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LC-MS Analysis of Ginsenosides in Different Parts of *Panax quinquefolius* and Their Potential for Coronary Disease Improvement

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Authors
Xuanming Zhang[1], Can Kong[1], Xixin Wang[1], Hairong Hou[1], Hongxia Yu[3], Lizhen Wang[1], Peihai Li[1], Xiaobin Li[1], Yun Zhang[1], Liwen Han[1,2], Kechun Liu[1]

Affiliations
1 Engineering Research Center of Zebrafish Models for Human Diseases and Drug Screening, Biology Institute, Qilu University of Technology (Shandong Academy of Sciences), Jinan, China
2 College of Pharmacy and Pharmaceutical Sciences, Shandong First Medical University (Shandong Academy of Medical Sciences), Jinan, China
3 Wendeng Daodishen Industry Co. Ltd., Weihai, China

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Georg Thieme Verlag KG, Rüdigerstraße 14,
70469 Stuttgart, Germany

Correspondence
Professor Liwen Han
Biology Institute, Qilu University of Technology (Shandong Academy of Sciences)
No. 28789 Jingshidong Road, Caishi Town, Licheng District, 250103 Jinan, China
Phone: + 86 05 31 82 60 53 31, Fax: + 86 05 31 82 60 53 52
hanliwen@sdfmu.edu.cn

Correspondence
Professor Kechun Liu
Biology Institute, Qilu University of Technology (Shandong Academy of Sciences)
No. 28789 Jingshidong Road, Caishi Town, Licheng District, 250103 Jinan, China
Phone: + 86 05 31 82 60 53 31, Fax: + 86 05 31 82 60 53 52
hliukch@sdas.org

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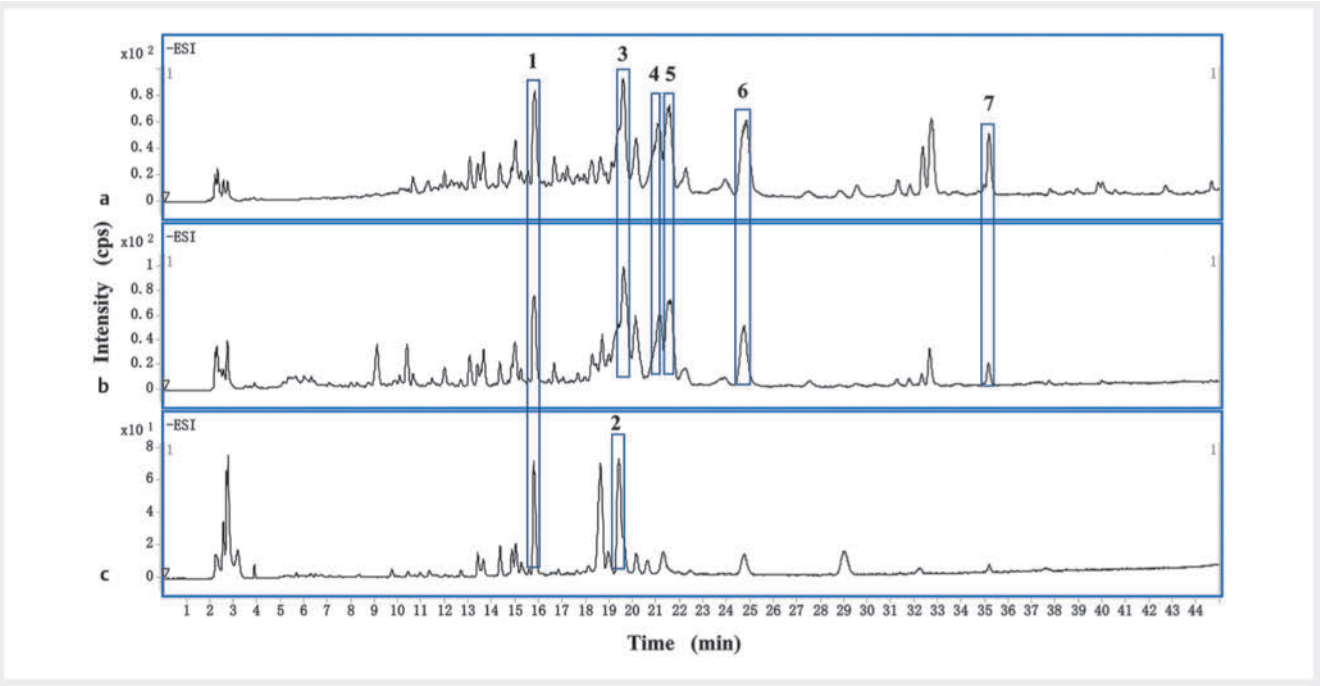
ABSTRACT

Seven main ginsenosides, including ginsenoside Re, ginsenoside Rb_1, pseudoginsenoside F_{11}, ginsenoside Rb_2, ginsenoside Rb_3, ginsenoside Rd, and ginsenoside F_2, were identified by LC-QTOF MS/MS from root, leaf and flower extracts of *Panax quinquefolius*. These extracts promoted intersegmental vessel growth in a zebrafish model, indicating their potential cardiovascular health benefits. Network pharmacology analysis was then conducted to reveal the potential mechanisms of ginsenoside activity in the treatment of coronary artery disease. GO and KEGG enrichment analyses elucidated that G protein-coupled receptors played a critical role in VEGF-mediated signal transduction and that the molecular pathways associated with ginsenoside activity are involved in neuroactive ligand–receptor interaction, cholesterol metabolism, the cGMP–PKG signaling pathway, etc. Moreover, VEGF, FGF2, and STAT3 were confirmed as the major targets inducing proliferation of endothelial cells and driving the pro-angiogenic process. Overall, ginsenosides could be potent nutraceutical agents that act to reduce the risks of cardiovascular disease. Our findings will provide a basis to utilize the whole *P. quinquefolius* plant in drugs and functional foods.



▶ **Fig. 1** LC-MS chromatograms of leaf (**a**), flower (**b**) and root (**c**) extracts from *P. quinquefolius*. The peaks were identified as ginsenoside Re (**1**), ginsenoside Rb_1 (**2**), pseudoginsenoside F_{11} (**3**), ginsenoside Rb_2 (**4**), ginsenoside Rb_3 (**5**), ginsenoside Rd (**6**), and ginsenoside F_2 (**7**).

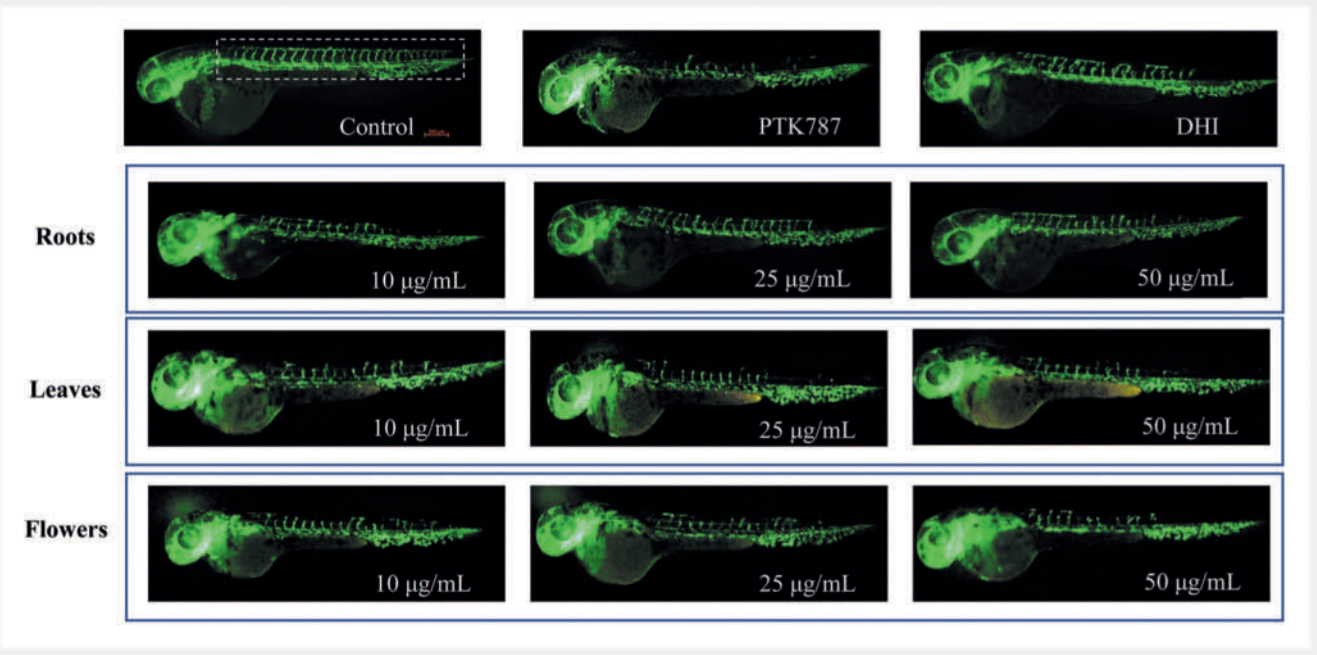
Introduction

The herbs *Panax quinquefolius, Panax ginseng*, and *Panax notoginseng*, which belong to the Panax genus (Araliaceae), are recognized as important natural sources of active ingredients for promoting health [1, 2]. *Panax quinquefolium* Linn. is a highly valued herbal remedy that is commonly used in drugs, dietary supplements, and food products. There has been a large demand for its root material in the Asian market. More than 95% of its wild types are consumed in mainland China and adjacent regions [3]. Historically, the plant was native to the USA and Canada. Since Wendeng (China) has a comparable latitude and similar climate to the producing areas in the USA, *P. quinquefolius* has been cultivated in the county in recent decades [4]. The active components of *P. quinquefolius* are known as ginsenosides. Previous studies have compared the chemical properties of *P. quinquefolius* and its congeneric species to unveil the differences in their ginsenoside compositions to enable better quality control [5–8]. Based on their skeleton, ginsenosides can mainly be divided into the protopanaxadiol types, protopanaxatriol types, oleanolic acid types, etc., which exhibit diverse pharmacological activities such as anti-tumor, cardiovascular protective, immunomodulatory, anti-inflammatory, antidiabetic, and hepatoprotective effects, as well as being useful tonics [9–12].

The leaves, stems and flower buds of *P. quinquefolius* contain many of the same active ingredients and have received much attention [13, 14]. Twenty marker ginsenosides have been characterized via untargeted metabolomics analysis as the most important diagnostic markers for differentiating the stem, leaf, flower bud, and root parts [15]. However, the components and bioactivity of the whole plants have not been investigated comprehensively. "Total ginsenosides" from *P. quinquefolius* stems and leaves have been added to the China Food Drug Administration standards (YBZ01382003). Systematic evaluation of the leaves, stems, and flower buds from *P. quinquefolius* will contribute to exploring the therapeutic basis of these plants and elaborating on the quality standards to promote their further utilization. In our study, liquid chromatography-quadrupole-time of flight mass spectrometry (LC-Q-TOF-MS) was applied for the analysis of ginsenosides in different parts (root, leaves, and flower buds) of *P. quinquefolius*. The compounds were characterized by accurate mass measurements, fragment ions, and comparing retention times to the reference standards. The relationships between the components and their cardio-protective effects were established via an *in vivo* zebrafish model, network pharmacology, and investigating the molecular mechanism in human umbilical vein endothelial cells (HUVECs). Our work lays a foundation for the utilization of the species in health-promoting products.

Results

The total ion chromatograms of root, leaf, and flower extracts (RE, LE, and FE) from *P. quinquefolius* are shown in ▶ **Fig. 1**, and seven ginsenosides were successfully identified by LC-MS/MS under Q/TOF conditions through a comparison with data from the literature [16–19]. In negative-ion mode, the MS/MS data of these ginsenosides were always acquired from the adduct ions, which provided valuable structural information. Ginsenoside Re (**1**) showed a $[M + Cl]^-$ ion at m/z 981.5102 and characteristic fragment ions at *m/z* 945 $[M - H]^-$, 783, 647, and 475. Ginsenoside Rb_1 (**2**) generated a $[M + Cl]^-$ ion at *m/z* 1143.5580 and corresponding fragment ions at *m/z* 1107 $[M - H]^-$, 945, 783 and 621. Pseudoginse-

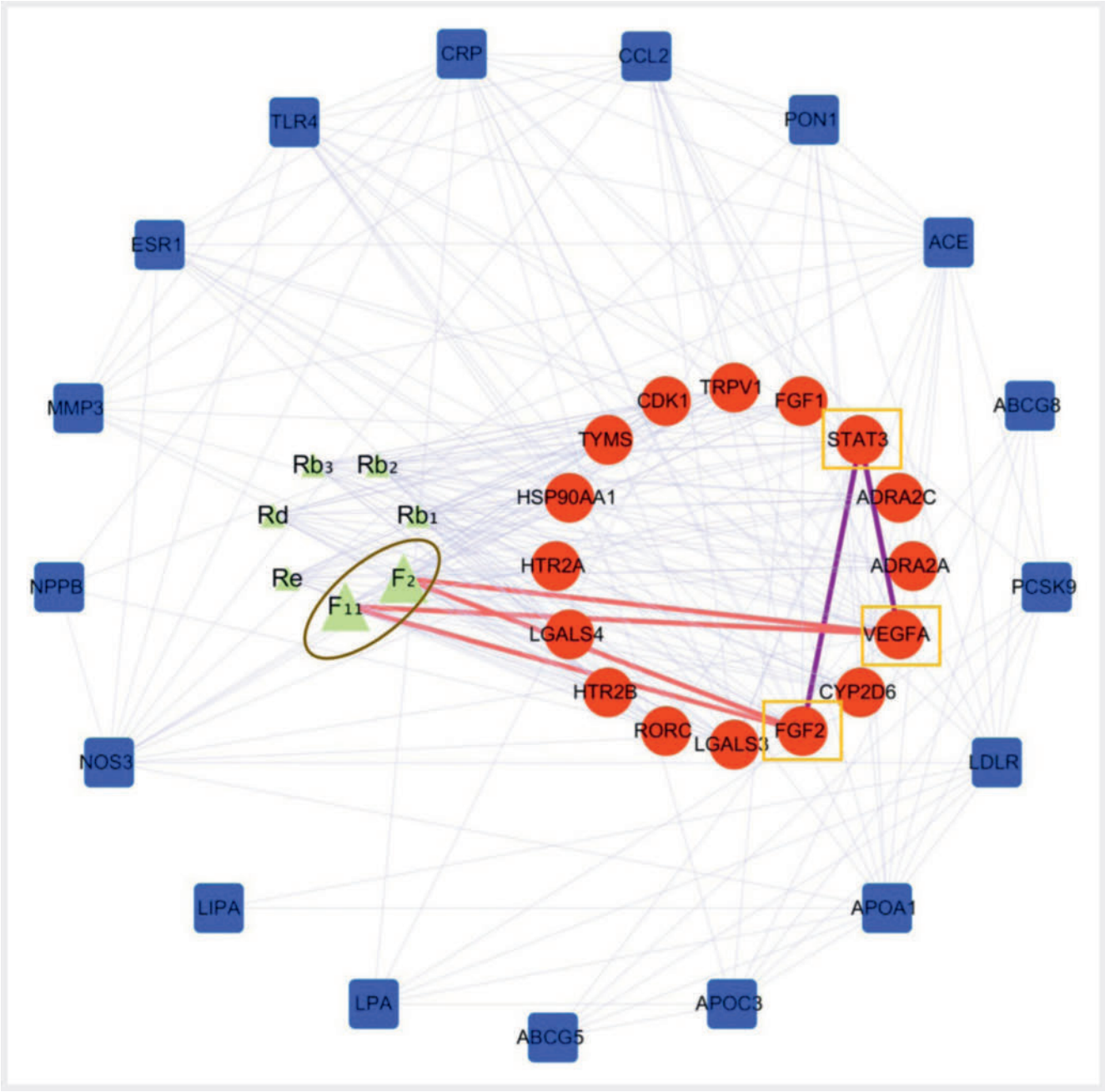


▶ **Fig. 2** Pro-angiogenic effects of RE, LE, and FE in transgenic zebrafish (Tg:vegfr2-GFP). The intersegmental vessels are indicated by the white square; Scale bar, 200 μm.

noside F_{11} (**3**) exhibited a $[M + Cl]^-$ quasi-molecular ion peak at *m/z* 835.4566 with daughter ions at *m/z* 799 $[M - H]^-$, 653, and 491. Ginsenoside Rb_2 (**4**) displayed a $[M + Cl]^-$ ion at *m/z* 1113.5393 and fragment ions at *m/z* 1077 $[M - H]^-$, 945, 783, and 621. Ginsenoside Rb_3 (**5**) produced a $[M + Cl]^-$ ion at *m/z* 1113.5513 along with fragment ions at m/z 1077 $[M - H]^-$, 945, 783, and 621. Ginsenoside Rd (**6**) showed a $[M + Cl]^-$ ion at *m/z* 981.5117 and main fragment ions at *m/z* 945 $[M - H]^-$, 783, and 621. Ginsenoside F_2 (**7**) demonstrated a signal corresponding to the $[M + Cl]^-$ ion at m/z 819.4597 and fragment ions at *m/z* 621. Lastly, all of the compounds were confirmed by LC–MS analysis of the reference substances (**Fig. 1S–7S**, Supporting Information). The ginsenosides Re and Rb_1 were detected at high levels in the roots, and the ginsenosides Re, Rb_2, Rb_3, Rd, F_2, and pseudoginsenoside F_{11} were abundant in the leaves and flowers. The details and structures of the identified ginsenosides are summarized in **Table 1S** and **Fig. 8S** (Supporting Information), respectively.

Coronary artery disease (CAD) is among the cardiovascular disease entities and will soon be the leading cause of death globally. Therapeutic angiogenesis is a promising strategy for revolutionizing the treatment of CAD, and the components for stimulating the growth of new blood vessels in the heart are highlighted in current clinical trials [20, 21]. Many proteins necessary for blood vessel growth in zebrafish are highly conserved and the same as those in mammals. Therefore, a transgenic zebrafish (Tg:vegfr2-GFP) model containing fluorescent blood vessels was considered to be an ideal tool for evaluating the effect of pro-angiogenesis compounds [22]. The zebrafish larvae had well-developed intersegmental blood vessels (ISVs) in the control, which were connected to the dorsal longitudinal anastomotic vessels. In contrast to the intact interstitial vessels, the vascular morphology of larvae showed severe damage after the PTK787 treatment, resulting in an obvious impairment of ISV formation in zebrafish. RE, LE, and FE were applied to determine whether angiogenesis could resume with the increase in treatment concentrations. After application, the extracts were shown to decrease the proportion of defective blood vessels and increase the proportion of normal blood vessels at proper concentrations. In particular, angiogenesis was more pronounced under 10 μg/mL LE, 25 μg/mL RE, and 10 μg/mL–25 μg/mL FE treatments, and these were statistically significant for restoring PTK787-induced ISV insufficiency (**Table 2S**, Supporting Information). The results indicated that RE, LE, and FE have the potential to promote angiogenesis; however, interestingly, the extracts exerted an inverted effect on protective vessels at concentrations greater than 50 μg/mL. Danhong injection (DHI), a Chinese patent compound injection, was used as the positive control.

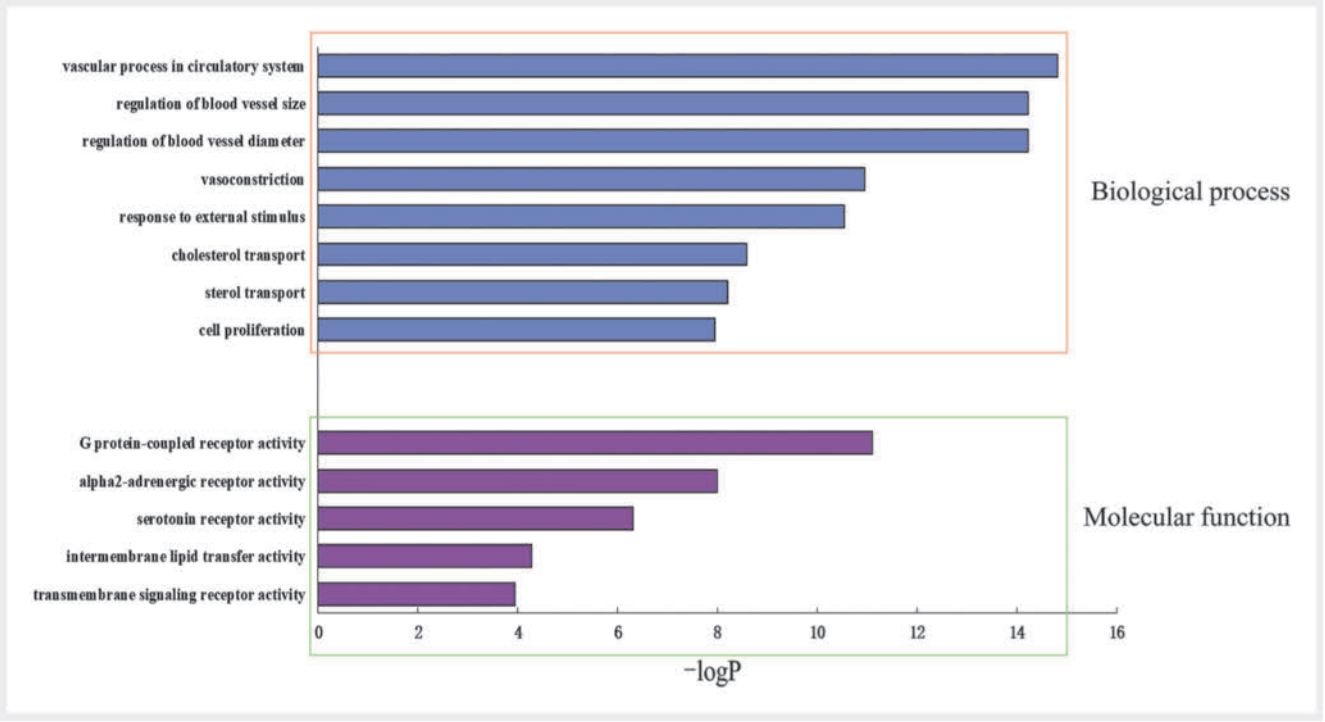
Network pharmacology has been proven to be a dominant paradigm by establishing a visualization network to understand the complex pharmacological action of effective substances in herbs [23]. After creating the intersections via an online Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/), 24 targets of the seven ginsenosides and 19 targets for CAD were gathered, respectively, to predict the links between the compounds and diseases through protein–protein interaction (PPI). "Degree" is the number of edges that a node shares with others, which is used to estimate the importance of the node in complex networks. As shown in ▶ **Fig. 3**, the analysis of the network revealed that VEGF (Degree, 22), FGF2 (Degree, 18) and STAT3 (Degree, 20) were visualized as the major targets of the compounds. Ginsenoside F_2 and pseudoginsenoside F_{11} were selected as the core compounds. Remarkably, both VEGF and FGF2 act, as angio-



▶ **Fig. 3** Construction of a candidate-target network for ginsenosides against CAD. The triangle nodes represent compounds; the circular nodes and rectangle nodes represent predicted targets and therapeutic targets, respectively.

genesis factors can be significantly affected by the two compounds, triggering the STAT3 targets and then hitting downstream genes for the treatment of CAD. The general network properties of the targets are shown in **Table 3S** (Supporting Information).

GO enrichment analysis was performed with the whole human genome as the background. The GO biological process and GO molecular function were used to classify the genes according to their functional annotation (▶ **Fig. 4**). Among biological processes, the candidate targets were mainly related to vascular processes in the circulatory system ($P = 1.51 \times 10^{-15}$), including regulation of blood vessel size, regulation of blood vessel diameter, vasoconstriction, etc. Among molecular functions, the targets were enriched in G protein-coupled receptors (GPCRs) activity, alpha2-adrenergic receptor activity, serotonin receptor activity, intermembrane lipid transfer activity, transmembrane signaling receptor activity, etc. GPCRs are proven to produce the most significant *P*-values, and indeed a number of discussions have shown that they play a critical role in VEGF-mediated signal transduction and are associated with the field of angiogenesis [24]. The KEGG pathway enrichment analysis was carried out to elucidate the molecular pathways involving these targets. The top 20 pathways are



▶ **Fig. 4** GO enrichment analysis displayed the targets from PPI network are enriched in various biological processes and molecular function.

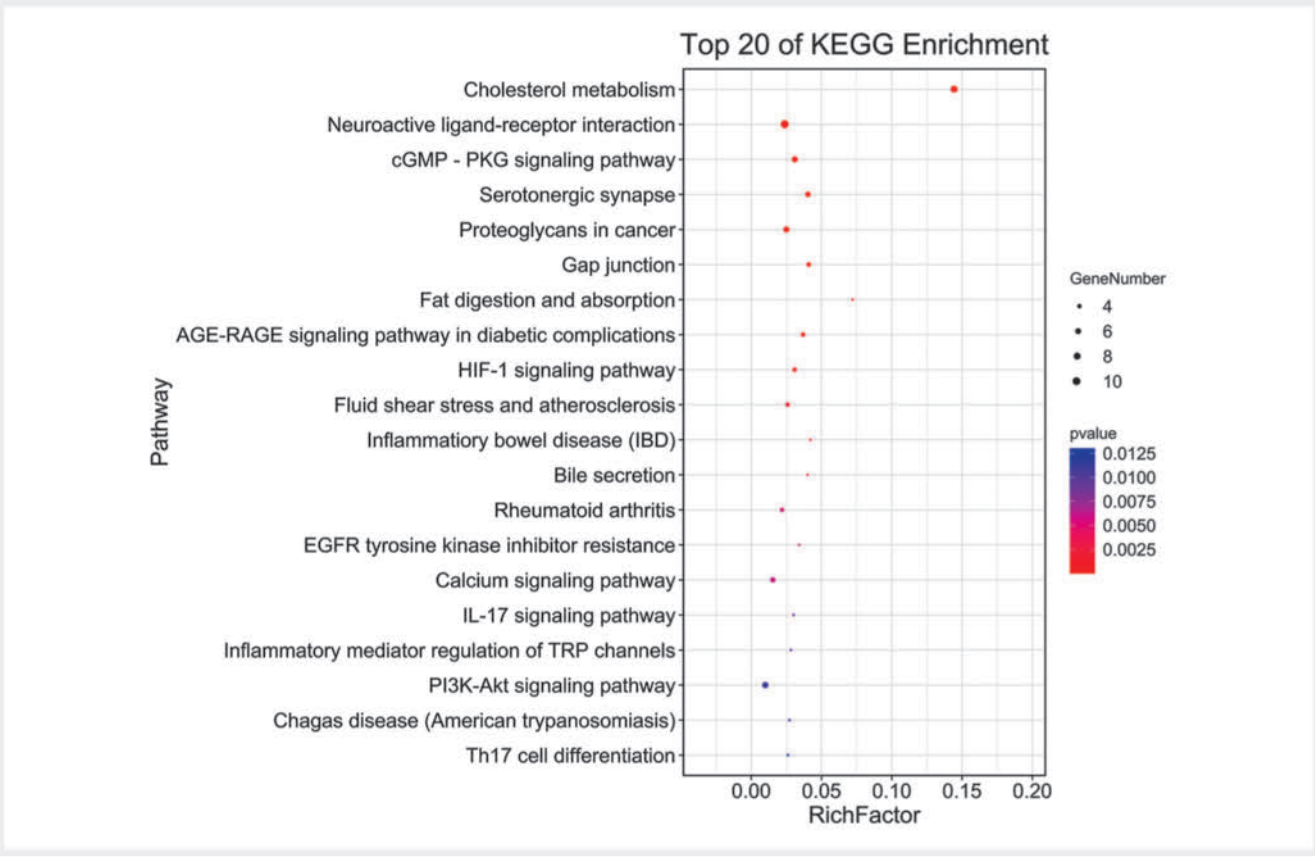
shown as the core pathways in ▶ **Fig. 5**. The results indicated that the KEGG pathways of the ginsenosides against CAD include neuroactive ligand–receptor interaction (10 genes), cholesterol metabolism (8 genes), the cGMP-PKG signaling pathway (6 genes), etc. Neuroactive ligand–receptor interactions have been suggested to be an important factor in response to angiogenesis, yielding a *P*-value of 1.55×10^{-6} among these terms. An explanation was that the growth of blood vessels and nerves promote each other and follow the same pathway [25]. Furthermore, we performed molecular biological assays involving VEGF, FGF2, and STAT3 to validate the effects of the compounds.

Because of their importance in angiogenesis, ginsenoside F_2 (protopanaxadiol-type) and pseudoginsenoside F_{11} (ocotillol-type) were investigated as the representative compounds for their effects on vascular growth. According to the protocol, the molecular mechanisms involved in the angiogenic process were generalized to HUVECs [26, 27]. In ▶ **Fig. 6a–b**, we observed that the exposure of HUVECs to ginsenoside F_2 and pseudoginsenoside F_{11} resulted in an obvious increase in cell numbers at concentrations of 2.5–5 µM and 5–10 µM, respectively, which exhibit statistically significant differences with *P*-values < 0.01. Cells treated with 20 ng/mL VEGF were used as the positive control. The results supported that HUVEC proliferation was allowed to proceed in the presence of the compounds at individual-level concentrations, whereas endothelial cell proliferation has been recognized as the key steps of the angiogenic process. However, similar to the zebrafish model, ginsenoside F_2 and pseudoginsenoside F_{11} also exerted an inverted effect on cell viability at higher concentrations (more than 10 µM and 25 µM, respectively). As featured in ▶ **Fig. 6c–d**, the compounds can activate expression of the proteins as predicted by bioinformatics. When compared with the control, the Western blotting data produced a dose-dependent increase in the VEGF, FGF2, and p-STAT3 levels at concentrations of 1–5 µM ginsenoside F_2 and 2.5–10 µM pseudoginsenoside F_{11}, respectively. Although the expression of p-STAT3 protein was obviously enhanced, the total level of STAT3 was not affected. Consequently, VEGF, FGF2, and p-STAT3 were recognized as the potential influencers of the ginsenosides, stimulating the proliferation of endothelial cells and driving the pro-angiogenic process.

Discussion

Ginsenosides accumulate at high levels in many parts of *P. quinquefolius*, such as the roots, leaves, and flower buds. In this study, the ginsenosides Re and Rb_1 were detected at high levels in the roots. Ginsenosides Rb_1 has been recognized as the top marker in the roots, which must meet the essential requirements for medical use in the Chinese Pharmacopoeia. Previous work showed that the leaves and flowers exhibited similar ginsenoside compositions, and pseudoginsenoside F_{11} was found to be the characteristic component in *P quinquefolium* [15]. Consistent with the conclusion, the protopanaxadiol-type ginsenosides Rb_2, Rb_3, Rd, F_2, protopanaxatriol-type ginsenosides Re, and ocotillol-type pseudoginsenoside F_{11} were identified from total-ion chromatograms of the leaves and flowers with high intensity. Because of the cultivation period of 4–6 years for the roots, the leaves and flowers can be harvested every year, and they are an important resource for obtaining these compounds.

There is evidence that *P. quinquefolius* can offer benefit to patients with heart failure and has been purported to improve car-



▶ **Fig. 5** KEGG analysis revealed the top 20 pathway terms associated with the targets from PPI network.

diac performance. The ginsenosides were demonstrated to be the active constituents that protect against myocardial infarction and thereby ameliorate cardiac dysfunction [28, 29]. The present study showed that RE, LE, and FE can restore vascular insufficiency in zebrafish at the specified concentrations and verified their positive association with angiogenic factors, illustrating their potential protective effects toward cardiovascular diseases. Neuroactive ligand–receptor interaction, cholesterol metabolism, and the cGMP–PKG signaling pathway were indicated as the top KEGG enrichments for the ginsenosides against CAD. Accumulating clinical evidence has confirmed that these pathways play a crucial role in angiogenesis, vascular protection, and vasodilatory effects [30–32].

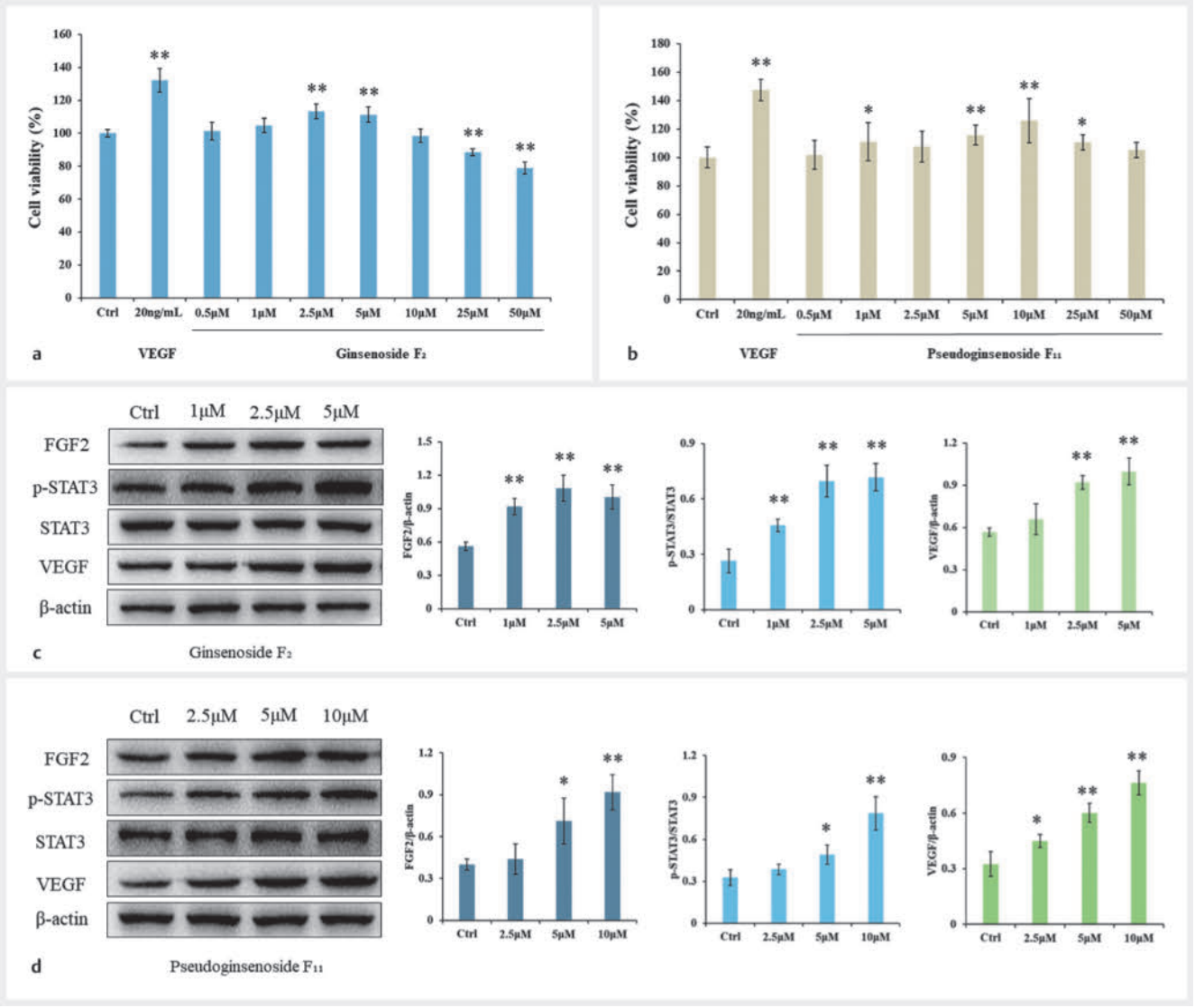
Angiogenesis involves endothelial cell differentiation, proliferation, migration, and cord formation for the formation of new capillaries sprouting from pre-existing vessels [33]. Many endothelium-specific molecules are associated with this process. For example, VEGF is present and is a key driver of angiogenesis signaling pathways. Recent evidence has demonstrated that VEGF significantly induces STAT3 activation, which is essential for vascular endothelial cell proliferation, vascular survival, or remodeling. Interestingly, STAT3 is also phosphorylated in response to FGF2 during angiogenic activation [34–36]. Based on our studies, we suggested that the overexpression of VEGF and FGF2 was induced by the ginsenosides and triggered the phosphorylation of STAT3. They were responsible for the proliferation of endothelial cells, leading to the angiogenic response. Our findings will provide a basis to facilitate the utilization of *P. quinquefolius* in nutraceutical agents and functional foods for CAD treatment.

We analyzed molecular signals at concentrations within the range shown to have a promoting effect on HUVEC proliferation. However, as previously reported, the ginsenosides were also found to inhibit the proliferation of HUVECs and served as the inhibitors against vascular growth at higher levels, while also being possibly involved in the expression of various factors associated with angiogenesis via the key mediator of VEGF. These qualities make *P. quinquefolius* potentially a very promising agent for controlling tumor growth [37, 38]. To better evaluate the biological characteristics and effects, the mechanisms involved in the cardiovascular activities of these ginsenosides should be further investigated.

Materials and Methods

Chemicals and reagents

Ginsenoside Re, ginsenoside Rb_1, pseudoginsenoside F_{11}, ginsenoside Rb_2, ginsenoside Rb_3, ginsenoside Rd, and ginsenoside F_2 were purchased from Shanghai Yuanye Biotechnology Co., Ltd. MS-grade water and acetonitrile were acquired from Watsons Ltd and Tedia Company Inc., respectively. All other chemicals used were analytical grade. Danhong injection (DHI, lot: 16011017)



▶ **Fig. 6** The ginsenosides stimulated the proliferation and induced VEGF, FGF2, and p-STAT3 activation in HUVECs. (A) Proliferation effects of ginsenoside F_2, (B) Proliferation effects of pseudoginsenoside F_{11}, (C) Ginsenoside F_2-mediated alterations in protein expression, (D) Pseudoginsenoside F_{11}-mediated alterations in protein expression. (The experiments were repeated six and three times for cell viability and western blot, respectively; error bars represent means ± SD, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control.)

was purchased from Danhong Pharmaceutical Co., Ltd. Human umbilical vein endothelial cells (HUVEC) were acquired from the American Type Culture Collection (ATCC).

Plant material extraction

Root, leaves, and flower buds of *P. quinquefolius* were obtained from Wendeng Daodishen Industry Co., Ltd. The samples were identified by Professor Kechun Liu, Biology Institute, Qilu University of Technology (Shandong Academy of Sciences). The plant materials were deposited at the Key Laboratory for Drug Screening Technology of the Biology Institute as a voucher specimen (No. SWS402B). One gram each of powdered root, leaf, and flower material was extracted at 50 °C with 8 mL of 50 % ethanol for 1 h using ultrasound.

LC–MS/MS analysis

The extract solutions were filtered through 0.45 μm nylon filters and subjected to LC-Q/TOF-MS analysis. LC-MS was performed on an XDB-C_{18} HPLC column (4.6 × 250 mm, 5 μm; Agilent), and the gradient conditions for solvents A (water) and B (acetonitrile) were as follows: 2–35 % B (0–15 min), 35 % B (15–25 min), 35–60 % B (25–40 min), and 60–90 % B (40–45 min). The following MS conditions were employed: ESI-negative-ion mode, nebulizer pressure at 35 psi, drying gas temperature of 325 °C, drying gas flow of 10 L/min, capillary voltage of 4000 V, and scanning range of 200–2000 m/z. High-resolution tandem mass spectrometry (HR–MS/MS) was performed for qualitative identification using standard compounds as a reference.

Zebrafish angiogenesis assay

Transgenic zebrafish (Tg : vegfr2-GFP) were obtained from the Zebrafish Drug Screening Platform, Qilu University of Technology (Shandong Academy of Sciences). After removal of the solvent, the residues of root, leaf, and flower bud extracts were evaluated for their angiogenesis activity. Zebrafish larvae 24 hpf (hour post-fertilization) were divided randomly into 24-well plates at a density of 10 larvae per well. The experiment consisted of a vehicle control group (embryo medium), a model group (0.1 µg/mL PTK787), a positive group (0.1 µg/mL PTK787 + 10 µL/mL DHI), and intervention groups (0.1 µg/mL PTK787 + 10, 25, 50, 100, or 150 µg/mL of each extract). All treatments, performed in triplicate, were maintained under standard culture conditions for a further 24 h. Subsequently, the zebrafish larvae were observed under a fluorescence microscope (SZX16, Olympus), and the angiogenic activity was assessed according to the integrity of the intersegmental vessels.

Target database construction and bioinformatics analysis

The chemical structures of ginsenoside were downloaded from the PubChem database (http://pubchem.ncbi.nlm.nih.gov/) and used for network pharmacology analysis. Swiss Target Prediction (http://www.swisstargetprediction.ch/) was used to screen the potential target of the active components. Considering the evidence for angiogenesis, the target genes associated with "Coronary artery disease" (CAD) were collected using the DisGeNET (http://www.disgenet.org/) and GeneCards (https://www.genecards.org/) databases. The candidate targets were inputted to String 11.5 (https://string-db.org/) to obtain the relevant information on protein interactions [39]. Furthermore, a compound-target network was constructed by employing Cytoscape 3.6.1. to study the therapeutic mechanism of CAD. GO and KEGG pathway enrichment analyses were executed using the OmicShare tools (https://www.omicshare.com/tools).

HUVEC proliferation and Western blot assay

The cells were cultured in RPMI-1640 medium under 5% CO_2 at 37 °C. An MTT assay was used to evaluate the effects of pseudoginsenoside F_{11} and ginsenoside F_2 on cell proliferation, which is responsible for angiogenesis. Furthermore, Western blot was performed as previously described with minor modifications [40]. Briefly, after lysing cells in RIPA buffer, the proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to polyvinylidene fluoride (PVDF) membranes. The primary antibodies and horseradish peroxidase-conjugated secondary antibodies were incubated with the membranes, and the protein bands were visualized using an enhanced chemiluminescence (ECL) substrate (cat No. 180–5001, Tanon) with the Tanon 5200 system.

The following antibodies were used: anti-FGF2 (1 : 1000, cat No. PA5-116495, Invitrogen), anti-STAT3 (1 : 1000, cat No. 60199-1-Ig, Proteintech), anti-p-STAT3 (1 : 1000, cat No. E121–31, abcam), anti-VEGF (1 : 1000, cat No. 66828-1-Ig, Proteintech), anti-β-actin (1 : 1000, cat No. 200068–8F10, ZenBio), HRP-conjugated goat anti-rabbit IgG (1 : 5000, cat No. A0208, Beyotime), and HRP-conjugated goat anti-mouse IgG (1 : 5000, cat No. A0216, Beyotime).

Statistics and analysis

In biological assays for multiple comparisons, ANOVA tests were performed using an online tool, Variance Calculator 20210415 (AB126 Software Park, http://www.ab126.com/shuxue/8016.html). Differences with a P value of < 0.05 were considered to be statistically significant.

Supporting information

Supplementary **Figure 1S–8S**: MS and MS/MS spectra of ginsenoside Re in negative mode; MS and MS/MS spectra of ginsenoside Rb_1 in negative mode; MS and MS/MS spectra of pseudoginsenoside F_{11} in negative mode; MS and MS/MS spectra of ginsenoside Rb_2 in negative mode; MS and MS/MS spectra of ginsenoside Rb_3 in negative mode; MS and MS/MS spectra of ginsenoside Rd in negative mode; MS and MS/MS spectra of ginsenoside F_2 in negative mode; Chemical structures of the identified ginsenosides.

Supplementary **Tables 1S–3S**: Retention time and characteristic ions of the ginsenosides evaluated using LC-MS/MS analysis; Angiogenic Activities in Zebrafish Larvae; The general network properties of the major targets.

Contributors' Statement

Conception and design of the work: X. Zhang, L. Han, K. Liu; data collection: X. Zhang, C. Kong, X. Wang, H. Hou, H. Yu; analysis and interpretation of the data: X. Zhang, X. Wang, L. Wang, P. Li, X. Li, Y. Zhang; statistical analysis: X. Zhang, C. Kong; drafting the manuscript: X. Zhang, X Wang, L. Wang, X. Li, Y. Zhang; critical revision of the manuscript: X. Zhang, L. Han, K. Liu.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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