Taiwanese Green Propolis and Propolin G Protect the Liver from the Pathogenesis of Fibrosis via Eliminating TGF-β-Induced Smad2/3 Phosphorylation

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ABSTRACT: Pathogenesis of fibrosis is a common process leading to chronic liver diseases and liver cirrhosis. New compounds for disease treatment and adjuvant therapy have been important issues in recent years. In this study, we isolated propolin G from Taiwanese green propolis (TGP) and investigated its antifibrotic effects by utilizing active hepatic stellate cells (HSCs) fibrosis model. Our results showed that TGP and propolin G inhibited α-SMA, collagen expression, and proliferation of HSC-T6 cells after TGF-β treatment. They also reduced the accumulation of extracellular matrix (ECM) components such as collagen Iα1 (Col Iα1) through down-regulating JNK signaling. Subsequently, mRNA and protein expression of Smad2/3 but no other Smad members was specifically down-regulated in the presence of propolin G. This effect also significantly induced apoptosis-associated expression of cleaved caspase-3 and caspase-7 proteins for fibrotic cell clearance. In in vivo experiments, we found that propolin G and TGP can reduce plasma alanine aminotransferase (ALT) activation and perhaps lead to the prevention of alcohol-induced liver cirrhosis. Furthermore, TGP can significantly decrease the malondialdehyde (MDA) level but has no influence on plasma or hepatic superoxide dismutase (SOD) and glutathione peroxidase (GPx) levels, suggesting TGP protects the liver from alcohol-induced injury through antioxidant-independent pathways. In conclusion, this study provides a new perspective of propolin G and TGP on liver protection, and its application has potential for health management by daily supplement or adjuvant therapy in related diseases.

KEYWORDS: propolin G, apoptosis, collagen, liver fibrosis, Smad2/3, α-SMA, TGF-β1

INTRODUCTION

Liver fibrosis is the outcome of multiple forms of chronic liver disease, most commonly viral hepatitis, excessive alcohol consumption, or chronic biliary obstruction. Liver fibrosis is an aberrant wound-healing response, which results in the formation of a hepatic scar. Histologically, fibrosis is characterized by the disruption of the hepatic architecture and excess deposition of extracellular matrix (ECM) components, resulting in increased fibrillar collagens, proteoglycans, and fibronectin1,2 because of their increased production and reduced degradation.4 The cells primarily responsible for the production of the ECM in the fibrotic liver are the hepatic stellate cells (HSCs), which can be activated and transformed into fibrogenic myofibroblast-like cells, with enhanced proliferation, fibrogenesis, and ECM synthesis.4 Therefore, inhibiting the activation and proliferation of HSCs could provide an effective strategy for antifibrosis.

Previous studies have identified several mediators of HSC transdifferentiation in vitro, including growth factors, lymphocytes, apoptotic fragments of hepatocytes, oxidative stress, and matrix components.4,5 Growth factors that are heavily involved in the process include transforming growth factor (TGF)-β. TGF-β is essential for the development of hepatic fibrosis in experimental animals. TGF-β1 acts through various signaling pathways, predominantly the canonical Smad pathway.5,6 The Smad proteins are divided into three functional classes: receptor-regulated (Smads 1, 2, 3, 5, and 8), common mediator (Co-Smad4), and inhibitor (Smads 6 and 7).5,6 TGF-β signaling occurs through transmembrane receptors that stimulate cytoplasmic Smad proteins, which modulate the transcription of target genes, including those encoding ECM proteins, such as procollagen-I and procollagen-III.7 Previous studies have shown that disruption of TGF-β/Smad signaling, predominantly because of down-regulated Smad3 or up-regulated Smad7 expression, produces resistance to tissue fibrosis in several organs8−10 and suggested that reducing TGF-β synthesis or inhibiting components of its complex signaling pathways could provide effective strategies for the treatment of liver fibrosis.5−4 Because Smad3 plays a crucial role in mediating the pathobiology of fibrotic disease, the inhibition of Smad3 signaling could also provide a useful intervention in fibrotic conditions.

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Six propolins, A–G, can be isolated from Taiwanese green propolis (TGP) and exert a broad spectrum of biological activities. TGP is widely used in Taiwanese traditional medicine and reportedly exhibits anticancer, antioxidant, and antimicrobial properties. Chen et al. used NMR and fast atom bombardment mass spectrometry to identify the chemical structure of propolin G and showed it to be identical to that of the compound nymphaeol C. In our previous study, we found several active ingredients with TGP, including propolin G. It was revealed that propolin G can efficiently induce apoptosis in brain cancer cell lines (glioma and glioblastoma) through mitochondrial- and caspase-dependent pathways. Using the 1,2-diphenyl-2-picrylhydrazyl (DPPH) assay, we also showed that propolin G displays free radical scavenging activity and that propolin G, TGP, and Brazilian propolis (BP) extracts exert neuroprotective activity against oxidative stress in rat primary cortical neurons. Because the apoptosis is an important response in physiological condition, especially in liver steatosis, we would like to investigate whether TGP and propolin G can modulate the process of liver fibrosis in hepatocytes. Propolis was collected from Chang-Hwa, a county in the middle of Taiwan. In the past, we collected propolis from northern, central, and southern Taiwan for analysis. We found that propolin G was most abundant from central Taiwan. Therefore, we utilized propolis from Chang-Hwa for this study. Therefore, we modified the previous purification method and used a prepared column and improved analysis method for mass propolin G collection (Figure 1). This study aimed to identify the underlying antifibrotic molecular mechanisms of TGP and propolin G to potentially facilitate the development of a novel treatment for liver fibrosis targeting Smad3 signaling components. According to our research, this study is the first to show that TGP and propolin G selectively down-regulate the expression of Smad2/3, but not Smads 4 and 7, mRNA, and protein in cultured rat HSCs. This process, in turn, significantly attenuates the TGF-β-induced deposition of Col Iα1 in the HSCs. Our results suggest that TGP and propolin G exert their antifibrogenic effects by directly or indirectly down-regulating Smad2/3 protein expression and phosphorylation through TGF-β signaling.

Alcoholic liver injury is a complex process involving several injury mechanisms and multiple cellular targets. Ethanol oxidation and ethanol-induced injury occur in hepatocytes; thus, determining the mechanism by which ethanol primes and sensitizes hepatocytes is very important. It was revealed that ethanol exposure inhibits hepatocyte regeneration in vitro and in vivo systems. Free radicals can bind to cellular molecules (i.e., nucleic acids, proteins, lipids, and carbohydrates), induce lipid peroxidation, damage the membranes of liver cells and organelles, cause the swelling and necrosis of hepatocytes and result in the release of cytosolic enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) into the circulating blood. It is well-known that superoxide dismutase...
(SOD), catalase (CAT), and glutathione peroxidase (GPx) act by scavenging the superoxide anion and H2O2 to prevent reactive oxygen species (ROS)-induced damage. The present research therefore has the objective to explore the antioxidant properties and hepatoprotective effects of Taiwanese green propolis against ethanol-induced liver injury in mice. In this study, it was revealed that administration of TGP inhibited plasma or hepatic malondialdehyde (MDA) and ALT activation, whereas TGP has no influence on plasma or hepatic SOD and GPx levels in ethanol-treated mice. Further study is necessary to evaluate the effect of TGP on liver fibrosis prevention and to determine the molecular mechanisms.

## MATERIALS AND METHODS

### Propolis Origins.
Taiwanese green propolis was collected from Taiwan. All samples were collected from May to July 2011, using propolis collectors. Propolis from the collectors at each location was gathered every month and kept at −20 °C until processed.

### Extraction and Isolation of Propolin G.
The TGP (50 g) was extracted with 95% ethyl alcohol (250 mL × 3), sonicated for 3 h, and left to stand for 21 h at 25 °C. The filtered ethanol extract was evaporated to dryness under reduced pressure to yield a brown gum (34.5 g), which was kept at −20 °C until used. The brown gum was dissolved in methanol and applied to an open silica column for the purification of propolin G. An n-hexane/EtOAc solvent system was used as the eluting solvent. Fraction 4 (n-hexane/EtOAc, 40:60) contained higher levels of propolin G than the other fractions. Fraction 4 was evaporated to dryness under reduced pressure to yield a yellow gum. The yellow gum was dissolved in methanol and carried out on a reversed-phase (RP) preparative high-performance liquid chromatography (HPLC)-UV. Fractions of retention times at 17.0 min for propolin G were collected. Conditions were as follows: column, Macherey-Nagel (VP 250/10 NUCLEODUR C18 HTEC, 5 μm); solvent system, methanol/water (88:12); flow rate, 3.5 mL/min; and detection, UV 280 nm. We identified the compound as propolin G (Figure 1). The purity of the propolin G was estimated to be no less than 95% by HPLC-UV on the basis of the peak area.

### Analysis Conditions.
The propolin profile in the TGP extracts was analyzed with a RP HPLC-UV. The conditions were as follows: column, ZORBAX SB-C18 (4.6 × 250 mm); Agilent, Santa Clara, CA, USA); solvent system, methanol/water (88.8:11.2); flow rate, 1.0 mL/min; and detection, UV 280 nm.

### Chemicals and Reagents.
TGP and propolin G were stored as a stock solution in DMSO, which was used after dilution with medium for each assay. Trizol reagent and RevertAidi MMLV reverse transcriptase were obtained from Fermentas Life Science (Lithuania); Taq polymerase, dNTPs, and DNA marker were from TaKaRa Biotechnology Co. (Dalian, China). Primers for rat PAI-1, Smad3, Smad2, Smad7, Smad4, type I, type II, Col I (product size 202 bp), and rat G3PDH (Genbank BC063166), forward 5′-′-CAT GGT GAA CCC TTC |′-′-CAT GGT GAA CCC TTC′, respectively. The resulting cDNA was subsequently subjected to 40 cycles of PCR. PCR products were quantitated to confirm that they were in the linear range of amplification. PCR products were quantitated by Quantum RNA18S internal standards (Ambio). The following primers were used in this study: rat α-SMA (Genbank L26110), forward 5′-′-TTT GTT ACT GCT GAT CGT GAG-3′, reverse 5′-CGG GGA AGT AGG TCG ATT TC-3′ (product size 202 bp); and rat G3PDH (Genbank BC063166), forward 5′-′-CAT GCC GAC GAC GCC AG -3′ (product size 452 bp). The conditions for RT-PCR were as follows: cDNA synthesis and pre-denaturation were at 42 °C for 60 min, followed by 94 °C for 10 min. PCR amplification sequence was performed for 40 cycles at 94 °C for 45 s and at 57 °C for 45 s. After amplification, each sample was applied to 1.2% agarose/ethidium bromide gel. The resolved PCR products were photographed under UV illumination.

### Western Blot.
For Western blot, treated cells were harvested by the addition of ice-cold lysis buffer as described above. Centrifuged lysates (30 μg) from each sample were analyzed by SDS–polyacrylamide gel electrophoresis and transferred to a PVDF membrane by semidry transfer. The membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween 20 and 3% BSA. Blots were probed overnight at 4 °C with the following primary antibodies: anti-α-SMA, anti-Smad2/3, anti-pho-Smad2/3, and anti-β-actin antibodies. This was followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody at a dilution of 1:1000 for 1 h. Detection was achieved by enhanced chemiluminescence (Amersham Pharmacia Biotech) followed by luminescent image analyzer (Image-Quant LAS4000). Digital data were further quantitated by scanning densitometry using ImageQuant TL with a 1-D Analyst software package for Macintosh. The relative expression fold was all normalized with internal control (GAPDH or tubulin).

### Animal Study.
The animal study was approved by the Review Boards of National Ilan University with approval no. 102-8. Briefly, mice were housed in the animal center of National Ilan University according to general guidelines at 25 ± 1 °C with a 12 h dark/light cycle. For hepatic injury experiments, male 7-week-old C57BL/6J mice (approximately 21 g) were obtained from the National Laboratory Animal Center, Taipei, Taiwan. The study protocol was in accordance with the animal experimental guidelines of the Council of Agriculture, Executive Yuan. Each experimental group consisted of six mice, and to determine the optimal concentration of TGP or propolin G for use in these experiments, dose-dependent cytotoxicity was initially performed with different concentrations of TGP or propolin G added to culture for 48 h. Cell viability was determined by incubating each well with 10 μL of WST-1 for 4 h at 37 °C. Metabolically active cells cleave WST-1 to water-soluble formazan, which is directly quantitated with an enzymatically linked immunosorbent assay plate reader (Bio-Rad 3550). Each experiment was performed at least twice, and treatment for the cell line was done in triplicate.

### Preparation of RNA and Protein Extracts.
HSC-T6 cells were plated at a confluence of approximately 75% in the wells of a 6-well dish and allowed to attach overnight. Cells were treated with 10 ng/mL TGF-β for 12 h before being treated with a variety of concentrations of propolis, propolin G, or SB-431542 for 12 h to determine RNA levels and for 24 h to analyze TGF-β-regulated protein expression. Treated cells were harvested, washed, and lysed in ice-cold buffer (250 mM Tris-HCl, pH 7.4, containing a mixture of protease inhibitors {100 2 g/mL phenylmethanesulfonyl fluoride, 1 μg/mL E-64, 0.5 μg/mL leupeptin, and 1.7 μg/mL pepatin A}). The debris was removed by centrifugation at 13000g for 20 min at 4 °C. Total protein amount in the extracts was measured using the Bio-Rad Bradford reagent. Total RNA was isolated from HSC-T6 cells as described by Chomczynski and Sacchi.

### Reverse Transcription Polymerase Chain Reaction Assay.
After treatment, RNA was harvested with Trizol (Invitrogen, Carlsbad, CA, USA). For each reaction, 1 μg of total RNA was reverse-transcribed to cDNA using an RT kit (MBI) for reverse transcription polymerase chain reaction (RT-PCR) (Ambion) as recommended by the manufacturer. The resulting cDNA was subsequently subjected to 40 cycles of PCR. PCR products were quantitated to confirm that they were in the linear range of amplification. PCR products were quantitated by Quantum RNA18S internal standards (Ambio). The following primers were used in this study: rat α-SMA (Genbank L26110), forward 5′-′-TTT GTT ACT GCT GAT CGT GAG-3′, reverse 5′-CGG GGA AGT AGG TCG ATT TC-3′ (product size 202 bp); and rat G3PDH (Genbank BC063166), forward 5′-′-CAT GCC GAC GAC GCC AG -3′ (product size 452 bp). The conditions for RT-PCR were as follows: cDNA synthesis and pre-denaturation were at 42 °C for 60 min, followed by 94 °C for 10 min. PCR amplification sequence was performed for 40 cycles at 94 °C for 45 s and at 57 °C for 45 s. After amplification, each sample was applied to 1.2% agarose/ethidium bromide gel. The resolved PCR products were photographed under UV illumination.

### Cell Culture.
HSC-T6 cells, derived from immortalized and activated HSCs transfected by SV40, were kindly donated by Professor Friedman from Mount Sinai Medical Center and were maintained at 37 °C in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS), 100 units/mL penicillin, and 100 μg/mL streptomycin under a 5% CO2 atm. These cells are spindle-shaped and have a bipolar appearance, with cytoplasmic filaments visible. They express the typical features of an activated rat HSCs transfected by SV40, were kindly donated by USA). All other reagents were analytically pure.

### Cell Viability Assays.
HSC-T6 cells were seeded overnight at 10000 cells/well in 96-well plates in DMEM, and the subconfluent cells in culture (75%) were washed twice with phosphate-buffered saline and serum-starved for 24 h with serum-free medium. To
each of them was administered TGP (200 and 400 mg/kg/day) for 28 days. For injury, mice were administered alcohol (5 g/kg) followed by sacrifice after 24 h. Blood was drawn from the heart for serum analysis, and livers were quickly excised to analyze the levels of SOD, catalase, GPx, and lipid peroxide. Serum was separated by centrifugation at 3000 \(g\) for 5 min for assays of ALT and ALS activity, and protein levels. These samples were preserved at \(-80^\circ\)C until biochemical assay.

**Statistical Analysis.** All values are given as means ± SE. Data analysis involved one-way ANOVA with subsequent Scheffé test. * and ** indicate significant differences at the levels of \(P < 0.05\) and \(P < 0.01\), respectively, compared to PBS alone.

### RESULTS AND DISCUSSION

**Effects of TGP or Propolin G on TGF-β1-Induced α-SMA mRNA and Protein Expression in HSC-T6 Cells.** To evaluate the cytotoxic effects of TGP and propolin G on HSC-T6 cells, we used the MTT assay to analyze HSC-T6 cell viability after treatment with TGP or propolin G. Our results showed that HSC-T6 cells can be treated with TGP (up to 2 ug/mL) or propolin G (up to 2 μM) for up to 48 h without any effects on cell viability (Figure 2A). The IC50 of TGP on HSC-T6 cell was 9.22 μg/mL at 24h and 7.61 μg/mL at 48 h while the IC50 of propolin G was 6.61 μM at 24h and 6.22 μM at 48h. We also evaluated the potential inhibitory effects of TGP and propolin G on TGF-β1-mediated biological effects in HSC-T6 cells. To do this, we pretreated the cells with 1.5 ng/mL TGF-β1 before exposing them to TGP or propolin G. Around IC50 concentration, the cell viability was elevated 24h after treatment, however, with prolong treatment for 48h, the cell viability was reduced when compared with those of 24h after treatment. Thus, TGP and propolin G treatments can attenuate TGF-β1-stimulated HSC cell viability in a dose-dependent manner (Figure 2B). We then analyzed the cells’ responsiveness to TGF-β1 by evaluating α-SMA mRNA and protein expression. We observed significant up-regulation of α-SMA mRNA and protein expression following the treatment of HSC-T6 cells with 1.5 ng/mL TGF-β1 for 24 h (Figure 2C).
Furthermore, we observed the reduction of TGF-β1-induced α-SMA protein expression especially after 4 μM propolin G treatment in the HSC-T6 cells (Figure 2D). However, although the inhibitory effect was more significant after 8 μM treatment, we cannot exclude the cytotoxicity effect. These results indicated that TGP and propolin G attenuated the TGF-β1-dependent up-regulation of α-SMA mRNA and protein expression in HSCs. This is the first study to show that TGP and propolin G can reduce the TGF-β1-induced accumulation of ECM in cultured HSC-T6 cells. One potential underlying mechanism of these effects might be the disruption of TGF-β1 signaling by selectively targeting Smad2/3. To evaluate this mechanism, we first investigated the cytotoxicity of TGP and propolin G to HSC-T6 cells using the MTT assay. Our results showed that TGP and propolin G have low cytotoxicity to HSCs, with an IC50 value of 100 μM (data not shown). The study by Well et al. described the antiproliferative activity of TGF-β in HSCs and rats, showing that the disruption of TGF-β1 signaling leads to increased HSC proliferation.6 In this study, nontoxic concentrations of TGP and propolin G minimally promoted HSC-T6 cell propagation (Figure 2), indicating that these compounds might antagonize TGF-β1 signaling.

TGP and Propolin G Down-regulate the TGF-β-Induced Expression of Profibrotic Gene and Inhibit TGF-β-Induced Smad2/3 and Smad2/3 Phosphorylation.

We used Western blotting to quantify the Col Iα1 protein level, in comparison with α-tubulin control. After pretreatment with TGF-β1 and treatment with TGP and propolin G in HSC-T6 cells, the expression level of Col Iα1 had no significant change after TGP or propolin G treatment except a slight reduction at
8 μM propolin G treatment, which might be due to cytotoxicity (Figure 3). Smad3 is considered the essential mediator of TGF-β signaling, because it directly activates genes encoding transcriptional regulators and signal transducers through the Smad2/3 DNA-binding motif characteristics of the immediate early target genes of TGF-β. In this study, we used RT-PCR
and Western blotting to evaluate the potential inhibitory effects of propolin G on TGF-β-mediated Smad2/3 and Smad2/3 phosphorylation in HSC-T6 cells. As shown in Figure 4A, TGF-β can significantly induce Smad2/3 phosphorylation in HSC-T6 cells in a time-dependent manner (from 15 to 120 min), suggesting that the phosphorylation of Smad3 by TGF-βRII is the initial step in signal transduction. We also evaluated the potential inhibitory effects of propolin G on TGF-β-induced Smad2/3 phosphorylation in HSC-T6 cells. We observed that propolin G reduced TGF-β-induced Smad3 phosphorylation in a dose-dependent manner in the HSC-T6 cells (Figure 4B). The HSCs are considered the primary sites of hepatic fibrogenesis, and studies have shown activated HSCs to be the key sources of excess ECM proteins in liver fibrosis.29,30 With regard to liver fibrosis, there are several pieces of evidence indicating that apoptosis played an important role in this process. For example, in CCl4-induced acute liver fibrosis, caspase and FasL were overexpressed in liver with caspase activation.31 In fact, in chronic hepatitis and liver damage, the progression of liver fibrosis was due to the reduced apoptosis because of other undamaged cells and growth factors contributing to tissue regeneration.32 During liver fibrogenesis, increased extracellular TGF-β initiates TGF-β-Smad3 signaling before activated Smad3/Smad4 complexes translocate into the nucleus and immediately bind and activate target genes, including Col Iα, TIMP-1, PAI-1, FN, and other ECM genes.33–36 PAI-1 is a specific target gene of Smad3 and is typically used as a marker of activated TGF-β-Smad2/3 signaling.37,38 In our study, TGP and propolin G significantly down-regulated TGF-β1-induced α-SMA mRNA and protein expression in HSCs (Figure 2), indicating that these compounds might directly or indirectly inhibit TGF-β1-Smad3/Smad2 signaling. These results were similar to those of Tang et al. that blockage of TGF-β-Smad2/3 signaling can efficiently protect liver from fibrosis.39 Furthermore, according to a study by Bissell et al., the down-regulation of PAI-1 protein expression could indirectly increase matrix degradation through increased plasmin activity.40 SB-431542 is a small molecular inhibitor of the type I TGF-β receptor through a specific and competitive A TGP-binding site kinase and inhibits the phosphorylation of Smad2/3 by binding TGF-βRII.38 This process, in turn, inhibits the exogenous TGF-β1-induced synthesis of FN, PAI-1, and Col Iα. In our study, TGP and propolin G down-regulated Smad2/3 mRNA and protein expression and reduced TGF-β1-induced Smad2/3 phosphorylation in HSC-T6 cells (Figure 4).

Propolin G Increases Phosphor-JNK MAPK Expression and Induces Apoptosis in TGF-β-Stimulated HSCs in...
TGF-β-Stimulated HSCs. To investigate the apoptotic signaling pathways underlying the effects of propolin G on HSC-T6 cells, we evaluated the expression of two signaling proteins associated with oxidative stress: phospho-JNK MAPK and phospho-Akt. The result indicated that p-P38 and p-JNK were significantly elevated 30 min and 2 h after TGF-β treatment; this was consistent with the previous study. However, we did not observe the elevation of p-ERK. We treated the HSCs with various concentrations of TGP (1–8 μg/mL) or propolin G (1–8 μM) prior to treatment with TGF-β for 15 min. Our Western blotting results showed that TGP and propolin G significantly up-regulated phospho-JNK and phospho-P38 MAPK expression in TGF-β1-stimulated cells (Figure 5). To investigate the effects of TGP and propolin G on apoptosis, we treated the HSCs with various concentrations of TGP (1–8 μg/mL) or propolin G (1–8 μM) for 24 h, before evaluating apoptosis. As shown in Figure 6A, TGP and propolin G activated caspase-7 and caspase-3 in a dose-dependent manner (Figure 6A). TGP and propolin G also activated caspase-9 and induced the cleavage of caspase-3 and PARP, in a time-dependent manner. Taken together, these results indicated that TGP and propolin G can not only attenuate TGF-β signaling via Smad2/3 inhibition but also enhance apoptosis through inducing p38 and JNK mediated caspase activity (Figure 6B). Because we cannot exclude the reduction of Smad2/3 phosphorylation by TGP or propolin G may due to cell apoptosis at this time, the results indicate TGP or propolin G can affect the process of fibrosis possibly through both TGF-β signaling inhibition and cell apoptosis below IC50 concentration. However, the issue of cell cytotoxicity at high dose in our findings should be carefully addressed when considering applications in the future when compared with other low toxicity compounds. On the other hand, these findings suggested that naringenin might reduce ECM components through a molecular mechanism different from SB-431542. Naringenin increases PI3K and MAPK ERK1/2 (p 38) activity in HepG2 cells in a manner similar to that of insulin. In previous studies, TGP and propolin G did not induce insulin receptor substrate phosphorylation. Although other studies have shown that insulin- and insulin-like growth factor-I-mediated signaling pathways modulate TGF-β signaling, the molecular details of the effects of these pathways on TGF-β signaling have yet to be fully elucidated. One mechanism by which anti-apoptotic signaling, mediated by PI3K-PKB, inhibits TGF-β signaling is the induction of interaction between PKB and Smad3, thus reducing the pool of Smad3 available for TGF-β signaling. The process is based on the down-regulating effects of insulin and insulin-like growth factors on TGF-β-regulated gene expression and can be reversed by using highly specific inhibitors of PI3K, LY294002, or wortmannin. Insulin significantly inhibits the phosphorylation of TGF-β1-induced Smad3, but not Smad2, whereas Smad2/3 protein expression remains unaffected by insulin treatment.

Ethanol Extract of Taiwanese Green Propolis against the Ethanol-Induced Hepatic Injury in Mice. We measured the activities of SOD and GPx and the MDA level in the plasma of mice. The MDA and ALT levels in EtOH-treated mice were significantly increased, as compared to that in the control group (Figure 7). Administering TGP (200 and 400 mg/kg/day) significantly inhibited the increase of MDA (Figure 7a) and GPx (Figure 7b) as well as SOD (Figure 7c). The SOD and GPx expression in the liver tissue was similar to plasma (data not shown). Furthermore, to examine whether the liver function can be rescued by TGP, we tested the ALT (Figure 7d) and AST (Figure 7e) to add support to the in vivo animal result. The data indicated that TGP can significantly reduce ALT and AST levels induced by ethanol, suggesting that TGP had a liver protection function. The production of ROS can also be evaluated indirectly by analyzing the level of MDA, a product of free radical-induced lipid peroxidation. To protect cells against oxidative damage induced by ROS, the antioxidant system in the body is activated, and endogenous antioxidant enzymes, such as SOD and GPx, scavenge ROS or prevent their formation. It has been reported that naturally fermented noni juice (NJ) contains polyphenols, polysaccharides, and some trace minerals. NJ promotes hepatoprotection against alcohol-induced injury due to regulations of lipid homeostasis, antioxidant status, alcohol metabolism (Chuang et al., 2013). TGP is widely used in Taiwanese traditional medicine and reportedly exhibits anticancer, antioxidant, antimicrobial properties. Interestingly, TGP has no influence on plasma or hepatic SOD and GPx levels but significantly decreased the MDA value. It is possible that TGP acts by several mechanisms, mediating a potential role in liver fibrosis retrieval in mice. Further study is necessary to evaluate the effect of TGP on liver fibrosis prevention and to determine the molecular mechanisms.

In conclusion, our results indicate that TGP and propolin G exert antifibrotic effects through the disruption of TGF-β- Smad2/3 signaling by directly or indirectly reducing Smad2/3 formation. These findings verify that Smad3 is a crucial mediator of TGF-β-modulated fibrogenic function and indicate that the inhibition of Smad2/3 protein function could potentially provide an effective strategy for the treatment of liver fibrosis. Although more detailed mechanistic studies are necessary to clarify the mechanisms of antifibrosis using TGP or propolin G, these results should encourage further studies to explore the potential antifibrosis effects of TGP or propolin G in chronic liver diseases.

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Author Contributions
Y.-L. S. Chen made contributions to concept and design and gave final approval of the manuscript to be published. C.-T. Chuang and C.-T. Chen performed experiments and collected the data for analysis. Y.-W. Chen, C.-Y. Hsieh, and K.-Y. Su provided the interpretation of data and were involved in drafting the manuscript.

Notes
The authors declare no competing financial interest.

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■ REFERENCES


