The aqueous extract of *Gentianella acuta* improves isoproterenol-induced myocardial fibrosis via inhibition of the TGF-β1/Smads signaling pathway

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Abstract. *Gentianella acuta* (*G. acuta*) is one of the most commonly used herbs in Chinese Mongolian medicine for the treatment of heart disease. Previously, it was found that *G. acuta* ameliorated cardiac function and inhibited isoproterenol (ISO)-induced myocardial fibrosis in rats. In this study, the underlying anti-fibrotic mechanism of *G. acuta* was further elucidated. Histopathological changes in the heart were observed by hematoxylin-eosin, Masson trichrome and wheat germ agglutinin staining. Relevant molecular events were investigated using immunohistochemistry and western blotting. The results revealed that *G. acuta* caused improvements in myocardial injury and fibrosis. *G. acuta* also inhibited collagens I and III and α-smooth muscle actin production in heart tissue. *G. acuta* downregulated the expression of transforming growth factor β1 (TGF-β1) and notably inhibited the levels of phosphorylation of TGF-β receptors I and II. Furthermore, *G. acuta* caused downregulation of the intracellular mothers against decapentaplegic homolog (Smads)2 and 4 expression and inhibited Smads2 and 3 phosphorylation. The results further demonstrated that the mechanism underlying anti-myocardial fibrosis effects of *G. acuta* was based upon the suppression of the TGF-β1/Smads signaling pathway. Therefore, *G. acuta* may be a potential therapeutic agent for ameliorating myocardial fibrosis.

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Introduction

Myocardial fibrosis, which is a morphological feature of ventricular remodeling, develops as a result of several different heart diseases, including myocardial infarction, hypertension, atherosclerosis and myocarditis (1,2). Excessive extracellular matrix (ECM) production often disrupts the tissues' physiological architecture (3). In the heart, the primary components of the ECM network structure are predominantly collagens I and III. Massive cardiac collagen deposition can lead to ventricular stiffness and diastolic and systolic dysfunction (4,5). These adverse changes can result in heart failure and even sudden cardiac death (4,6,7). Treatment strategies for myocardial fibrosis are still a focus of clinical attention.

Transforming growth factor β1 (TGF-β1) has been confirmed as a primary regulator of myocardial fibrosis (8,9). TGF-β1 exerts its biological functions by binding to the active TGF-β receptors type I and II (TβRI and II), which causes phosphorylation of TβRI and II (9). The activated receptors subsequently phosphorylate receptor-regulated mothers against decapentaplegic homolog (Smad) proteins and increase the common-regulated Smad protein expression. These Smad proteins are the primary downstream signaling molecules of TGF-β1 (10). It is well known that the upregulated TGF-β expression resulting from tissue injury plays a role in the tissue repair process and scar formation (11). In terms of post-infarct remodeling, TGF-β1 is involved in myocardial fibrosis via ECM synthesis upregulation by fibroblasts; as a consequence, TGF-β1/Smads is considered to be the primary signaling pathway that contributes to myocardial fibrosis (12). Therefore, the inhibition of TGF-β signaling is a promising approach for inhibiting myocardial fibrosis.

Gentianella acuta (*G. acuta*), which belongs to the Gentianaceae, is one of the most commonly used herbs in Mongolian medicine to cure hepatitis, jaundice, fever and headache. In China, this herb is widely distributed in Hebei, Inner Mongolia, Shanxi and Shandon. The whole grass of *G. acuta* has been used to treat angina by the local herdsmen in Inner

Mongolia for a long time. In these regions, *G. acuta* was called 'guixincao'. Ewenki people had a habit of drinking 'guixincao' tea and used it to treat angina by chewing or water decoction (13). Certain experimental results have indicated that *G. acuta* exerts anti-inflammatory, antioxidant, antibacterial and antiarrhythmic effects, thereby preventing cardiovascular and cerebrovascular diseases (14-16). Numerous studies have shown that *G. acuta* and its active components have a protective effect on the heart (16,17). In the authors' previous study, it was found that *G. acuta* significantly improved cardiac function and inhibited myocardial fibrosis in a rat model (18). However, the detailed mechanism underlying *G. acuta's* anti-myocardial fibrotic effects is not clear. The aim of the present study was to investigate whether the mechanism underlying *G. acuta's* anti-myocardial fibrosis effects is based on the TGF-β1/Smads signaling pathway, which will provide evidence for clinical application.

Materials and methods

Primary drugs and reagents. Isoproterenol (ISO) was purchased from Tokyo Chemical Industry, Ltd. Bellidifolin, swertianolin and demethylbellidifolin were acquired from Chengdu Alfa Biotechnology, Co., Ltd. Masson trichrome and wheat germ agglutinin (WGA) staining kit were purchased from Wuhan Servicebio Technology, Ltd. Antibodies for α-smooth actin (α-SMA; cat. no. ab32575), TGF-β1 (cat. no. ab92486), Smad4 (cat. no. ab40759), phosphorylated (P)- and total Smads2 (cat. nos. ab53100 and ab40855) and 3 (cat. nos. ab52903 and ab40854) were purchased from Abcam. The antibody for TβRI (cat. no. GB11271) was purchased from Wuhan Servicebio Technology, Ltd. The antibody for TβRII (cat. no. A11788) was purchased from ABclonal Biotech Co., Ltd. Antibodies for collagens I (cat. no. AF7001), III (cat. no. AF0136), phospho-TβRI (cat. no. AF8080) and II (cat. no. AF8191) were purchased from Affinity Biosciences. Antibody for GAPDH (cat. no. 60004-1-Ig) was obtained from Wuhan Sanying Biotechnology. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (cat. no. ZDR-5306) and HRP-conjugated anti-mouse IgG (cat. no. ZDR-5307) antibodies were obtained from Beijing Zhongshan Golden Bridge Biotechnology, Co., Ltd.; OriGene Technologies, Inc. The SP9000 immunohistochemical staining kit was purchased from Beijing Zhongshan Golden Bridge Biotechnology, Co., Ltd.; OriGene Technologies, Inc.

Preparation of plant material. The whole plant of *G. acuta* was purchased from the market in Inner Mongolia and authenticated by Professor Zheng Yu-Guang (Department of Pharmacognosy, School of Pharmacy, Hebei University of Chinese Medicine). According to body surface area conversion equations (human: Rat=1: 6.3) for translating dosages from human to animal (19,20), the clinical dosage of *G. acuta* (6 g/d) in adults is ~ equal to the daily dosage of 0.6 g/kg in rat. The dosages of *G. acuta* adopted in the experiments are 0.3, 0.6 and 1.2 g/kg, respectively. *G. acuta's* dried whole plant was ground and soaked in a 20-fold volume of distilled water for 20 min. The mixture, which was decocted twice at 100℃ for 30 min, was then filtered and concentrated at 80℃. The dark brown aqueous extracts were stored in a refrigerator at 4℃ until use.

Animal protocol. A total of 50 male Sprague-Dawley rats (5 weeks old) were obtained from the Experimental Animal Centre of Hebei Medical University (Shijiazhuang, China) and maintained in an animal facility (22-23℃; 55-60% humidity) on a 12-h light/ dark cycle with free access to food and water. All experiments were conducted in accordance with the P.R. China legislation for the use and care of laboratory animals. The animal care and study protocols were approved by the Institutional Animal Care and Use Committee of Hebei University of Chinese Medicine (no. 1703440).

The myocardial fibrosis model was established as described previously (21,22). Briefly, ISO (5 mg/kg/day) was injected subcutaneously into rats for seven days. A total of 50 rats were equally divided into the following five groups at random: i) Control group (Control); ii) model group (ISO); iii) low-dose drug intervention group (ISO+*G. acuta* 0.3 g/kg); iv) middle-dose drug intervention group (ISO+*G. acuta* 0.6 g/kg); and v) high-dose drug intervention group (ISO+*G. acuta* 1.2 g/kg). All drugs were administered via oral gavage and the treatment period lasted 21 days beginning from the day after the first ISO injection. On the 22nd day, the rats were anaesthetized and then sacrificed. All the samples were cut from the same location of heart, the mid left ventricular (2.5 mm above the apex). The tissues were then fixed for 24 h at room temperature with 4% paraformaldehyde for histopathological analyses and immunohistochemical measurements, and the fresh myocardial tissues were stored at -80℃ for western blotting analysis.

Morphological detection of heart. The heart tissues were fixed in 4% paraformaldehyde, embedded in paraffin wax and cut into 5-μm sections. Hematoxylin and eosin (H&E), Masson trichrome and WGA staining were performed in accordance with the manufacturer's protocol in order to observe histopathological changes and collagen deposition in the heart tissues. Sections were dehydrated, made transparent and sealed. Staining was visualized and images were captured using light microscopy and fluorescence microscope (Leica Microsystems GmbH).

Immunohistochemical staining. Immunohistochemical staining was performed for detection of collagens I and III, α-smooth muscle actin (α-SMA), TGF-β1, Smads2, 3 and 4 expression according to the manufacturer's protocol of the SP9000 immunohistochemical staining kit. Briefly, heart samples were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 5-μm sections. The sections were then de-paraffinized, incubated with 3% hydrogen peroxide for 10 min at room temperature in order to block endogenous peroxidase activity and then incubated for 30 min at room temperature with 3% goat serum (OriGene Technologies, Inc.) to block nonspecific binding. Next, the sections were incubated with a primary antibody (collagen I, 1:100; collagen III, 1:100; α-SMA, 1:500; TGF-β1, 1:50; Smad2, 1:50; Smad3, 1:1,000; and Smad4, 1:100) at 4℃ overnight. After washing with phosphate-buffered saline (PBS), the sections were incubated with secondary antibodies from the SP9000 kit for 15 min each at room temperature. Finally, the sections were incubated with 3, 3'-diaminobenzidine for 2 min at room temperature and observed under a light microscope (Leica Microsystems GmbH).

Western blotting. The heart tissues were washed twice with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail, phenylmethyl sulfonyl fluoride, and phosphatase inhibitors. The protein concentration was measured using a bicinchoninic acid Protein Assay kit (Wuhan Servicebio Technology, Ltd.). An equal amount of protein (20 μg/lane) was separated using 10% SDS-PAGE and then transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk for 90 min at room temperature and then individually incubated with the appropriate primary antibody (collagen I, 1:1,000; collagen III, 1:1,000; α-SMA, 1:5,000; TGF-β1, 1:1,000; TβRI, 1:1,000; P-TβRI, 1:3,000; TβRII, 1:1,000; P-TβRII, 1:3,000; Smad2, 1:2,000; P-Smad2, 1:1,000; Smad3, 1:10,000; P-Smad3, 1:2,000; and Smad4, 1:5,000) at 4°C overnight. Subsequently, the membranes were incubated with the appropriate HRP-conjugated secondary antibody (HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG, both 1:20,000) at room temperature for 60 min. The immunoreactive bands were detected using the enhanced luminol-based ECL reagent kit (Invitrogen; Thermo Fisher Scientific, Inc.) and the Fusion FX5 Spectra multifunction laser-scanning system. The expression of each protein of interest was normalized to that of GAPDH and was analyzed using Image J 3.0 software (National Institute of Health).

High performance liquid chromatography (HPLC) analysis. The sample solutions were prepared by dissolving 0.2 g of *G. acuta* in 50 ml water. The aqueous extracts of *G. acuta* were evaluated by HPLC analysis. The standard solutions of i) swertianolin, ii) demethylbellidifolin, and iii) bellidifolin were prepared to concentrations of 0.02 mg/ml. HPLC analysis was performed on a XBridge C18 (4.6x250 mm, 5 μm, Waters Corporation) maintained at 35°C. Mobile phases A and B were acetonitrile and 0.1% acetic acid, respectively. The flow rate was set at 1.0 ml/min. The detection wavelength was set at 254 nm to identify xanthones from *G. acuta* (Fig. 1).

Statistical analysis. Each experiment was repeated three times. Data are expressed as the mean ± standard error of the mean or mean ± standard deviation. All statistical analyses were performed with SPSS 21.0 statistical software (IBM, Corp.). Differences were analyzed using one-way analysis of variance followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

G. acuta ameliorates ISO-induced myocardial fibrosis and cardiac remodeling in rats. Morphological examination of heart tissue sections stained with H&E revealed that an ISO-induced myocardial fibrosis rat model had been successfully established. Compared with the control group, heart tissues from ISO-treated rats exhibited widespread myocardial structural disorder and necrotic degeneration. Compared with the ISO group, *G. acuta* treatment produced a marked improvement in ISO-induced myocardial necrosis and structural disorder (Fig. 2A and B). Masson trichrome staining revealed that the collagen deposition was significantly increased in the

ISO-treated rat hearts. However, *G. acuta* treatment caused a significant reduction in collagen deposition compared with the ISO group (P<0.05; Fig. 2A and C). WGA staining indicated that ISO could significantly induce cardiomyocytes injury and hypertrophy (P<0.01), and treatment with *G. acuta* could inhibit myocardial hypertrophy (Fig. 2A and D). These results indicate that *G. acuta* treatment caused an inhibition of collagen deposition and ameliorated ISO-induced myocardial fibrosis and left ventricle remodeling.

G. acuta inhibits the expression of collagens I, III and α-SMA induced by ISO. In order to further confirm *G. acuta's* inhibitory effects on myocardial fibrosis, the content and distribution of collagen types I and III, the two major components of the ECM, were quantified using immunohistochemical staining and western blotting. Immunohistochemical staining revealed that the expression of myocardial collagens I and III were markedly enhanced in the ISO group vs. the control group. *G. acuta* treatment decreased ISO-induced collagens I and III expression enhancement (Fig. 3A-C). Western blotting results indicated that *G. acuta* treatment inhibited the collagens I and III expression (Fig. 3E and G). These findings demonstrate that *G. acuta* treatment caused a decrease in collagens I and III expression in the process of ISO-induced myocardial fibrosis.

A crucial event in myocardial fibrosis is the conversion of cardiac fibroblasts (CFs) to myofibroblasts (MFs). MFs express contractile proteins, including α-SMA. Therefore, the expression of α-SMA, which is a typical marker of MFs differentiation, was analyzed using immunohistochemical staining and western blotting. In the control group, α-SMA-positive staining was observed in the blood vessels. In comparison with the control group (without ISO), an abundance of ISO-induced brown particles was present in the fibrotic areas, while *G. acuta* treatment ameliorated the ISO-induced positive brown particles (Fig. 3A and D). Western blotting results indicated that the expression level of α-SMA was significantly downregulated by *G. acuta* (P<0.01; Fig. 3F and H).

These findings indicate that *G. acuta* treatment caused inhibition of ISO-induced myocardial fibrosis by reducing collagens I, III and α-SMA expression in the heart tissue.

G. acuta treatment inhibits ISO-induced TGF-β1 expression and the phosphorylation of TβRI and II. TGF-β1 is the major regulator of the fibrogenic process. In order to identify whether TGF-β1 is involved in *G. acuta*-mediated attenuation of myocardial fibrosis, TGF-β1 levels were examined in the heart tissue. Immunohistochemical analysis revealed that the expression levels of TGF-β1 were increased in the ISO group compared with the control group. However, compared with the ISO group, *G. acuta* treatment caused a significant decrease in the ISO-induced TGF-β1 expression levels (P<0.01; Fig. 4A and B). Western blotting results showed that TGF-β1 expression was significantly decreased in *G. acuta* treatment group (P<0.01; Fig. 4C). These results indicate that *G. acuta* treatment inhibited ISO-induced TGF-β1 expression in heart tissues.

Phosphorylation of the TGF-β receptor is responsible for the initiation of multiple TGF-β signaling pathways. In order to confirm whether *G. acuta* affects TGF-β receptors, the
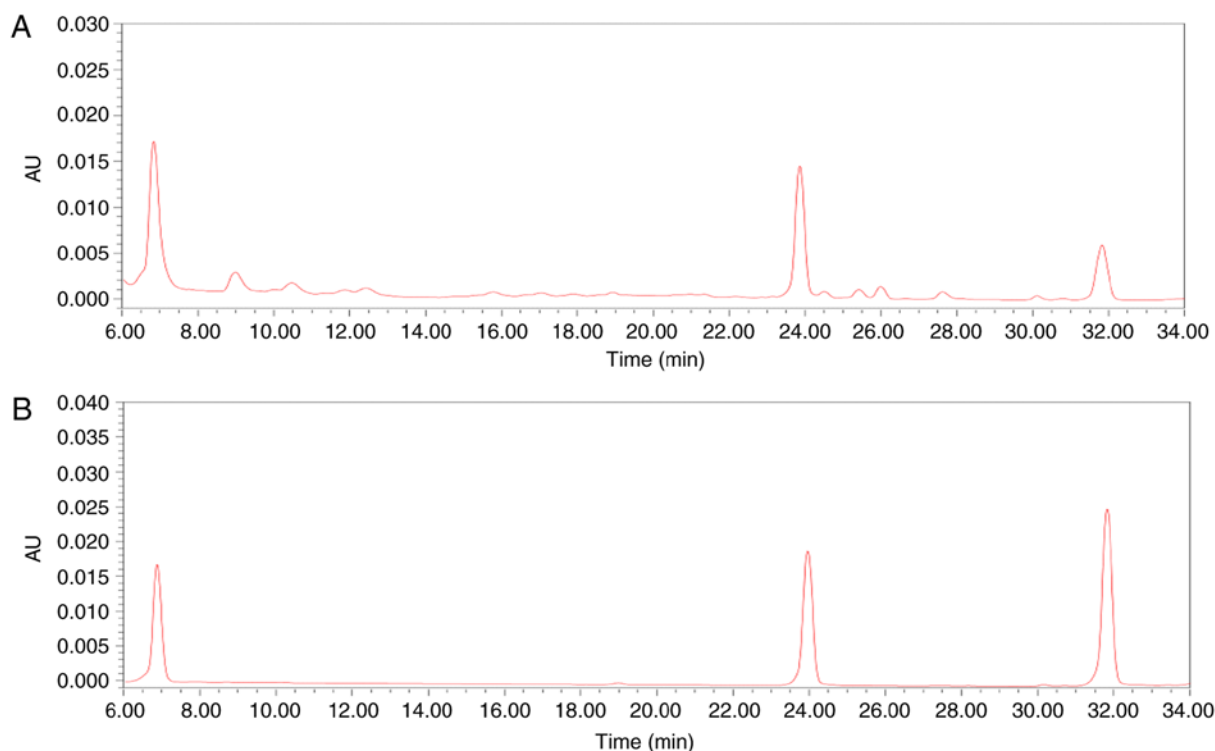
Figure 1. Analysis of xanthone compounds in *G. acuta* using high performance liquid chromatography. (A) The aqueous extracts of *G. acuta*. (B) The mixed standard solutions. The peaks were numbered as 1: Swertianolin, 2: Demethylbellidifolin and 3: Bellidifolin. *G. acuta*, *Gentianella acuta*.
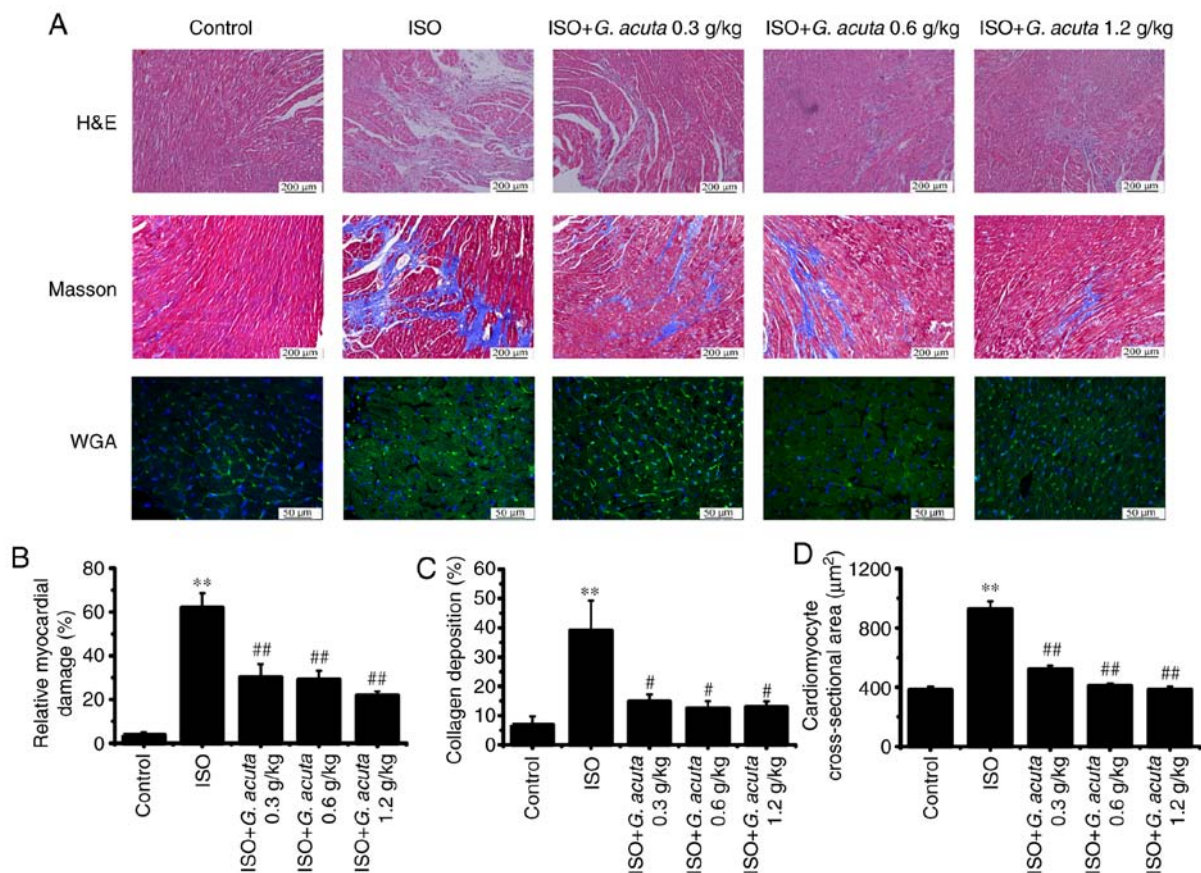

Figure 2. *G. acuta* ameliorates ISO-induced myocardial fibrosis and cardiac remodeling in rats. (A) H&E staining to detect morphological changes; Masson trichrome staining to detect myocardial fibrosis; scale bars=200 µm; WGA staining to access cardiomyocyte cross-sectional area; scale bars=50 µm. (B) Bar graphs show the percentage of relative myocardial damage. (C) Bar graphs show the percentage of collagen deposition. (D) Bar graphs show the cardiomyocyte cross-sectional area. Data are shown as the mean ± standard deviation. **P<0.01 vs. the Control, #P<0.05 and ##P<0.01 vs. ISO. ISO, isoproterenol; H&E, hematoxylin and eosin; WGA, wheat germ agglutinin; *G. acuta*, *Gentianella acuta*.

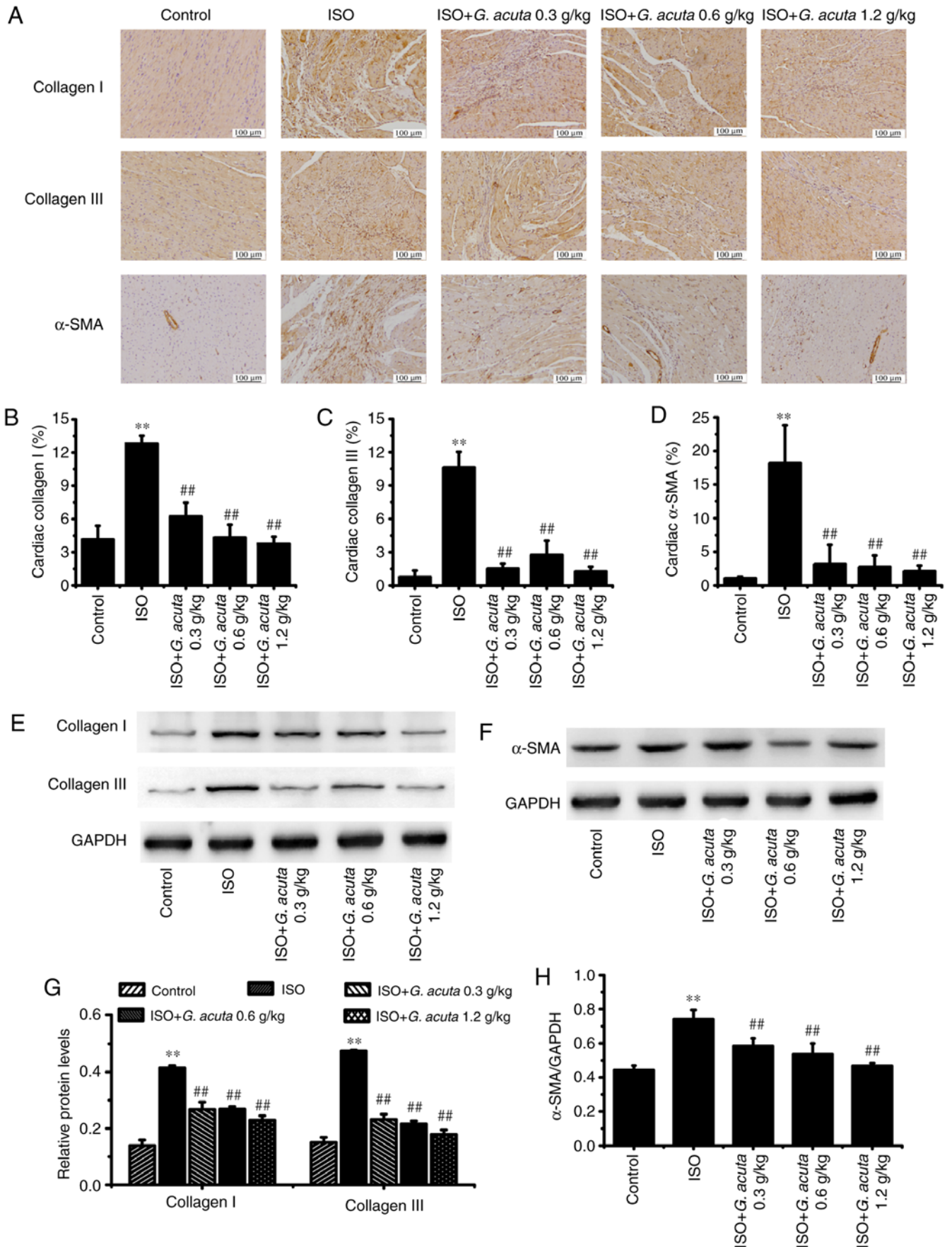Figure 3. *G. acuta* inhibits the expression of collagens I, III and α-SMA induced by ISO. (A) Immunohistochemical staining for collagens I, III and α-SMA, scale bars=100 μm. Bar graphs show the percentage of (B) collagens I, (C) III and (D) α-SMA. (E) Western blotting detection of collagens I, III and (F) α-SMA. (G) Bar graphs show fold-changes for the collagens I and III and (H) α-SMA expression as analyzed by western blotting. GAPDH was used as a loading control (n=3). Data are shown as the mean ± standard error of the mean. **P<0.01 vs. the Control, ##P<0.01 vs. ISO. α-SMA, α-smooth muscle actin; ISO, isoproterenol; *G. acuta*, *Gentianella acuta*.
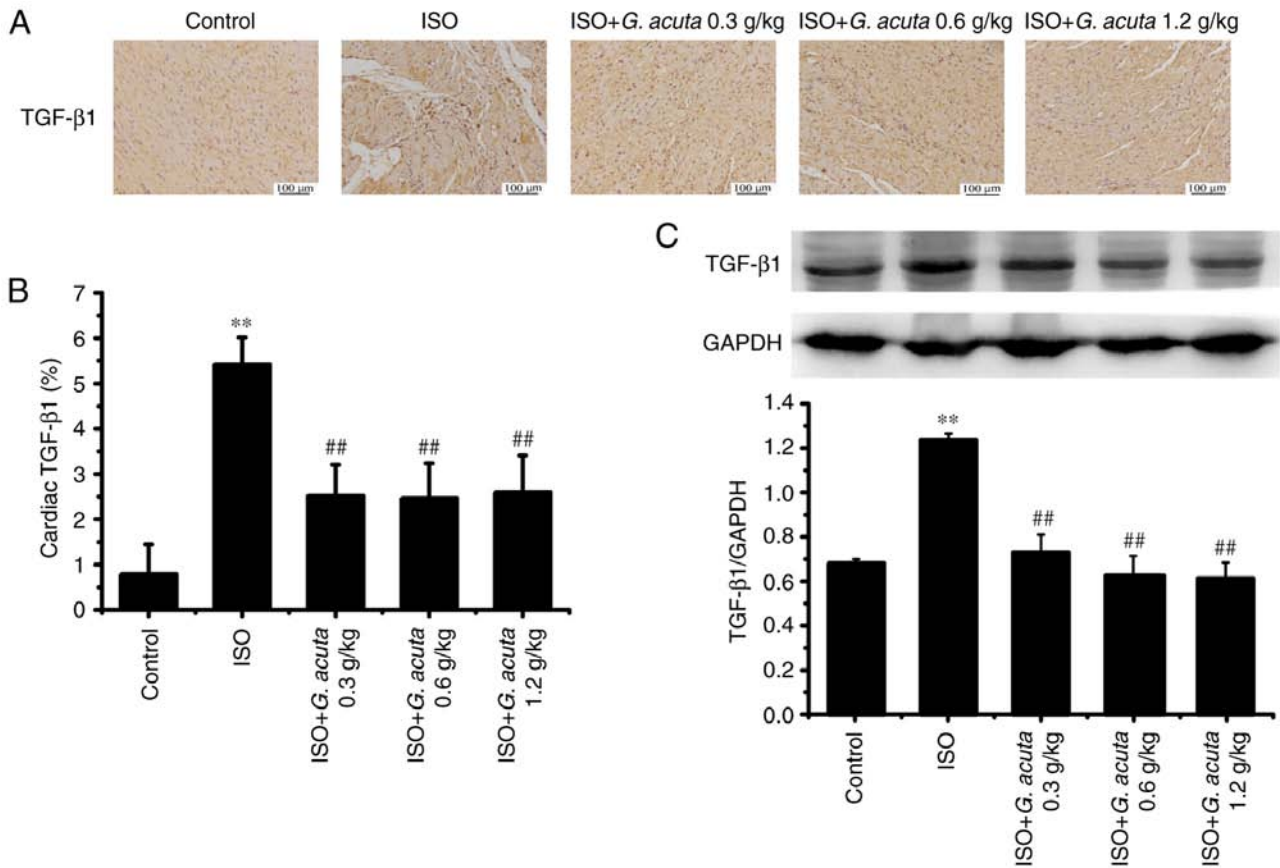
Figure 4. *G. acuta* causes a reduction in ISO-induced TGF-β1 expression. (A) Immunohistochemical staining to detect TGF-β1, scale bars=100 μm. (B) Bar graphs show the percentage of TGF-β1. (C) Western blotting to detect TGF-β1 and bar graphs show fold-changes of TGF-β1 expression as analyzed by western blotting. GAPDH was used as a loading control (n=3). Data are shown as the mean ± standard error of the mean. **P<0.01 vs. the Control, $^{##}$P<0.01 vs. ISO. TGF-β1, transforming growth factor; ISO, isoproterenol; *G. acuta*, *Gentianella acuta*.

expression and phosphorylated levels of TβRI and II were examined by western blotting. The results indicated that TβRI expression and levels of TβRI and II phosphorylation were significantly upregulated in the ISO group compared with the control group (P<0.01). Moreover, *G. acuta* treatment abolished upregulation of these ISO-induced proteins expression and phosphorylation levels (Fig. 5). These results demonstrate that *G. acuta* attenuated TβRI expression and inhibited the phosphorylation and activation of TβRI and II.

G. acuta inhibits ISO-activated TGF-β1/smads signaling. The TGF-β1/Smads signaling pathway plays a critical role in myocardial fibrosis. Smad proteins are the primary downstream molecules of TβRI. In order to investigate whether *G. acuta* caused a reduction in the ISO-induced expression and activation of Smads, immunohistochemical staining and western blotting were performed. In comparison with the control group, the immunoreactivities of Smads2, 3 and 4 were significantly increased in the ISO group. Moreover, *G. acuta* treatment reduced the increase in ISO-induced Smads2, 3 and 4 expression (Fig. 6A). Western blot analysis showed that *G. acuta* treatment caused a decrease in the Smads2 and 4 expression. In addition, compared with the control group, Smads2 and 3 phosphorylation levels were significantly increased in the ISO group (P<0.01). *G. acuta* treatment caused a significant reduction in Smads2 and 3 phosphorylation levels, compared with the ISO group

(P<0.05; Fig. 6B-E). These results demonstrate that *G. acuta* treatment attenuated Smads2 and 4 expression and inhibited activation of ISO-induced Smads2 and 3 via phosphorylation. In conclusion, the present data suggested that *G. acuta* caused an inhibition of myocardial fibrosis through the regulation of the TGF-β1/Smads signaling pathway (Fig. 7).

Discussion

In mammals, the ability to regenerate the heart after birth is limited and the adult mammalian heart rarely exhibits the capacity for self-renewal. Myocardial fibrosis is a morphological feature of ventricular remodeling in a variety of heart diseases involved in myocardial infarction (23). Accompanying the fibrosis, normal structural components of heart tissue can be disrupted. Employment of ISO has been popular for the induction of myocardial fibrosis in rats. A previous study showed that the pathophysiological change of left ventricular induced by ISO could persist up to 21 days after a single subcutaneous injection (24). In the authors' previous study, it was confirmed that *G. acuta* could decrease ISO-increased heart rate, improve cardiac function and ameliorate myocardial fibrosis (18). However, the detailed mechanism of *G. acuta* on myocardial fibrosis was not clear. In this study, H&E and Masson trichrome staining demonstrated that *G. acuta* alleviated cardiac structural disorder and myocardial fibrosis induced by ISO, which were consistent
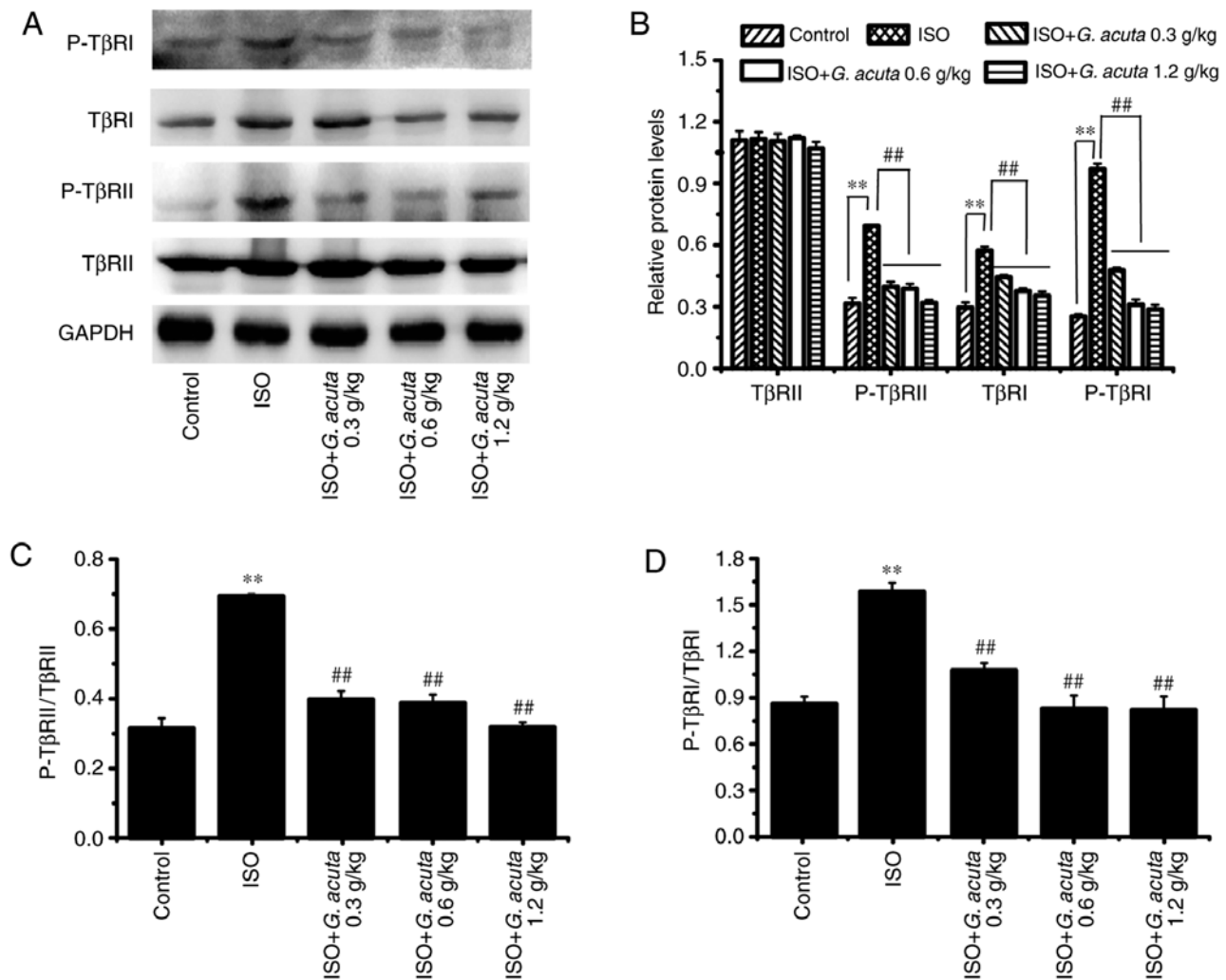
Figure 5. *G. acuta* causes a reduction in ISO-induced TβRI levels and the phosphorylation of TβRI and II. (A) Western blotting analysis of TβRII, phosphory-lated (P)-TβRII, TβRI, P-TβRI. (B) Bar graphs show fold-changes of the TβRII, P-TβRII, TβRI, P-TβRI expression, as analyzed by western blotting. GAPDH was used as a loading control (n=3). (C) Bar graphs show fold-changes of P-TβRII/TβRII and (D) P-TβRI/TβRI. Data are shown as mean ± standard error of the mean. $^{**}P<0.01$ vs. the Control and $^{##}P<0.01$ vs. ISO. ISO, isoproterenol; *G. acuta*, *Gentianella acuta*; P-TβR, phosphorylated- TGF-β receptor.

with the authors' previous study (18). In addition, WGA staining determined that *G. acuta* also inhibited ISO-induced myocardial hypertrophy. These results determined that *G. acuta* treatment ameliorated ISO-induced myocardial fibrosis and ventricular remodeling.

ECM is a crucial element and regulator in numerous biological tissues (25). In the heart, the collagen matrix plays an important role in supporting muscle cells and blood vessels via connecting cells and muscle to govern the architecture and maintain an important diastolic and systolic myocardial stiffness (26,27). Adverse cardiac remodeling and myocardial fibrosis lead to the alteration of ECM in heart tissue. Fibrillar collagen replaces dead cardiomyocytes and scar tissue formation occurs in the infarct zone along with the excessive deposition of collagen in the interstitium remote to the infarc-tion site (28). One of the key events in myocardial fibrosis is the transformation of myocardial CFs into MFs. MFs are the primary cells involved in the repair of pathological tissue, which produce collagen during the fibrotic process (29,30). High α-SMA expression is a key MFs feature (31). In this study, it was determined that ISO induced the excessive

deposition of collagen and *G. acuta* decreased the collagen deposition by immunohistochemical staining, that results were in consistent with the authors' previous study (18). It was also verified that *G. acuta* downregulated collagen expression by western blotting. Furthermore, *G. acuta* treatment amelio-rated the expression of α-SMA in the context of ISO-induced myocardial fibrosis. These data suggest that *G. acuta* inhib-ited the conversion of CFs to MFs, reduced collagen synthesis and improved myocardial fibrosis.

TGF-β family members consist of three isoforms (TGF-β1, TGF-β2 and TGF-β3), which are involved in numerous cellular processes, including cell differentiation, organ development, wound healing and immune regulation (10). However, the distinct biological functions of different isoforms *in vivo* are still under debate. Accumulating evidence has revealed that TGF-β1 plays a vital role in fibrogenesis and myocardial remodeling (32). TGF-β1 mediates the process of ECM production, which can induce CF differentiation and promote collagen synthesis (33). In this study, the present results showed that TGF-β1 expression was upregulated by ISO in the heart, which was attenuated by *G. acuta* treatment. The results were
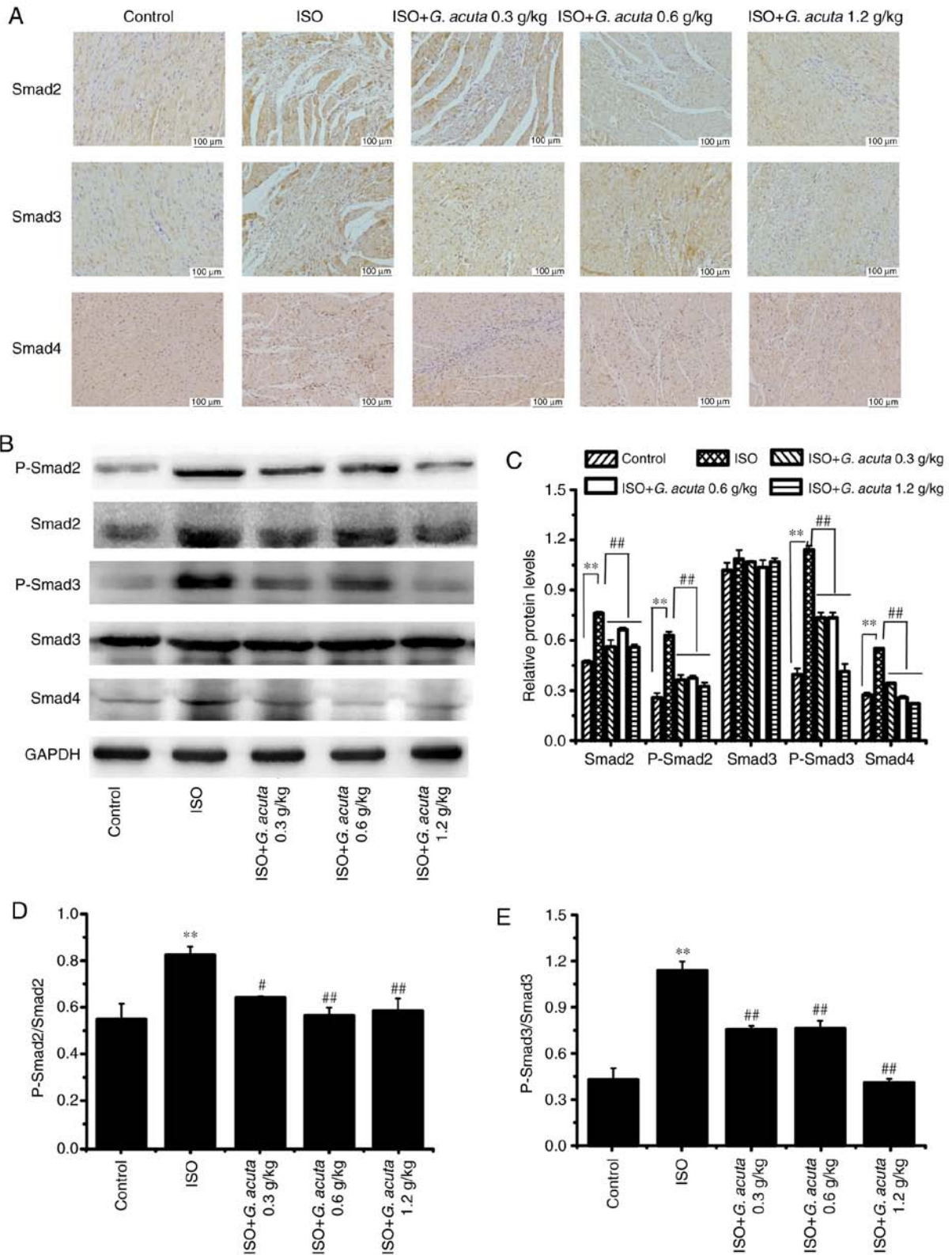
Figure 6. *G. acuta* causes inhibition of ISO-activated TGF-β1/Smads signaling. (A) Immunohistochemistry staining to detect Smads2, 3 and 4; scale bars=100 μm. (B) Western blotting analysis of Smad2, P-Smad2, Smad3, P-Smad3 and Smad4 expression. (C) Bar graphs show fold-changes of Smad2, P-Smad2, Smad3, P-Smad3 and Smad4 expression as analyzed by western blotting. GAPDH was used as a loading control (n=3). (D) Bar graphs show fold-changes of P-Smad2/Smad2 and (E) P-Smad3/Smad3. Data are shown as mean ± standard error of the mean. **P<0.01 vs. control, #P<0.05 and ##P<0.01 vs. ISO. P-Smad, phosphorylated-mothers against decapentaplegic homolog; ISO, isoproterenol; *G. acuta*, *Gentianella acuta*.

consistent with the authors' previous study (18). Therefore, anti-fibrotic effects of *G. acuta* may be mediated by inhibiting the TGF-β1 signaling at least partly.

TGF-β1 signaling regulates tissue fibrosis and tissue scarring by activating specific downstream serine/threonine kinase receptors. TβRII is the specific downstream serine/threonine
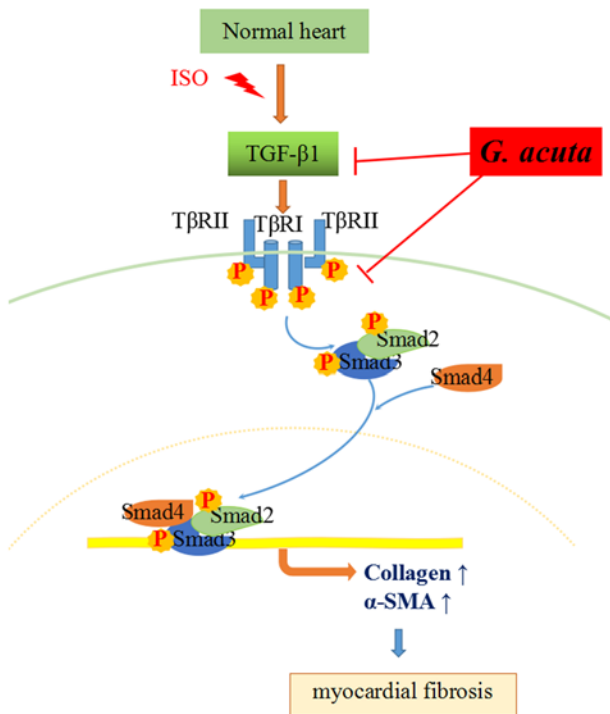
Figure 7. The proposed antifibrotic mechanism of *G. acuta* in ISO-induced myocardial fibrosis in rats. Myocardial injury activates the TGF-β/Smads pro-fibrotic signaling pathway. *G. acuta* inhibits TGF-β1 expression, downregulates TβRI expression and TβRI and II phosphorylation levels, suppresses Smad expression and phosphorylation activation, and causes a decrease in ISO-induced α-SMA, collagens I and III expression in heart tissues. The results indicate that *G. acuta* improves ISO-induced myocardial fibrosis via inhibition of the TGF-β1/Smads signaling pathway. P-Smad, phosphorylated-mothers against decapentaplegic homolog; ISO, isoproterenol; *G. acuta*, *Gentianella acuta*; P-TβR, phosphorylated- TGF-β receptor.

kinase receptor of TGF-β1. TGF-β1 combines with TβRII, which results in TβRII autophosphorylation (34). It is essential for its kinase activity and regulation of downstream signaling to regulate tissue fibrosis and tissue scarring. As a result, the activated TβRII further binds and phosphorylates TβRI (32). Activation of this receptor complex leads to downstream signaling molecule activation and then signal transmission into the cells. In the present study, *G. acuta* treatment alleviated the ISO-induced TβRI and II phosphorylation. These results indicated that *G. acuta* could block the TGF-β signaling pathway activation.

TGF-β signaling includes canonical and non-canonical (Smad and non-Smad-based, respectively) signaling pathways (12). Numerous studies have demonstrated that the Smad-dependent pathway contributes to myocardial fibrosis in the TGF-β-induced signaling pathway (10,12,35,36). Smad proteins are the core downstream signaling molecules of TGF-β1 and they are mediated by the TGF-β receptor. Smads2 and 3 can be phosphorylated and activated. The latter becomes a transcription complex by further combining with Smad4. After this event, the complex moves into the nucleus to complete the process of intracellular signaling transduction via mediation of the target genes such as collagen and α-SMA, leading to myocardial fibrosis (37). In the present study, it was demonstrated that *G. acuta* caused a decrease in Smads2 and 3 phosphorylation levels and inhibited Smads2 and 4 expression

in ISO-induced myocardial fibrosis. It was confirmed that *G. acuta* inhibited myocardial fibrosis via TGF-β1/Smads signaling pathway suppression.

An increasing number of studies have demonstrated that *G. acuta* exerts a protective effect on the heart (15,16). In the authors' previous study, it was found that *G. acuta* caused inhibition of myocardial fibrosis (18). In the present study, the mechanism underlying the anti-fibrotic effect was further clarified to be based upon the TGF-β1/Smads signaling pathway. Phytochemical analysis showed that *G. acuta* contains numerous kinds of constituents, such as xanthones, flavonoids, monoterpenes, lignans and phenolic acids, and xanthones were elucidated to be main constituents in the plant (14-16,38,39). Recently, a few studies have explored the effects of the primary active components of this plant on anti-myocardial injury (14-16) and xanthones from *G. acuta* can protect cardiomyocytes from injury (16,40). The present study first revealed that the aqueous extracts of *G. acuta*, a form of clinical application, contains three xanthones components by HPLC (13). The above results indicated that xanthones were a kind of major bioactivity substances in *G. acuta*. However, except for xanthones, it has been reported that phenolics, flavonoids and lignan glycosides also have a protective effect on the heart (17,41). Therefore, the effect of *G. acuta* on myocardial fibrosis may be the potential synergistic effect of various compounds. In addition, because of that, it is difficult to find a suitable plant drug or single compound as a positive control that was the main limitation of the present study. Based upon the present study, it was hypothesized that the inhibitor of TβR may be a potential positive control, such as Galunisertib or LY2109761, which exhibited preclinical anti-fibrotic effects (42,43).

For the first time, the present study demonstrated that *G. acuta* treatment causes a reduction in TGF-β1 and TβRI expression and TβRI and II phosphorylation levels, thereby suppressing the Smads2 and 4 expression and Smads2 and 3 activation. This suppression further inhibits the ISO-induced α-SMA and collagens I and III expression in rats. These findings demonstrate that *G. acuta* has potential anti-fibrotic function via inhibition of the TGF-β1/Smads signaling transduction pathway. It will be necessary to confirm the specific active ingredients that are responsible for the therapeutic effects of *G. acuta*.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Authors' contributions

As the corresponding author, AL contributed to the conception and design of the study. HY contributed to the study's design, performed the experiments, analyzed the data and prepared the manuscript for writing. GX performed western blot analysis. JHS established the experimental animal model. CZ contributed significantly to HPLC analysis and identification of the effective constituent xanthones. YZ, JNS, YFL and YL were involved in analyzing the data.

Ethics approval and consent to participate

All experiments were approved by the Institutional Animal Care and Use Committee of Hebei University of Chinese Medicine (Shijiazhuang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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