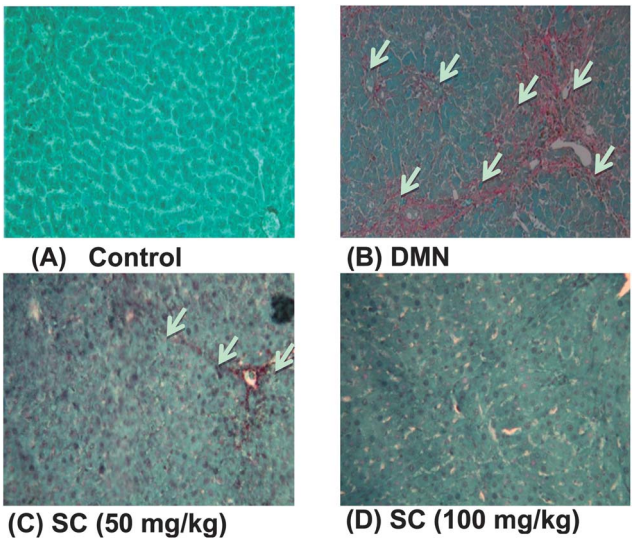

Fig. 2 Phyto-power dietary supplement (SC) reduces collagen deposition in dimethylnitrosamine (DMN)-treated animals. A representative set of Sirius red staining of the liver tissue sections is shown: control group (A), DMN rats (B), DMN rats receiving 50 mg kg^{-1} (C) or 100 mg kg^{-1} (D) of SC supplementation. The arrows indicate the areas of DMN-induced collagen deposition.

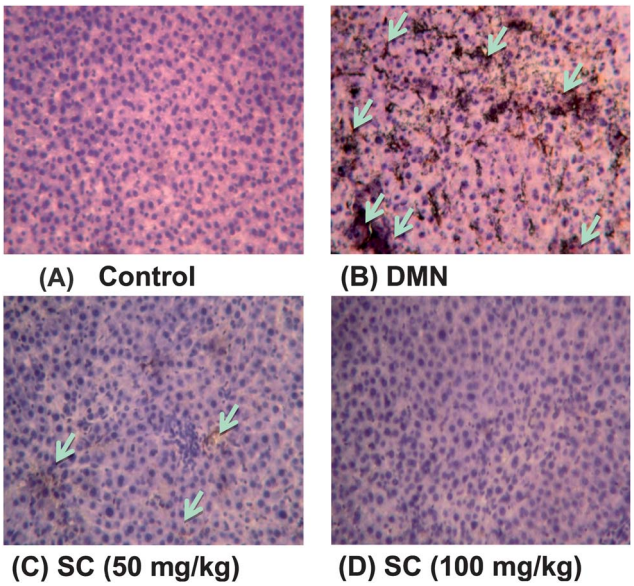

Fig. 3 Phyto-power dietary supplement (SC) reduces α-smooth muscle actin (α-SMA) formation in dimethylnitrosamine (DMN)-treated animals. A representative set of immunochemical staining of the liver tissue sections for α-SMA is shown: control group (A), DMN-treated animals (B), DMN rats receiving 50 mg kg^{-1} (C) or 100 mg kg^{-1} (D) of SC supplementation as indicated. The arrows indicate the accumulation of DMN-induced α-SMA expression.

the development of liver fibrosis and the activation of hepatic stellate cells, potentially by inhibiting the TGF-β1/Smad signaling pathway.

4 Discussion

Oxidative stress levels that override antioxidant defense often triggers liver damage.[7] Excess ROS activates HSC, recruits pro-inflammatory cytokines, and initiates downstream responses. Increased expression of α-SMA and collagen I as well as the secretion of inflammatory mediators facilitate fibrotic processes.[9,10] Other players involved in fibrosis include matrix metalloproteinase family members, where MMP2 has been shown to be responsible for hepatic fibrogenesis.[19,20] The current study employed the DMN-induced liver injury model, which closely resembles the development of liver damage in humans, including histopathological manifestations and biochemical alterations.[21,22] Using the animal model of DMN-induced liver injury, we found that DMN intoxication inhibited an animal's growth, damaged liver function, activated TGF-β1/Smad signaling, increased the expression of α-SMA, MMP2, and collagen, and instigated hepatic fibrogenesis. However, with oral administration of the phyto-power dietary supplement, the DMN-induced damage was relieved by increasing the relative weight of the liver and spleen, improving the biochemical indices of the liver function and histopathological appearance of hepatic morphology.

Most commercial curcumin preparations from turmeric often contain curcumin (77%), demethoxycurcumin (curcumin II) at 17%, and bis-demethoxycurcumin (curcumin III) at 3%.[13] Curcumin used in our study was Curcumin C3 Complex®, which also contains these curcuminoids in similar ratios as described. The biological activity of these compounds vary; some data suggest that a mixture of curcuminoids exhibits synergistic effects over individual components.[14,23]

Curcumin has been widely used for studying the mechanism of liver injury.[24] The antifibrotic effect of curcumin has been

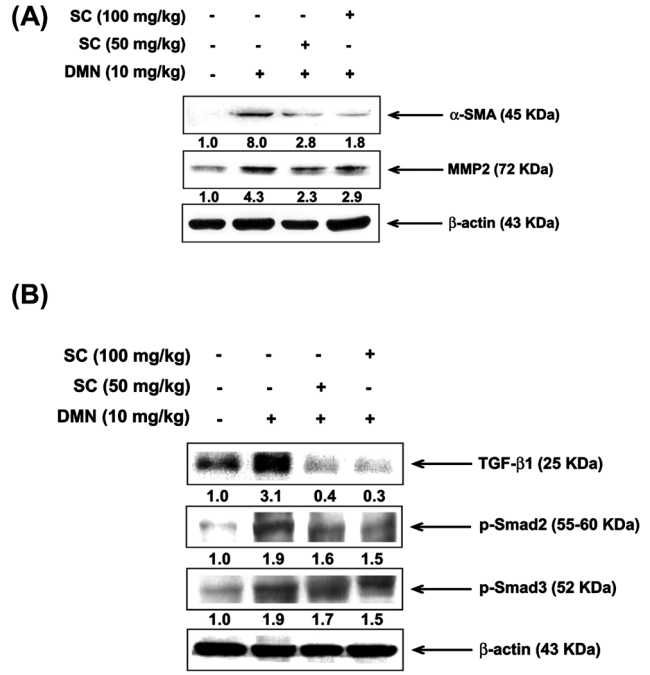

Fig. 4 Phyto-power dietary supplement (SC) supplementation inhibits α-SMA and MMP2 expression (A) and suppresses TGF-β/Smad signaling (B) in dimethylnitrosamine (DMN)-treated rats. DMN was given intraperitoneally at a dose of 10 mg kg^{-1} body weight three times a week (Mon, Wed, and Fri) for four consecutive weeks to each group except the control group. DMN (10 mg kg^{-1}), DMN alone; SC (50 mg kg^{-1}), DMN with 50 mg kg^{-1} per day SC supplementation by oral gavage daily; SC (100 mg kg^{-1}), DMN with 100 mg kg^{-1} per day SC supplementation by oral gavage daily. Total liver cell lysates were analyzed for the expression of α-SMA, MMP2, TGF-β1, p-Smad2, and p-Smad3 by Western blotting analysis; β-actin served as a loading control.

shown in hepatic stellate cells.[25] Curcumin can induce apoptosis, suppress the gene expression of extracellular matrix proteins, activate peroxisome proliferator-activated receptor-γ, and inhibit the growth of hepatic stellate cells.[25] In experimental models of liver damage, curcumin inhibits oxidative stress and inflammation in an animal model of carbon tetrachloride-induced liver injury[26] and suppresses inflammatory responses in the development of thioacetamide-induced liver cirrhosis.[27]

Despite the promising features of safety and efficacy as shown in animal studies and clinical trials, developing suitable formulations of curcumin is limited by its low bioavailability and poor water solubility.[13] It is noteworthy that the dose of curcumin used in the previous animal studies was mostly between 50 and 400 mg kg^{-1}, whereas the amount of curcumin used in the current study (C3 Curcumin Complex®) was only 1.25 and 2.5 mg kg^{-1} body weight in 50 mg and 100 mg of the fermented soy and curcumin mix, respectively. Therefore, the combination of fermented soy and a small amount of curcuminoids appears to be an applicable solution to the poor solubility and color staining action of curcumin in formulations.

Biologically active components of soy, including soy protein, isoflavones, saponins and plant sterols, have been studied for their beneficial effects against chronic diseases.[16,17] However, in contrast to curcumin, sparse studies have been documented for the effect of soy on liver injury. Most reports focused on the roles of isoflavones, including a protective effect on the experimental steatohepatitis animal model[28] and carbon tetrachloride-induced liver damage,[29] and inhibition of cell proliferation and induction of apoptosis in experimental hepatic carcinoma.[30]

Many fermented soybean products, such as miso, natto, soy sauce, and tempeh, have drawn attention due to their health benefits, which appear to be responsible for the lower incidence rate in a number of chronic diseases in Asia.[17,18] Consumption of fermented soy milk lowers liver total cholesterol and triglyceride levels under CCl_4-induced oxidative stress.[31] Hu *et al.* reported that a commercial product of fermented soybean extracts exhibits strong antioxidant activity *in vitro* and induces higher levels of antioxidant enzymes in rats.[32] Chungkookjang, a Korean fermented soy product, has also been found to sequester free radicals and exhibit cytoprotective effects.[33]

In our study, the inhibitory effect of DMN toxicity on liver injury was probably due to the anti-oxidant and anti-inflammatory properties of the fermented soy and curcumin mix. The development of liver fibrosis and cirrhosis is essentially a series of oxidative stress and inflammatory responses, including the activation of HSC and the secretion of pro-inflammatory cytokines. Our data suggest that the phyto-power dietary supplement exhibits a protective effect against liver injury, which may be potentially applicable to the clinical prevention and treatment of liver damage.

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