Bellidifolin from *Gentianella acuta* (Michx.) Hulten protects H9c2 cells from hydrogen peroxide-induced injury via the PI3K-Akt signal pathway

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**ABSTRACT**

Cardiovascular disease is the most common disease in the world and the first among the causes of human death. Its morbidity and mortality increase annually, but no effective treatment is available. Therefore, new drugs should be developed to treat cardiovascular disease. *Gentianella acuta* (Michx.) Hulten (G. acuta) is an important Mongolian medicine in China and elicits protective effects on cardiovascular health. In this study, liquid chromatography-mass spectrometry (LC-MS) combined with network pharmacology was used to screen the main active ingredients and confirm that bellidifolin was one of the main components for the treatment of ischemic heart disease. Then, rat myocardial (H9c2) cells injury model induced by hydrogen peroxide (H\(_2\)O\(_2\)) in vitro was established to verify the effect of bellidifolin on oxidative stress stimulation, including determination of antioxidant enzyme activity and apoptosis. Transcriptome sequencing, qRT-PCR, and western blot were performed to further verify the antioxidant stress mechanism of bellidifolin. Results showed that bellidifolin pretreatment decreased the rate of apoptosis and the levels of lactate dehydrogenase (LDH), creatine kinase (CK), and alanine aminotransferase (ALT). Conversely, it increased the contents of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in a dose-dependent manner, indicating that bellidifolin caused a protective effect on cardiomyocyte injury. Bellidifolin minimized the H\(_2\)O\(_2\)-induced cell injury by activating the PI3K-Akt signal pathway and downregulating glycogen synthase kinase-3β (GSK-3β) and p-Akt1/Akt1. Therefore, this work revealed that G. acuta has a good development prospect as an edible medicinal plant in cardiovascular disease. Its bellidifolin component is a potential therapeutic agent for cardiovascular disease induced by oxidative stress damage.

1. Introduction

Cardiovascular disease has become an important disease that threatens human life and health all over the world [1,2]. Atherosclerosis, myocardial ischemia–reperfusion, myocardial infarction are the common clinical diseases [3]. Cardiomyocyte injury is closely related to the occurrence of these diseases [4]. Oxidative stress and apoptosis-induced cardiomyocyte injuries are important mechanisms of cardiovascular disease [5,6]. Oxidative stress impairs the function of mitochondria [7] and promotes the excessive production of reactive oxygen species (ROS) [8,9]. Excessive ROS cause serious damage to cardiomyocytes and lead to apoptosis by destroying the balance of oxidant enzyme activity and antioxidant enzyme activity.

**Key words:** Bellidifolin, Network pharmacology, Liquid chromatography-mass spectrometry, H\(_2\)O\(_2\)-induced injury, Oxidative stress, Protective effect

**Abbreviations:** G. acuta, *Gentianella acuta* (Michx.) Hulten; H\(_2\)O\(_2\), hydrogen peroxide; LDH, lactate dehydrogenase; CK, creatine kinase; ALT, alanine aminotransferase; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; GSK-3β, glycogen synthase kinase-3β; ROS, reactive oxygen species; PPI, protein-protein interaction; GO, gene ontology; PDB, protein data bank; KEGG, kyoto encyclopedia of genes and genomes; qRT-PCR, quantitative reverse transcription PCR; TP53, tumor protein P53; Akt1, protein kinase B1; TNF, tumor necrosis factor; VEGFA, vascular endothelial growth factor A; IL-6, interleukin 6; DEGs, differentially expressed genes; AMPK, AMP-activated protein kinase; cAMP, cyclic adenosine monophosphate; BP, biological processes; MF, molecular functions; CC, cellular components.

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oxidation and antioxidation [10–12]. Cardiomyocytes are highly differentiated cells; when they apoptose, cardiac dysfunction and heart failure occur [13,14]. Therefore, the intervention of oxidative stress to reduce cardiomyocyte injury has been widely investigated in the treatment of cardiovascular diseases [15].

The use of plants to treat diseases can be traced back to hundreds of BC, and the development and utilization of medicinal plants is an important pillar of medical and health care all over the world [16]. Their active components have the advantages of lower toxicity and less side effects than synthetic compounds. Therefore, natural antioxidants have been widely investigated [17]. Medicinal plants are rich in antioxidants [18], which are an important source of active components against ischemic heart disease. In recent years, there are more and more studies on the use of natural products to treat cardiomyocyte injury [19,20]. The isolation and identification of the active components in medicinal plants and the clarification of their cardioprotective mechanism can help us to obtain new ideas for the development of cardioprotective medicinal plants.

Gentianella acuta (Michx.) Hulten (G. acuta) is a commonly used medicine in Mongolian and Tibetan medicine [21,22]. It belongs to Gentianaceae and is mainly distributed in northern China, Mongolian plateau, Siberia, and the Far East of Russia [23]. According to “Inner Mongolia Phytopharmacology,” it has the functions of clearing heat, promoting the gallbladder, and reducing jaundice. It mainly contains xanthone, schizoroidoid, and other compounds. Modern studies have shown that bellidifolin, a xanthone compound, can play a protective role in the heart [24,25]. However, studies have yet to determine whether bellidifolin exerts protective effects against oxidative cytotoxicity as a result of its antioxidant property. Therefore, this study was performed to investigate the potential of bellidifolin against H2O2-induced oxidative stress in cultured H9c2 cells for the treatment of ischemic heart disease. This article was also studied to provide a new basis for the treatment of myocardial cell injury.

2. Materials and methods

2.1. Preparation of G. acuta and bellidifolin

G. acuta was collected from Genhe City, Inner Mongolia Autonomous Region. A powdered G. acuta sample was passed through an 80-mesh sieve, and 0.1 g of this sample was placed in a 50 mL conical flask. Methanol (10 mL, 70% MeOH) was added, and ultrasound (40 kHz, 20 min) was applied to produce the crude extract. The sample was then allowed to cool naturally to 25 °C, vortexed (30 s), centrifuged (4500 rpm, 4 °C, 10 min), and filtered through a microporous membrane (0.22 μm).

Bellidifolin was extracted from G. acuta. The whole grass of G. acuta was crushed, dried, refluxed, and extracted with 95% ethanol three times and concentrated under vacuum with a rotary evaporator. The crude mixture was extracted with ethyl acetate in a Soxhlet extractor until a colorless extract was obtained. The extract was concentrated, and the same amount of anhydrous ethanol was added to produce yellow precipitate. After being dried, the precipitate (25 g) was dissolved in ethyl acetate and mixed with the same amount of silica gel (200 mesh) until the silica particles became dry and uniform again. The sample was separated through silica gel column chromatography (150 g, 200–300 mesh silica gel) by using petroleum ether:ethyl acetate (7:3) through the elution of 2–3 column volumes. The liquid was collected, and the solvent was removed under reduced pressure to obtain the target compound (2 g).

2.2. LC-MS analysis of G. acuta

The SCIEX X500 QTOF system was used in this assay. Chromatographic separation was performed on the Acquity UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm; Waters) column. Acetonitrile (solvent B) and water (containing 0.1% formic acid, solvent A) were used as the mobile phase. The gradient was set at 5–95% B for 0–60 min. The flow rate was 0.3 mL/min, the column temperature was 40 °C, and the sample quantity was 5 μL.

Mass spectrometry data were acquired using the SCIEX X-500R QTOF (SCIEX, USA) equipped with an ESI interface in the positive ion modes. Parameters used were as follows: temperature, 500 °C; spray voltage, 5500 V (positive ion mode); curtain gas, 30 ps; and CAD gas, 7 psi. MS data were generated across a mass range of 100–1500 Da. The stepped normalized collision energy settings were 20, 40, and 60 V. All MS spectra were analyzed using SCIEX OS software (SCIEX, USA).

2.3. Network pharmacology and molecular docking analysis

The compounds in G. acuta, which appears in the results of LC-MS analysis and literature reports were found in Pubchem (https://pubchem.ncbi.nlm.nih.gov/) to obtain the SMILES number. And the corresponding targets of these compounds were searched in SwissTargetPrediction (http://www.swistargetprediction.ch/). Gene names were then converted to protein names on UniProt (https://www.uniprot.org/). Disease targets were obtained from two existing resources, namely, GeneCards (https://www.genecards.org/) and DisGeNET (http://www.disgenet.org/), with the keyword “ischemic heart disease.” Disease and drug targets were inputted to the jvenn online database (http://jvenn.toulouse.inra.fr/app/index.html) to obtain the intersection target. A Protein-Protein Interaction (PPI) network was constructed with the STRING online database (https://cn.string-db.org/). The Kyoto Encyclopedia of Genes and Genomes(KEGG) and Gene Ontology(GO) terms associated with biological processes (BP), molecular functions (MF), and cellular components (CC) were annotated and visualized using the Metascape database. Based on these compounds and targets information, the protein structure of the corresponding target was obtained from the Protein Data Bank (PDB) database (http://www.rcsb.org/). Water and small-molecule ligands were removed using PyMOL 2.4.1, and hydrogenation was performed with Autodock 1.5.6. Subsequently, the file was converted to a pdbqt format. With Autodock Vina 1.1.2, molecular docking was calculated, docking results through PyMOL were visualized, and a molecular docking mode diagram was established.

2.4. Reagents

High-glucose Dulbecco’s modified Eagle medium (DMEM), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT), frozen dimethyl sulfoxide (DMSO) stock solution, trypsin, TRIzol, RIPA, BCA kit and Annexin V-FITC kit were purchased from Solarbio (Beijing, China). Fetal bovine serum (FBS), penicillin, streptomycin, and phosphate-buffered saline (PBS) were purchased from HyClone (Logan, UT, USA). PrimeScript™ RT reagent kit was purchased from TaKaRa (Japan). Ultrapure water was purchased from Gen Pure (Germany), and 30% H2O2 was purchased from Nanjing Reagent (Nanjing, China). Lactate dehydrogenase (LDH), superoxide dismutase (SOD), aspartate aminotransferase (AST), creatine kinase (CK), and glutathione peroxidase (GSH-PX) assay kits were purchased from Nanjing JiaCheng Bioengineering Institute (Nanjing, China). A PrimeScriptRT reagent kit (Perfect Real Time) was obtained from TaKaRa Biotechnology Co. (Japan). Antibodies against Akt1, p-Akt1, GSK-3β, and GAPDH were obtained from Affinity Biosciences (Cincinnati, OH, USA). Secondary antibodies were purchased from Beyotime (Shanghai, China).

2.5. Cell culture and treatment

H9c2 cells were purchased from Wuhan Procell Life Technology Co., Ltd. (Wuhan, China). The cells were cultured in DMEM containing 10% FBS and 100 μg/mL streptomycin and 100 U/mL penicillin at 37 °C, 5% CO2, and 95% saturated humidity. Cell growth was observed daily. H9c2
cells were harvested at the logarithmic growth stage for subsequent analyses and divided into control, \( \text{H}_2\text{O}_2 \) intervention, and bellidifolin groups.

2.6. Establishment of the \( \text{H}_2\text{O}_2 \)-induced injury model

Cells at the logarithmic growth stage were digested with trypsin, diluted with DMEM to a density of \( 2 \times 10^4 \) cells/mL, inoculated in 96-well plates for 24 h, assigned to the control and \( \text{H}_2\text{O}_2 \) intervention groups for the peroxidation challenge and cultured for 1, 2, and 3 h. Subsequently, 5 mg/mL pre-constituted MTT solution was added to the plates that were then incubated for 4 h. After incubation, the culture supernatant was removed, and DMSO (150 \( \mu \)L) added to each well. The contents were mixed and dissolved, and absorbance was measured at 570 nm. The cell survival rate was calculated to determine the optimal injury time and \( \text{H}_2\text{O}_2 \) concentration.

2.7. Cell viability assay

\( \text{H}9c2 \) cells were seeded in 96-well plates at a density of \( 2 \times 10^4 \) cells/mL in 100 \( \mu \)L of media and incubated at 37 \( ^\circ \)C for 24 h. Next, bellidifolin was dissolved in DMSO (<0.01%) and then diluted with DMEM to obtain 10 \( \mu \)L of solutions of different concentrations (20, 40, and 80 \( \mu \)M). The cells were treated with these solutions for 24 h. Next, \( \text{H}_2\text{O}_2 \) (300 \( \mu \)mol/L) was added and allowed to incubate for 3 h (no addition in the control group). MTT (5 mg/mL, 10 \( \mu \)L) and DMSO (150 \( \mu \)L) were added, and a model was established. Lastly, cell viability was determined using MTT reduction assay, and absorbance was measured at 570 nm. Cell activity was also evaluated through real-time cellular analysis (RTCA). RTCA monitors the cell proliferation in real time, records the cell index (CI), and plots the output with time as the abscissa and CI as the ordinate. For the RTCA, E-Plate 16 was placed in the xCELLigence RTCA system, and a reading was recorded every 15 min for up to 40 h. Changes in CI after bellidifolin treatment indicated the effect of the compound on \( \text{H}9c2 \) cells.

2.8. Detection of LDH, AST, and CK activities in the culture medium

\( \text{H}9c2 \) cells were seeded in a 6-well plate at a density of \( 2 \times 10^4 \) cells/mL and treated as described above. Cell culture supernatant was collected, and the activities of LDH, AST, and CK were determined using a microplate reader (Thermo, USA) according to the procedure described in the kit.

2.9. Detection of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in \( \text{H}9c2 \) cells

The cells in each group were digested, collected, and suspended in PBS (1 mL). The activities of SOD and GSH-Px were determined according to the instructions of the kit.

2.10. Apoptosis assay

Cell suspensions were prepared after trypsin digestion; the cells were centrifuged at 1000 \( \times \)g for 5 min. The supernatant was discarded, and the pellet was washed with PBS pre-cooled to 4 \( ^\circ \)C. The washed cells were centrifuged again (1000 \( \times \)g, 5 min), and the obtained pellet was suspended in a 2 \( \mu \)L flow tube by using the binding buffer (300 \( \mu \)L) from the Annexin V-FITC kit to obtain a final cell concentration of \( 5 \times 10^6 \) cells/mL. Next, 5 \( \mu \)L of Annexin V-FITC was first added to the suspension and incubated in the dark for 10 min and then with 5 \( \mu \)L PI; then, the specimen was stored in the dark for 5 min. Lastly, apoptotic cells were detected using a flow cytometer (BD, USA) by analyzing the cell suspensions within 1 h, and the apoptosis rate was calculated for each group.

2.11. RNA-seq transcriptome analysis

\( \text{H}9c2 \) cells were divided into control, \( \text{H}_2\text{O}_2 \) intervention (300 \( \mu \)mol/L, \( \text{H}_2\text{O}_2 \)), and bellidifolin (20, 40, and 80 \( \mu \)M) groups. After 12 h, \( \text{H}_2\text{O}_2 \) was added, and the plates were incubated for 3 h. The cells were then collected, and RNA was extracted using the TRIzol reagent. Sequencing was commissioned to Sangon Biotech Co., Ltd. (Shanghai, China). An Illumina HiSeq 2500 sequencing platform was used for high-throughput sequencing, including RNA library construction, cluster generation, reference genome comparison, screening for differentially expressed transcripts, and functional analysis (GO and KEGG functional enrichment analyses). RNA-seq data were then analyzed using DESeq to identify differentially expressed genes (DEGs) with the following screening criteria: q-value \( \leq 0.05 \) and difference multiple fold change \( \geq 1.5 \).

2.12. Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from the cultured \( \text{H}9c2 \) cells by using TRIzol reagent and reverse-transcribed with a PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) in accordance with the manufacturer’s instructions. The aliquots of the obtained cDNA samples were then amplified through PCR with the following schedule: 40 cycles at 95 \( ^\circ \)C for 30 s, primer annealing at 95 \( ^\circ \)C for 5 s, and extension at 60 \( ^\circ \)C for 34 s. The specifically designed primers are listed in Table 1. All primers were tested, the fluorescence signals were recorded, and the relative values were compared with those of the control group.

2.13. Western blotting

Cells from all groups were washed twice with PBS and digested with RIPA containing PMSF and phosphatase inhibitors. The liquid supernatant was collected after centrifugation at 13,500 \( \times \)g for 10 min at 4 \( ^\circ \)C. The protein concentration in the supernatant was determined using the BCA kit. Equal amounts of protein (60 \( \mu \)g) were separated on 10% SDS-PAGE and subsequently transferred to PVDF membranes. Afterward, the membranes were blocked with 5% (w/v) nonfat dry milk in TBST at room temperature for 1 h and incubated at 4 \( ^\circ \)C overnight with the following primary antibodies: anti-Akt (Cat. No.: AF0836, 1:1000), anti-p-Akt (Cat. No.: AF8355, 1:500), anti-GSK-3β (Cat. No.: AF5016, 1:1000), and anti-GAPDH (Cat. No.: AF7021, 1:2000). The membranes were then washed three times for 10 min each with TBST and incubated with horseradish peroxidase (HRP)-conjugated affinity-pure goat anti-rabbit IgG (H+L, 1:2000). Lastly, the membranes were washed three times on a shaking table and treated with enhanced chemiluminescent reagent (ECL Advance western blotting detection kit; GE Healthcare Life Sciences) in accordance with the manufacturer’s instructions. Western blot signal strength was measured using Western Bright™ ECL spray and quantitatively assessed using Syngene’s (Cambridge, UK) GeneTools.

2.14. Statistical analysis

All experiments were repeated three times, and data were analyzed using GraphPad Prism 9. Experimental results were expressed as means ± standard deviation (SD), and the groups were confirmed using the t-test or one-way ANOVA. \( P < 0.05 \) was considered statistically significant.

### Table 1

| Gene  | Forward  | Reverse  |
|-------|----------|----------|
| Akt   | GAAGGAGAACGCCACAGGCTC | CCGTGTGTTGAAAGGGCAGG |
| GSK-3β| GAGAAGATCTGCTGACCACTCA | ACAGAGCTGCGACACCATA |
| β-actin | CTCGGAGACATGGAGAAAA | AAGGAAGCTGAGAAGTGC |
3. Result

3.1. Qualitative analysis of G. acuta via LC-MS

SCIEX OS software was used to process the data of all samples, and the obtained data were compared with TCM MS/MS Library 2.1 to identify each compound. The specific conditions are as follows: mass range, 100–1500 Da; mass error, < 5 ppm; difference isotope ratio, < 5%; and library hit score, >80. A total of 8 compounds were identified in a positive ion mode in this method.

TCM MS/MS Library 2.1 is not comprehensive enough because it only contains 1295 compounds. Therefore, fragmentation laws and secondary fragmentation were also performed to assist in compound identification. In a positive ion mode, the parent ion is \( m/z \) 275.0559 \([\text{M} + \text{H}]^+\) when the element composition function of the OS software was used. Therefore, the molecular formula of this compound may be \( \text{C}_{17}\text{H}_{30}\text{O}_{16}\). In collision-induced dissociation, four possible dissociation modes were set: a) –CH\(_2\) in the methoxy group on the A ring is removed, resulting in the secondary fragment ion of \( m/z \) 260.0326 \([\text{M} + \text{H} - \text{CH}\text{H}]^+\); b) the removal of CO to produce the secondary fragment ion of \( m/z \) 247.0611 \([\text{M} + \text{H} - \text{CO}]^+\); c) remove a molecule of H\(_2\text{O}\) and C ring C-C (5–6, 7–8) fragmentation to produce \( m/z \) 232.0370 \([\text{M} + \text{H} - \text{C}_2\text{H}_2\text{O}_2]^+\); and d) H\(_2\text{O}\) that loses a molecule on the C ring and C-C (2–3, 8b-1) on the A ring is broken to obtain the fragment ion of \( m/z \) 214.0270 \([\text{M} + \text{H} - \text{C}_2\text{H}_2\text{O}_3]^+\). Based on the inferred cleavage rule, compound 9 was identified to be bellidifolin (Fig. 1 and Table 2). The specific information of the compounds identified by the above methods is shown in Table 2.

3.2. Network pharmacological analysis of G. acuta for the treatment of ischemic heart disease

According to the identification results of LC-MS and literature reports, 37 active components and 210 potential targets were obtained (Table 3). Genecards and DisGeNET databases were used to search the targets with “ischemic heart disease”, and 4064 targets were obtained. The target of G. acuta and ischemic heart disease were input into jveen, and 135 common targets were determined (Fig. 2A). Compounds, compound targets, and disease targets were collated and imported into Cytoscape 3.7.1 to construct a drug active component–target network consisting of 249 nodes and 701 edges (Fig. 2B). According to the degree value analysis, the top five compounds are luteolin, swertiabissxanthone-i, cinnamic acid, 1,3,5-trihydroxyxanthone, and bellidifolin. Then, 135 common targets were imported into the STRING database to construct the PPI network and analyzed visually with Cytoscape 3.7.1 (Fig. 2C). According to the ranking of degree values, the top five targets were tumor protein P53 (TP53), protein kinase B1 (Akt1), tumor necrosis factor (TNF), vascular endothelial growth factor A (VEGFA), and interleukin 6 (IL-6). Drug–disease common targets was subjected to KEGG enrichment analysis using the Metascape database. This analysis showed that the included pathways were those in cancer, prostate cancer, endocrine resistance, PI3K/Akt signaling pathway, and proteoglycans in cancer among others (Fig. 2D).

3.3. Molecular docking

Four genes, TP53, Akt1, TNF, and GSK-3β, were selected from the PI3K-Akt signaling pathway and PPI network. Docking results are shown in Table 4. The lower the free energy of the binding between a ligand and a receptor, the more stable the binding conformation and greater the possibility of action. The results showed that bellidifolin had high affinity to TP53, Akt1, TNF, and GSK-3β. After docking results were imported into PyMOL v2.4.1, the software conducts visual analysis. The docking mode of the compound and target is shown in Fig. 3. The structure of the compound and interacting amino acids is represented.

Table 2

| NO. | Molecular Formula | Retention Time (min) | Mass (m/z) | Identification                  |
|-----|------------------|----------------------|-----------|---------------------------------|
| 1   | \( \text{C}_{13}\text{H}_{20}\text{O}_{15} \) | 6.50                 | 357.1180  | Gentiopterin<sup>a</sup>        |
| 2   | \( \text{C}_{13}\text{H}_{20}\text{O}_{15} \) | 5.70                 | 359.1336  | Sweroside<sup>a</sup>           |
| 3   | \( \text{C}_{13}\text{H}_{20}\text{O}_{15} \) | 6.53                 | 423.9921  | Mangiferin<sup>a</sup>          |
| 4   | \( \text{C}_{13}\text{H}_{20}\text{O}_{15} \) | 8.61                 | 437.1082  | 7-O-Methylmangiferin<sup>a</sup>|
| 5   | \( \text{C}_{13}\text{H}_{20}\text{O}_{15} \) | 7.82                 | 449.1069  | Isooertizin<sup>a</sup>         |
| 6   | \( \text{C}_{13}\text{H}_{20}\text{O}_{15} \) | 9.23                 | 433.1121  | Isovitexin<sup>a</sup>          |
| 7   | \( \text{C}_{13}\text{H}_{20}\text{O}_{15} \) | 8.49                 | 287.0557  | Luteolin<sup>a</sup>            |
| 8   | \( \text{C}_{13}\text{H}_{20}\text{O}_{15} \) | 43.45                | 457.3678  | Ursolic Acid<sup>a</sup>        |
| 9   | \( \text{C}_{13}\text{H}_{20}\text{O}_{15} \) | 24.52                | 275.0559  | Bellidifolin                    |

<sup>a</sup> Compared with TCM MS/MS library 2.1

Fig. 1. Cracking laws of bellidifolin.
via a stick diagram in which amino acids are represented in pink, and interconnections by hydrogen bonds are indicated by black dotted lines. The name of the connected amino acid and hydrogen bond distance are also marked in Fig. 3. The results showed that the binding energy of bellidifolin to the targets was less than −5 kcal·mol\(^{-1}\), indicating that bellidifolin had a high affinity to the targets.

### 3.4. Establishment of \(\text{H}_2\text{O}_2\)-induced cell injury model

As shown in Fig. 4, compared with that in the control group, the groups treated with 50, 100, 200, 300, and 400 \(\mu\text{M}\) \(\text{H}_2\text{O}_2\) showed a dose-dependent increase in the proportion of damaged cells (\(P < 0.05\)). After peroxidation for 1, 2, and 3 h, the group treated with 400 \(\mu\text{M}\) \(\text{H}_2\text{O}_2\) showed the highest cell death, whereas about 50% of the cells survived after 3 h of treatment with 300 \(\mu\text{M}\) \(\text{H}_2\text{O}_2\). Therefore, 300 \(\mu\text{M}\) \(\text{H}_2\text{O}_2\) and a treatment time of 3 h were selected as the optimal conditions for establishing the \(\text{H}_2\text{O}_2\)-induced injury model.

Cells grown for 24 h were incubated with 20, 40, and 80 \(\mu\text{M}\) bellidifolin for an additional 12 h and subsequently treated with \(\text{H}_2\text{O}_2\) to induce oxidative stress. As shown in Fig. 5, after \(\text{H}_2\text{O}_2\) treatment, the cell index (CI) of the model and bellidifolin groups was significantly lower than that of the control group, and the CI of the bellidifolin groups increased in a dose-dependent manner compared with that of the \(\text{H}_2\text{O}_2\) intervention groups.

### 3.5. Cell viability assay

MTT assay results revealed that bellidifolin had no toxicity at 20, 40, and 80 \(\mu\text{M}\) with treatment times of up to 24 h, and the survival rates of the different groups did not differ significantly from that of the control group (\(P > 0.05\)). As shown in Fig. 6, compared with that of the control group, the survival rate of \(\text{H}_9\text{C}_2\) cells in the \(\text{H}_2\text{O}_2\) intervention group was significantly reduced (\(P < 0.05\)). The cell viability in bellidifolin groups and \(\text{H}_2\text{O}_2\) intervention group was lower than that in the control group, while the cell viability in bellidifolin groups was significantly higher than that in the \(\text{H}_2\text{O}_2\) intervention group (\(P < 0.05\)). And the cell viability also increased with the increase of bellidifolin concentration. Because the bellidifolin concentrations used were lower than toxic levels, bellidifolin toxicity was ruled out as the possible cause of the reduced cell viability in the bellidifolin groups relative to that in the control group.

3.6. Effect of bellidifolin on the antioxidant enzyme activities

To analyze whether bellidifolin could alleviate the oxidative stress in \(\text{H}_9\text{C}_2\) cells, we assayed the activities of SOD, AST, CK, GSHPx, and LDH. Compared with that in the control group, the \(\text{H}_2\text{O}_2\) intervention group showed significant differences in enzyme activities, indicating that the model was successfully established (\(P < 0.05\)). As shown in Fig. 7 A, B, and C, the LDH, AST, and CK levels were lower in the bellidifolin groups than in the \(\text{H}_2\text{O}_2\) intervention group (\(P < 0.05\)). Furthermore, the activities of SOD and GSHPx (Fig. 7 D and E) were substantially increased in the bellidifolin groups compared with those in the \(\text{H}_2\text{O}_2\) intervention group (\(P < 0.05\)). These results demonstrated the strong in vitro antioxidant activity of bellidifolin against \(\text{H}_2\text{O}_2\)-induced oxidative damage.

3.7. Bellidifolin protected \(\text{H}_9\text{C}_2\) cells from \(\text{H}_2\text{O}_2\)-induced apoptosis

We used the Annexin V-PI method to analyze the effects of bellidifolin on the apoptosis of \(\text{H}_9\text{C}_2\) cells. As shown in Fig. 8, the exposure of \(\text{H}_9\text{C}_2\) cells to \(\text{H}_2\text{O}_2\) (300 \(\mu\text{M}\)) for 3 h induced the apoptosis of approximately 47.75% of cells. Pretreatment with bellidifolin at different concentrations decreased the proportion of apoptotic cells in a dose-dependent manner (\(P < 0.05\)), suggesting that bellidifolin could protect \(\text{H}_9\text{C}_2\) cells from \(\text{H}_2\text{O}_2\)-induced apoptosis.

3.8. RNA-seq transcriptome analysis of \(\text{H}_9\text{C}_2\) cells treated with bellidifolin

Cellular RNA was extracted from the \(\text{H}_2\text{O}_2\) intervention group (\(\text{H}_2\text{O}_2\)-treated, \(n = 3\)), bellidifolin groups (\(\text{H}_2\text{O}_2\) and bellidifolin 20, 40, and 80 \(\mu\text{M}\) treatment, \(n = 3\)), and control group. Model-3 and bellidifolin-1 had poor repeatability and belong to outlier groups. Thus, they were removed during analysis. In DEGs, the genes between the \(\text{H}_2\text{O}_2\) intervention group (M) and the bellidifolin groups (A) showed 69 DEGs, including 20 upregulated and 49 downregulated genes. Between the \(\text{H}_2\text{O}_2\) intervention group (M) and control group (C), there are 828 DEGs, including 161 upregulated and 667 downregulated genes, were identified. Seventeen genes overlapped, as shown by the Venn diagram in Fig. 9A. According to the enrichment of differential genes, 37 related signaling pathways were found in M vs. A and 175 in M vs. C, including 31 identical signaling pathways. The enrichment analysis of the first 20 of the 31 pathways is shown in Fig. 9B. The pathways associated with cardiomyocites or oxidative stress included adrenergic signaling in cardiomyocytes, cardiac muscle contraction, AMP-activated protein kinase (AMPK) signaling pathway, cyclic adenosine monophosphate (cAMP) signaling pathway and PI3K/Akt signaling pathway. The volcano plots of M vs. A GO analysis is shown in Fig. 9C. The enriched BP of the targets were mainly associated with cellular component organization or biogenesis and positive regulation. CC were mainly distributed in organelle and supramolecular fiber. For the enriched MF, target proteins were mainly associated with binding and enzyme regulatory activities.

(A) KEGG enrichment analysis of DEGs of M vs. C and M vs. A. (B) DEGs Venn diagram. Different colors represent various combinations of
3.9. Effect of bellidifolin on GSK-3β comparisons. (C) GO enrichment analysis of DGEs of M vs. A. Molecular docking affinity.

| Compound | Binding energy/ (kcal mol⁻¹) |
|----------|-----------------------------|
|          | Akt1 | TNF | GSK-3β | TP53 |
| Bellidifolin | -8.2 | -8.3 | -8.2 | -5.1 |

comparisons. (C) GO enrichment analysis of DGEs of M vs. A.

Fig. 2. Network pharmacology prediction of the G. acuta treatment for ischemic heart disease. (A) Venn of drugs and disease (210 drug targets and 4064 disease targets). (B) Drug-active component–target network. The composition of G. acuta is shown in blue (37 in total); relevant targets are indicated in purple (135 in total). (C) Core target protein interaction network. The targets are arranged according to degree value, and the outermost target has a high degree value. (D) KEGG enrichment analysis.

Table 4 Molecular docking affinity.

4. Discussion

The pathogenesis of cardiovascular disease is complex [26], and cardiomyocyte injury is the pathophysiological basis of various cardiovascular diseases [13,27]. The occurrence of ischemic heart disease is due to the excessive oxygen consumption of myocardium [28] or excessive blood viscosity [29,30], which leads to insufficient blood supply and oxygen supply to cardiomyocytes, necrosis of cardiomyocytes or the damage of myocardial blood supply and oxygen supply function, which leads to the accumulation of reactive oxygen free radicals in vivo [31]. Oxidative stress plays an important role in the development and progression of ischemic heart disease. Oxidative stress is caused by an imbalance in oxidative and antioxidant effects, thereby producing large amounts of oxidized intermediate products. ROS include superoxide anion (•O₂⁻), hydroxyl radical (•OH), and H₂O₂. Excessive ROS production in cardiomyocytes can damage the mitochondria, stimulate the mitochondria to release cytochrome C, and activate caspase family members, such as caspase-9 and caspase-3, eventually leading to cell death via apoptosis [32,33]. H₂O₂ intervention is mostly used to generate an appropriate model for in vitro cytotoxicity studies and can partially simulate the heart pathophysiological process of the observed damage in vivo [34]. Therefore, we established H₂O₂-induced H9c2 cells in vitro.

The effects of G. acuta on ischemic heart disease have been comprehensively studied from chemical and pharmacological perspectives. In this study, 12 compounds, including the xanthone compound bellidifolin, in G. acuta were identified through LC-MS and compared with the literature records. According to previous studies [35–37], xanthone compounds have significant antioxidant effects. As a representative xanthone compound, bellidifolin was verified to have significant antioxidant properties in our previous experimental study [38]. In addition, the results of network pharmacological analysis showed that bellidifolin ranked highly for the treatment of ischemic heart disease. Therefore, the antioxidant mechanism of bellidifolin was studied in detail. Bellidifolin pretreatment significantly reduced the degree of myocardial injury, reduced cytotoxicity, and increased cell survival in H₂O₂-intervention models. The combination of the common pathways enriched by M vs. A and M vs. C in RNA-seq and analyzed through network pharmacology showed that the PI3K-Akt signal pathway is the key mechanistic pathway through which bellidifolin acted against the oxidative stress of cardiomyocytes. The level of myocardial enzyme activity in serum is related to the amount of myocardial cell necrosis. At present, the increased activities of LDH, CK, and AST in serum are often used as early diagnostic indicators of myocardial ischemia [39]. In this study, compared with that in the H₂O₂ intervention group, the bellidifolin groups had significantly increased SOD and GSH-Px activities in vitro [-8.2, -8.3, -8.2, -5.1]. The mRNA levels of GSK-3β and Akt significantly decreased in bellidifolin groups. (Fig. 10 C, D, and E). Akt1, and GSK-3β were detected through qRT-PCR and western blot, respectively, to investigate the changes in the PI3K-Akt signal pathway. As shown in Fig. 10 A and B, the mRNA levels of GSK-3β and Akt significantly decreased in the bellidifolin groups compared with those in the H₂O₂ intervention group, and significant differences were observed among the groups. The protein expression levels of GSK-3β and p-Akt1/Akt1 significantly decreased in bellidifolin groups. (Fig. 10 C, D, and E).
toxic effects on H9c2 cardiomyocytes in a H₂O₂-induced state.

PI3K-Akt signaling pathway is a key signaling cascade that plays a protective role in myocardial ischemia–reperfusion injury in vivo and in vitro. Akt overexpression is detrimental to the heart [40], and ROS can activate Akt through angiotensin II [41,42]. As a serine/threonine protein kinase, Akt can cooperate with phosphoinositide-dependent protein kinase 1/2 (PDPK1/2) to promote the binding of phosphatidylinositol triphosphate (PIP3) with itself and the transfer of Akt from the
cytoplasm to the plasma membrane [43]. At the same time, the phosphorylation of Akt at Ser473 and Thr308 activates the Akt protein kinase activity. GSK-3β is an important signaling protein located downstream of the Akt pathway, activated Akt activates GSK-3β. The Akt-related pathway can induce the phosphorylation of Ser9 of GSK-3β, thereby inactivating GSK-3β, activating downstream target molecules, reducing the myocardial infarction area, inhibiting myocardial cell apoptosis, and protecting the heart [44,45]. In the present study, the p-Akt1/Akt1 ratio and GSK-3β expression significantly increased in the H2O2 intervention group. The p-Akt1/Akt1 ratio and GSK-3β expression decreased after bellidifolin treatment, indicating that p-Akt1/Akt1 and GSK-3β are related to H2O2-induced cardiomyocyte toxicity, and bellidifolin protection of H2O2-induced H9c2 cells may be associated with the PI3K-Akt signaling pathway. However, a PI3K-Akt pathway inhibitor was not used in this study to block this pathway, and the exact link should be confirmed in further studies.

5. Conclusions

Edible medicinal plants contain various active ingredients which have a wide range of application prospects, and generally have the characteristics of low toxicity because of their edible properties. Many edible medicinal plant extracts, derivatives, and analogs have been developed and used to regulate health. Edible medicinal plants should be widely studied to determine the active components and mechanism of action, identify their safety and efficacy. *G. acuta* as an edible medicinal plant is commonly used in Mongolian and Tibetan medicine to make tea as a tonic for heart protection. However, its main active components and mechanism are unclear. This study provides strong evidence of the *in vitro* protective effects of bellidifolin on myocardial injury and explains its potential protective mechanisms. The results of our rigorous experiments and analysis showed that bellidifolin possesses potent antioxidant effects and functions by activating the PI3K-Akt signal pathway to improve the antioxidant capacity, inhibit cardiomyocyte apoptosis, and prevent cytotoxicity. These findings support the development of clinical application of bellidifolin for the treatment of ischemic heart disease and...
Fig. 8. Bellidifolin protected H9c2 cells from H2O2-induced apoptosis (means ± SD, n = 3). ***P < 0.001 compared with the control group; ###P < 0.001 compared with H2O2.

Fig. 9. Transcriptomic analysis of bellidifolin-treated H2O2-induced H9c2 cells.
provide a basis for the development of novel medicines. However, further research in vivo should be performed to support these results.

CRediT authorship contribution statement

Siqi Li: Conceptualization, Writing – original draft, Writing – review & editing. Congying Huang: Conceptualization, Writing – original draft, Writing – review & editing. Xing Li: Methodology, Writing – review & editing. Xiangxi Meng: Writing – review & editing. Rong Wen: Writing – review & editing. Minhui Li: Supervision, Project administration, Funding acquisition. Chunhong Zhang: Supervision, Project administration. Xiaodong Zhang: Supervision, Project administration.

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Fig. 10. Expression of GSK-3β and Akt. (A), (B): Validation of RNA-seq data by qRT-PCR. (C), (D), (E): Validation of protein expression by western blot. (means ± SD, n = 3). ***p < 0.001, **p < 0.01 compared with the control group; ****p < 0.001, ***p < 0.01, *p < 0.05 compared with H2O2.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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Conflict of Interest

The authors declare no conflicts of interest.

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