

Rosmarinic acid attenuates heart failure-related injury through modulation of ferroptosis-related pathways: Network pharmacology and cellular validation

Yili Yao¹, Jianghong Ling^{2*} and Xiaolong Wang¹

¹Department of (Internal Medicine) Cardiovascular, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 200021, China

²Department of Gastroenterology, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 200021, China

Abstract: Background: Ferroptosis-related oxidative injury has been implicated in the progression of heart failure (HF). Rosmarinic acid (RA) has antioxidant and anti-lipid peroxidation properties, but its role in HF-associated ferroptosis-related cardiomyocyte injury remains unclear. **Objectives:** This study aimed to investigate whether RA attenuates Ang II-induced cardiomyocyte injury by modulating ferroptosis-related oxidative injury and GPX4/xCT-associated defense responses. **Methods:** RA-related targets were retrieved from TCMSP, while HF-related and ferroptosis-related genes were obtained from GeneCards/OMIM and FerrDB. Intersection targets were analyzed using PPI network construction, GO and KEGG enrichment analyses, RA-target-disease-pathway network construction and molecular docking. An Ang II-induced H9c2 cardiomyocyte injury model was established for validation. Cell viability was assessed by CCK-8 assay; ANP and BNP mRNA levels were measured by RT-qPCR; MDA content was measured to evaluate lipid peroxidation; and GPX4 and xCT protein expression was examined by Western blotting. **Results:** Twenty-six RA-related targets, 3311 HF-related genes and 1393 ferroptosis-related genes were identified. Intersections yielded 397 HF-associated ferroptosis targets and 9 RA-related candidate targets. Network analysis highlighted PPARG, PTGS2, MAPK1, CDKN1A and CASP3 as important targets. Enrichment analyses indicated associations with oxidative stress, apoptotic regulation, iron ion response, inflammatory signaling, lipid and atherosclerosis, TNF, IL-17 and AGE-RAGE pathways. Molecular docking suggested favorable binding of RA to these targets, especially PPARG and PTGS2. In Ang II-treated H9c2 cells, RA restored cell viability, reduced ANP and BNP expression, decreased MDA accumulation and increased GPX4 and xCT expression; these effects were partially reversed by erastin. **Conclusion:** RA may attenuate Ang II-induced cardiomyocyte injury by modulating ferroptosis-related oxidative injury and GPX4/xCT-associated defense responses. These findings provide preliminary computational and cellular evidence supporting RA as a candidate compound for further investigation in HF-related cardiomyocyte injury.

Keywords: Ferroptosis; GPX4; Heart failure; Network pharmacology; Rosmarinic acid; xCT

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INTRODUCTION

Ferroptosis, which depends on iron and is driven by lipid peroxidation, constitutes a key pathogenic driver in cardiovascular diseases (Yu *et al.*, 2021). Featuring mitochondrial shrinkage, elevated membrane density, and cristae reduction, ferroptosis displays a distinct morphological profile that differs from that of apoptosis, necrosis and autophagy (Tang *et al.*, 2021). Driven by labile iron-catalyzed reactive oxygen species (ROS) accumulation, ferroptosis initiates at the molecular level via the Fenton reaction, leading to the lethal peroxidation of polyunsaturated fatty acids in membrane phospholipids (Li *et al.*, 2024). GPX4 and SLC7A11 (xCT) constitute the core defense system against ferroptosis. Glutathione (GSH) synthesis depends on SLC7A11-mediated cystine import. Lipid peroxide reduction to harmless alcohols requires GPX4, utilizing GSH. These processes

collectively inhibit lipid peroxidation and ferroptosis (Gong *et al.*, 2025). MDA, the terminal product of lipid peroxidation, serves as an important biomarker of ferroptosis when elevated (Yang *et al.*, 2024). Recent work has established that ferroptosis contributes significantly to HF (Zhang *et al.*, 2025), rendering it an attractive therapeutic target for cardioprotection.

Rosmarinic acid (RA) is a natural polyphenolic compound widely distributed in Lamiaceae and Boraginaceae plants, including rosemary (*Rosmarinus officinalis*), Danshen (*Salvia miltiorrhiza*) and perilla (*Perilla frutescens*) (Noor *et al.*, 2022). Chemically, RA derives from caffeic acid and 3,4-dihydroxyphenyllactic acid. Its two catechol structures enable potent free radical scavenging. They also provide anti-lipid peroxidation activity (Girgenti *et al.*, 2025). Growing evidence demonstrates that RA exhibits remarkable antioxidant, anti-inflammatory, anti-apoptotic and cardioprotective effects (Atefipour *et al.*, 2024). Notably, previous studies have shown that RA elevates the

*Corresponding author: e-mail: jh_ling870@163.com

expression of ferroptosis defense genes, including GPX4 and xCT, and suppresses lipid peroxidation, suggesting that it may exert cytoprotective effects by modulating ferroptosis-related proteins (Liu *et al.*, 2026). However, whether RA can alleviate HF-related cardiomyocyte injury by modulating ferroptosis-related oxidative stress remains unclear. In particular, the potential upstream targets and pathways linking RA to downstream GPX4/xCT-associated ferroptosis defense responses in HF-related cellular injury have not been systematically investigated.

Angiotensin II (Ang II), the pivotal effector of the renin-angiotensin-aldosterone system (RAAS), drives the development of pathological cardiac hypertrophy and HF (Tuo *et al.*, 2024). Ang II induces cardiomyocyte hypertrophy through multiple mechanisms, including oxidative stress generation, calcium overload and activation of pro-hypertrophic signaling pathways such as NF- κ B (Ren *et al.*, 2021). The Ang II-induced cardiomyocyte model is commonly used as an in vitro model to investigate cardiac hypertrophy and screen for cardioprotective drugs, as it simulates cell injury and pathological alterations associated with HF (Ren *et al.*, 2021). It is embryonic rat heart tissue that serves as the origin of the H9c2 cell line, a widely adopted cardiomyocyte model, retains cardiac-specific characteristics and responds to hypertrophic stimuli in a manner similar to primary cardiomyocytes, representing a well-established cellular model for studying cardiomyocyte injury and drug effects (Shi *et al.*, 2024).

Given the established role of ferroptosis in HF pathogenesis and the well-documented anti-lipid peroxidation activity of RA, along with its ability to upregulate ferroptosis defense genes such as GPX4 and xCT, it was hypothesized that RA may ameliorate Ang II-induced cardiomyocyte injury by modulating ferroptosis-related oxidative injury and GPX4/xCT-associated defense responses. The specific mechanistic question addressed in this study was whether RA attenuates Ang II-induced cardiomyocyte injury primarily by modulating downstream GPX4/xCT-associated ferroptosis defense responses, and whether network pharmacology could further identify potential upstream targets and pathways that may contribute to these effects. To address this research gap, this study employed an integrated approach combining network pharmacology prediction with cellular experimental validation, using Ang II-induced H9c2 cardiomyocytes as a model to systematically investigate the effects of RA on cardiomyocyte viability, hypertrophic markers (ANP and BNP) expression, lipid peroxidation levels (MDA content) and the expression of key ferroptosis proteins (GPX4 and xCT). Furthermore, interventions with ferrostatin-1 (Fer-1) and erastin were conducted to determine whether RA exerts cardioprotective effects by modulating ferroptosis-related pathways. These findings are expected to provide preliminary experimental evidence

and theoretical support for the development of RA as a potential cardioprotective compound for HF-related injury.

MATERIALS AND METHODS

Compound target prediction

RA (MOL007132) was predefined as the compound of interest in this study. The TCMSP database (Ru *et al.*, 2014) was used to retrieve RA-related targets through the “Related Targets” module. RA was not selected based on oral bioavailability or drug-likeness thresholds, as this study focused on a predefined single compound rather than screening active components from a herbal mixture. After deduplication, RA-related candidate targets were obtained for subsequent intersection analysis.

Disease target acquisition and intersection screening

HF-related targets were retrieved from GeneCards (Stelzer *et al.*, 2016) and OMIM (Amberger *et al.*, 2015) using the keywords “heart failure” and “cardiac failure”. For GeneCards, targets with a relevance score >10 were retained. Ferroptosis-related genes were retrieved from FerrDB V3 (Zhou and Bao, 2020; Zhou *et al.*, 2023), and only marker and driver genes were selected. After merging and deduplication, HF-related targets were intersected with ferroptosis-related genes to identify HF-associated ferroptosis targets. These targets were further intersected with RA-related targets using Venny 2.1 (Oliveros, 2007) to obtain potential RA targets involved in HF-associated ferroptosis.

Protein-protein interaction network construction

The intersection targets were imported into the STRING database (Szklarczyk *et al.*, 2023). The PPI network was constructed with “Homo sapiens” as the species and a confidence score threshold of ≥ 0.4 . The PPI results were then imported into Cytoscape 3.9.1 for visualization. The CytoHubba plugin was used to calculate degree values and identify relatively important nodes in the PPI network.

GO and KEGG analysis

GO and KEGG enrichment analyses were performed for the RA-HF-ferroptosis intersection targets using the clusterProfiler 4.6.0 package in R 4.3.0. Gene symbols were converted to Entrez IDs before enrichment analysis. GO enrichment analysis covered three categories: biological process (BP), cellular component (CC) and molecular function (MF). KEGG pathway enrichment analysis was performed using the KEGG pathway database. The entire human genome annotation from the org.Hs.eg.db package was used as the background gene set. Enrichment significance was calculated using the hypergeometric test and P values were adjusted for multiple comparisons using the Benjamini-Hochberg method. Terms or pathways with $P < 0.05$ and adjusted P value/FDR < 0.05 were considered significantly enriched. When more than 15 significant terms or pathways were

identified, the top 15 entries, ranked by adjusted *p*-value, were selected for visualization. GO bar plots and KEGG enrichment plots were generated using the ggplot2 package (v3.4.0).

RA-target-disease-pathway network construction

RA, the intersection targets, HF, ferroptosis and significantly enriched KEGG pathways were integrated to construct an RA-target-disease-pathway network. The network was visualized using Cytoscape 3.9.1 and the relationships among RA, candidate targets, diseases and pathways were analyzed to illustrate the potential multi-target and multi-pathway regulatory characteristics of RA.

Molecular docking evaluation

Candidate targets selected from the RA-target-disease-pathway network, including PPARG, PTGS2, MAPK1, CDKN1A and CASP3, were used for molecular docking analysis. The protein crystal structures of these targets were obtained from the RCSB PDB database (Berman *et al.*, 2000). PyMOL was used for protein preprocessing, including removal of water molecules, ligands and redundant conformations. The two-dimensional structure of RA was downloaded from TCMSP and submitted to CB-Dock2 (Liu *et al.*, 2022) for blind docking. Each protein-ligand pair was automatically docked five times and the conformation with the lowest binding energy was selected for visualization and analysis.

Cell culture

H9c2 rat cardiomyocytes were obtained from the Cell Bank of the Chinese Academy of Sciences (CBCAS/SIBS, Shanghai, China; catalog no. GNR 5) and used between passages 5 and 15. Cells were grown in high-glucose DMEM (Procell Life Science and Technology, Wuhan, China) fortified with 10% FBS (Gibco, Thermo Fisher Scientific) and 1% penicillin-streptomycin (Biosharp). Cells were maintained at 37°C in a humidified CO₂ incubator (Thermo Fisher Scientific, Waltham, MA, USA) with 5% CO₂, with medium renewal every 2–3 days. Upon reaching 80%–90% confluence, cells were passaged. Only logarithmic-phase cells with optimal viability were used for experiments.

Cell grouping

H9c2 cells were plated into 96-well plates (Corning Inc.) at 5×10^3 cells/well. After 24 h, randomization was performed to allocate cells to various treatments. To establish the working concentration of RA, cells were incubated with increasing concentrations of RA (5, 10, 50, 100 μ M; Aladdin Reagent, Shanghai, China) or DMSO vehicle (Sigma-Aldrich) for 24 h and 50 μ M was chosen for subsequent experiments. This concentration was also consistent with a previous study in H9c2 cardiomyocytes, which used 50 μ M RA to attenuate doxorubicin-induced cardiomyocyte injury following concentration screening (Xie *et al.*, 2025). Thereafter, cells were divided into five experimental cohorts: (i) Untreated control; (ii) Ang II

(Wang *et al.*, 2022): Exposure to 1 μ M Ang II (Shanghai Yuanye Bio-Technology) for 24 h; (iii) Ang II+RA: 1 h pretreatment with 50 μ M RA followed by 24 h Ang II stimulation; (iv) Ang II+Fer-1 (Sun *et al.*, 2022): 1 h pretreatment with 10 μ M Fer-1 (Sigma-Aldrich, Merck KGaA) prior to 24 h Ang II incubation; (v) Ang II+RA+Erastin: Sequential pretreatment with 50 μ M RA (1 h) and 5 μ M erastin (MedChemExpress, Monmouth Junction, NJ, USA) (1 h), then co-incubation with Ang II for 24 h. The concentration of erastin was selected based on previous NRCM ferroptosis studies showing that 5 μ M erastin effectively induces ferroptosis-related injury while maintaining sufficient cell viability for rescue analysis (Cheng *et al.*, 2024).

CCK-8 assay

Upon completion of the experimental treatments in Experiments 1 and 2, cell viability was assessed using the CCK-8 assay according to the manufacturer's instructions and previously described protocols (Sun *et al.*, 2022). Each well was supplemented with 10 μ L of CCK-8 working solution (Beyotime Biotechnology) and incubated at 37°C for 1–2 h. Absorbance readings at 450 nm were then obtained using a microplate reader (Synergy H1 Hybrid Multi-Mode Reader, BioTek Instruments/Agilent Technologies, Winooski, VT, USA).

MDA assay

Following the MDA detection kit manual (Cat. No. A003-1, Nanjing Jiancheng), cell precipitates were collected and processed after completion of the treatments in Experiment 2. Using the TBA method, MDA content was determined. Briefly, cell disruption was performed by sonication (200 W, 3-s pulses with 5-s intervals, 30 cycles). Following centrifugation of lysates (12,000 rpm, 10 min, 4°C). With the TBA reagent (95°C, 40 min), the supernatant was incubated using a microplate reader (Synergy H1 Hybrid Multi-Mode Reader, BioTek Instruments/Agilent Technologies), and absorbance at 532 nm was measured after cooling and centrifugation. MDA content, expressed as nmol/mg protein, was calculated based on the determined protein concentration (BCA protein assay kit, Beyotime Biotechnology).

RT-qPCR

Upon completion of Experiment 2, with TRIzol reagent (Thermo Fisher Scientific), total RNA was extracted from cells. RNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). With PrimeScript RT Master Mix (RR036A), reverse transcription of 2 μ g RNA to cDNA was conducted (37°C for 15 min, 85°C for 5 s), followed by cooling to 4°C. Subsequently, quantitative PCR was performed in a 20 μ L reaction containing SYBR Green qPCR mix (Bio-Rad). Using Primer-BLAST (NCBI) (Bai *et al.*, 2025), gene-specific primers for ANP, BNP, and GAPDH (as a reference) were designed. The sequence is shown in table 1.

Table 1: Primer sequences (5'-3').

Genes	Sense (5'-3')	Antisense (5'-3')
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
ANP	ACAATGCCGTGTCCAACGCAGA	CTCCTTCTGCATCCTGTCAGC
BNP	TCTGGCTGCTTTGGGAGGAAGA	CCTTGTGGAATCAGAAGCAGGTG

Western blot

Western blotting was performed as previously described with minor modifications (Sun *et al.*, 2022; Wang *et al.*, 2022). After treatment, cells were lysed in RIPA lysis buffer (Beyotime) containing 1% PMSF, 1% protease inhibitor, and 1% phosphatase inhibitor on ice for 30 min. The lysate was centrifuged at 12,000 rpm for 15 min at 4 °C and the supernatant was collected. 30 µg of protein samples were subjected to 12% SDS-PAGE electrophoresis, after protein concentration determination using the BCA protein concentration assay kit (Beyotime), followed by wet transfer onto PVDF membranes (Merck Millipore) at 300 mA for 90 min. The membranes were blocked for 1 h at room temperature after being treated with 5% skim milk (Biosharp). Primary antibody incubation was carried out overnight at 4°C using GPX4 (ab125066, 1:1000), xCT (ab307601, 1:1000) and β-actin (66009-1-Ig, 1:2000) antibodies. This was followed by three washes with TBST (10 min each). Secondary antibody incubation was then performed for 1 h at room temperature with HRP-conjugated goat anti-rabbit IgG (1:5000) or goat anti-mouse IgG (1:5000) (Proteintech). Using the ECL ultra-sensitive chemiluminescence reagent (Biosharp), the bands were visualized after washing with TBST, and images were captured with the ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Hercules, CA, USA). Densitometric analysis was performed using Image Lab software (v6.1, Bio-Rad) and relative protein expression levels were calculated as the ratio of target protein to β-actin.

Statistical analysis

All experiments were performed with three independent biological replicates. Data were reported as the mean ± SD. Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Group comparisons were performed by one-way ANOVA followed by Tukey's post hoc test. A P-value < 0.05 was deemed to indicate statistical significance.

RESULTS**Screening of RA targets related to heart failure-associated ferroptosis**

RA-related drug targets were retrieved from the TCMSP database and 26 RA-related candidate targets were obtained after deduplication. Heart failure-related targets were collected from GeneCards and OMIM, yielding 3311 heart failure-related genes. Ferroptosis-related genes were retrieved from FerrDB by selecting marker and driver genes, yielding 1393 ferroptosis-related genes. The

intersection between heart failure-related genes and ferroptosis-related genes identified 397 heart failure-associated ferroptosis targets (Fig. 1A). Further intersection of these 397 heart failure-associated ferroptosis targets with the 26 RA-related drug targets identified 9 potential RA targets involved in heart failure-associated ferroptosis (Fig. 1B). These 9 intersection targets were then used to construct the PPI network. The resulting network consisted of PTGS2, PPARG, CASP3, CDKN1A, MAPK1, AR, SNCA, CCL2 and CCL3 (Fig. 1C). Among them, PTGS2, PPARG, CASP3, CDKN1A and MAPK1 showed relatively higher network connectivity, suggesting that these targets may represent important candidate nodes through which RA participates in the regulation of heart failure-associated ferroptosis.

GO functional and KEGG pathway enrichment analysis of intersection targets

GO and KEGG enrichment analyses were performed based on the 9 RA-heart failure-ferroptosis intersection targets. GO biological process analysis showed that these targets were mainly enriched in regulation of cellular catabolic process, gliogenesis, regulation of apoptotic signaling pathway, positive regulation of DNA metabolic process, intrinsic apoptotic signaling pathway, response to oxidative stress, cellular response to oxidative stress, response to ischemia, response to iron ion, intracellular iron ion homeostasis, cardiac muscle cell apoptotic process and cardiac chamber development (Fig. 2A). These enriched biological processes are closely related to oxidative stress, iron homeostasis, apoptosis-related injury and cardiomyocyte pathological responses. KEGG pathway analysis showed that the intersection targets were mainly enriched in human cytomegalovirus infection, lipid and atherosclerosis, TNF signaling pathway, microRNAs in cancer, IL-17 signaling pathway, efferocytosis, thyroid cancer, AGE-RAGE signaling pathway in diabetic complications and other inflammation- or cell death-related pathways (Fig. 2B). These findings suggest that RA may regulate heart failure-associated ferroptosis through multiple biological processes, including oxidative stress, inflammatory signaling, apoptotic regulation, and iron-related cellular responses.

Construction of the RA-target-disease-pathway integrated network

To maintain consistency with the single-compound focus of this study, an RA-target-disease-pathway integrated network was constructed using RA, the 9 intersection targets, heart failure, ferroptosis and significantly enriched KEGG pathways (Fig. 3).

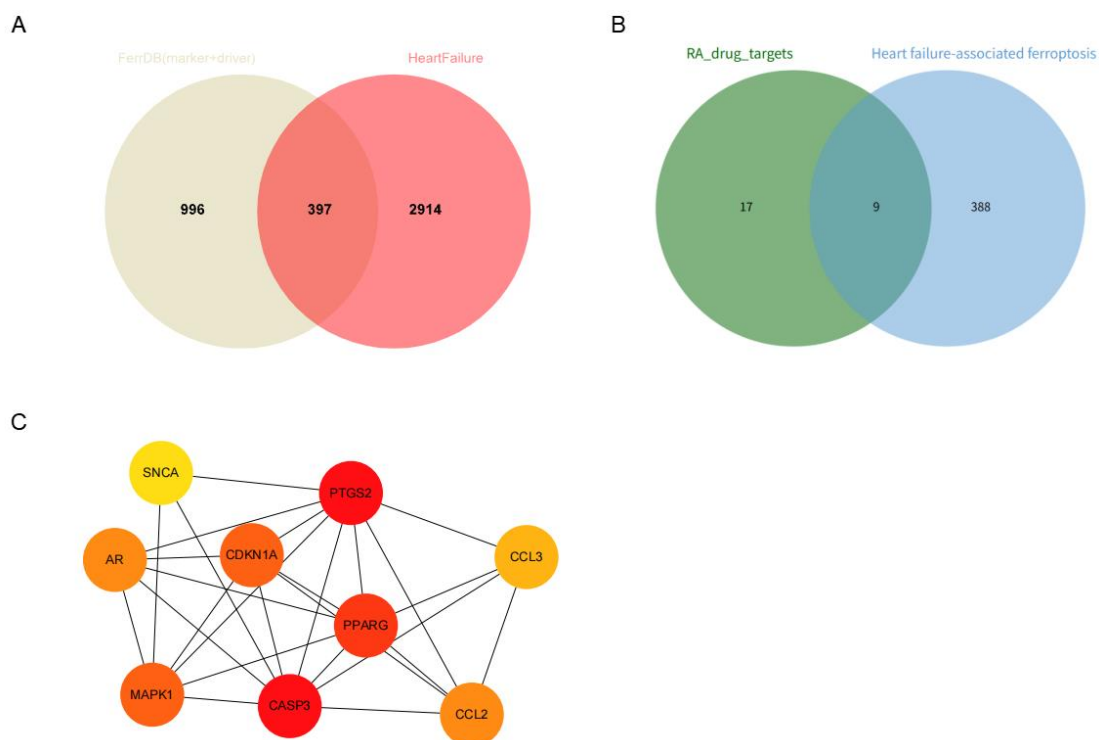


Fig. 1: Screening of RA-associated targets related to heart failure-associated ferroptosis. (A) Venn diagram showing the intersection between ferroptosis-related genes retrieved from FerroDB marker and driver categories and heart failure-related genes, yielding 397 heart failure-associated ferroptosis targets; (B) Venn diagram showing the intersection between RA-related drug targets and heart failure-associated ferroptosis targets, yielding 9 potential RA targets involved in heart failure-associated ferroptosis; (C) PPI network of the 9 intersection targets, including PTGS2, PPARG, CASP3, CDKN1A, MAPK1, AR, SNCA, CCL2 and CCL3. Node size and color intensity indicate the degree value.

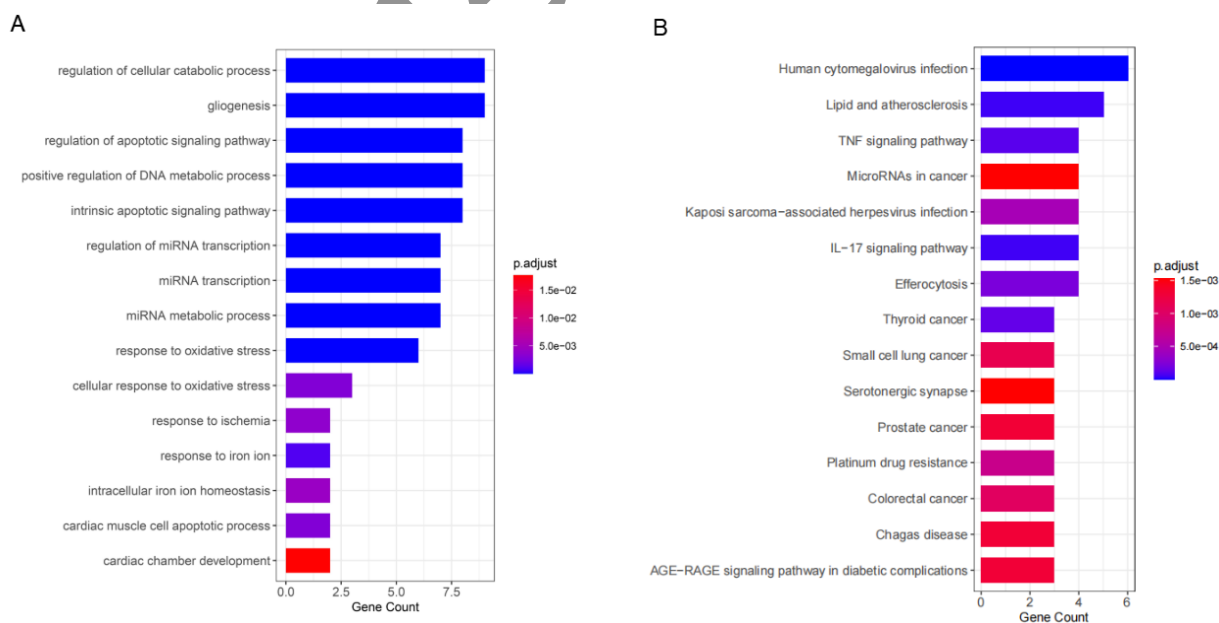


Fig. 2: GO and KEGG enrichment analyses of RA-associated intersection targets. (A) GO biological process enrichment analysis of the 9 RA-associated intersection targets. Bar length represents gene count and color indicates the adjusted P value; (B) KEGG pathway enrichment analysis of the 9 RA-associated intersection targets. Bar length represents gene count and color indicates the adjusted P value.

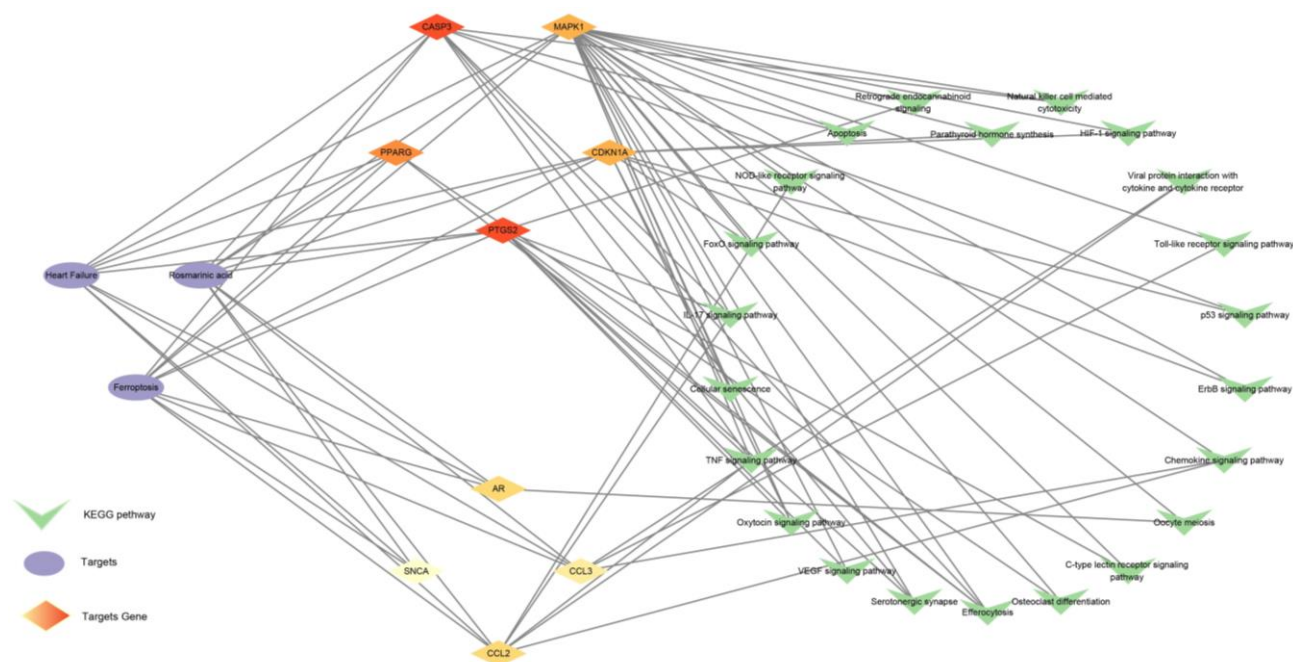


Fig. 3: RA-target-disease-pathway integrated network. The network diagram illustrates the relationships among RA-associated candidate targets, heart failure, ferroptosis, and enriched KEGG pathways. Target genes are shown as diamond nodes, diseases are shown as oval nodes and KEGG pathways are shown as V-shaped nodes. The network highlights the potential multi-target and multi-pathway regulatory characteristics of RA in heart failure-associated ferroptosis.

The network showed that RA was connected with multiple candidate targets, including PPARG, PTGS2, MAPK1, CDKN1A, CASP3, AR, CCL2 and SLC2A1, which were further associated with heart failure, ferroptosis and enriched signaling pathways. Among these targets, PPARG, PTGS2, MAPK1, CDKN1A and CASP3 were selected for molecular docking analysis due to their network relevance and potential involvement in inflammation, apoptosis, lipid metabolism, and stress-related cellular responses. This integrated network supports the multi-target and multi-pathway regulatory characteristics of RA in heart failure-associated ferroptosis.

Molecular docking validation of RA with candidate targets

Molecular docking was performed to evaluate the predicted binding interactions between RA and selected candidate targets. The docking results showed that RA exhibited favorable binding affinities with PPARG, PTGS2, MAPK1, CDKN1A and CASP3, with binding energies of -9.1, -9.1, -8.0, -7.9 and -7.3 kcal/mol, respectively (Table 2, Figs. 4). Among these targets, PPARG and PTGS2 showed the lowest binding energies, suggesting relatively stronger predicted binding affinities with RA. The docking conformations indicated that RA could fit within the predicted binding pockets of these proteins and engage multiple amino acid residues. Because these candidate targets are closely associated with inflammation, apoptosis, lipid metabolism, oxidative stress

and cellular stress responses, they may represent upstream regulatory nodes through which RA influences downstream ferroptosis-related oxidative injury. However, molecular docking should be interpreted as predictive evidence only and further experimental validation is required to confirm direct target engagement and biological relevance.

Effects of RA on Ang II-induced cardiomyocyte injury and ferroptosis-related markers

CCK-8 results showed that RA at concentrations of 5–100 μ M had no significant cytotoxicity in H9c2 cells (Fig. 5A). To further determine the working concentration of RA under Ang II-induced injury, H9c2 cells were treated with Ang II in combination with different concentrations of RA. Compared with the control group, Ang II significantly reduced cell viability ($P < 0.001$). Compared with the Ang II group, 5 μ M RA did not significantly restore cell viability, whereas 50 μ M RA did ($P = 0.002$). However, increasing the concentration to 100 μ M did not produce a stronger protective effect than 50 μ M RA (Fig. 5B). Therefore, 50 μ M RA was selected as the intervention concentration for subsequent experiments because it showed clear cytoprotective efficacy without detectable cytotoxicity.

RT-qPCR results (Fig. 5C) showed that ANP and BNP mRNA levels were significantly higher in the Ang II arm than in the control group (both $P < 0.001$) and that pretreatment with RA (50 μ M) prominently reduced their expression (both $P < 0.001$ vs. Ang II).

Table 2: Molecular docking binding energies of rosmarinic acid with candidate targets.

Target	MOL ID	Score
PPARG	MOL011865	-9.1
PTGS2	MOL011865	-9.1
MAPK1	MOL011865	-8.0
CDKN1A	MOL011865	-7.9
CASP3	MOL011865	-7.3

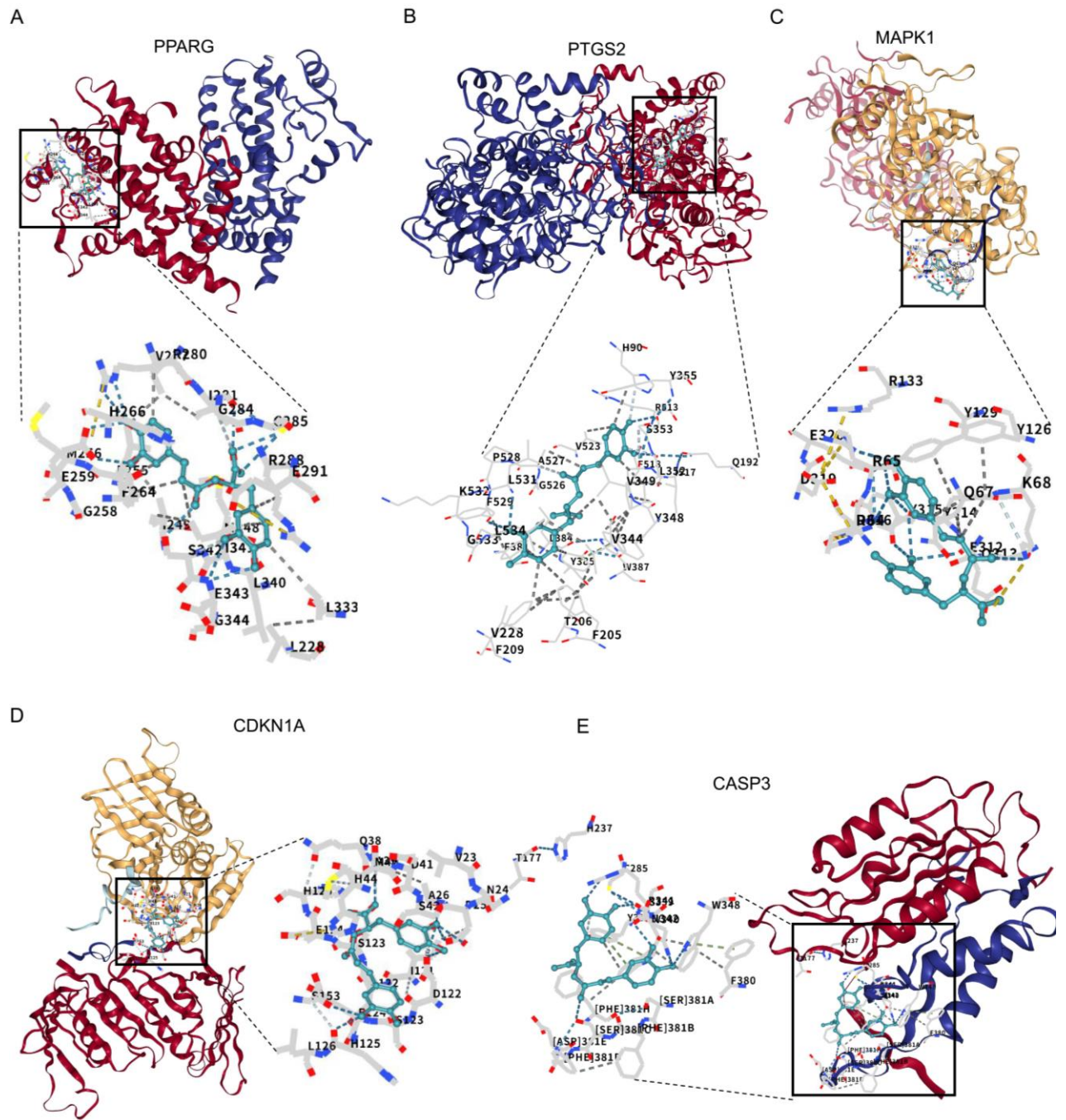


Fig. 4: Molecular docking validation of RA with candidate targets. Docking conformations of RA with (A) PPARG; (B) PTGS2; (C) MAPK1; (D) CDKN1A and; (E) CASP3. The enlarged panels show the predicted binding pockets and amino acid interactions between RA and each target.

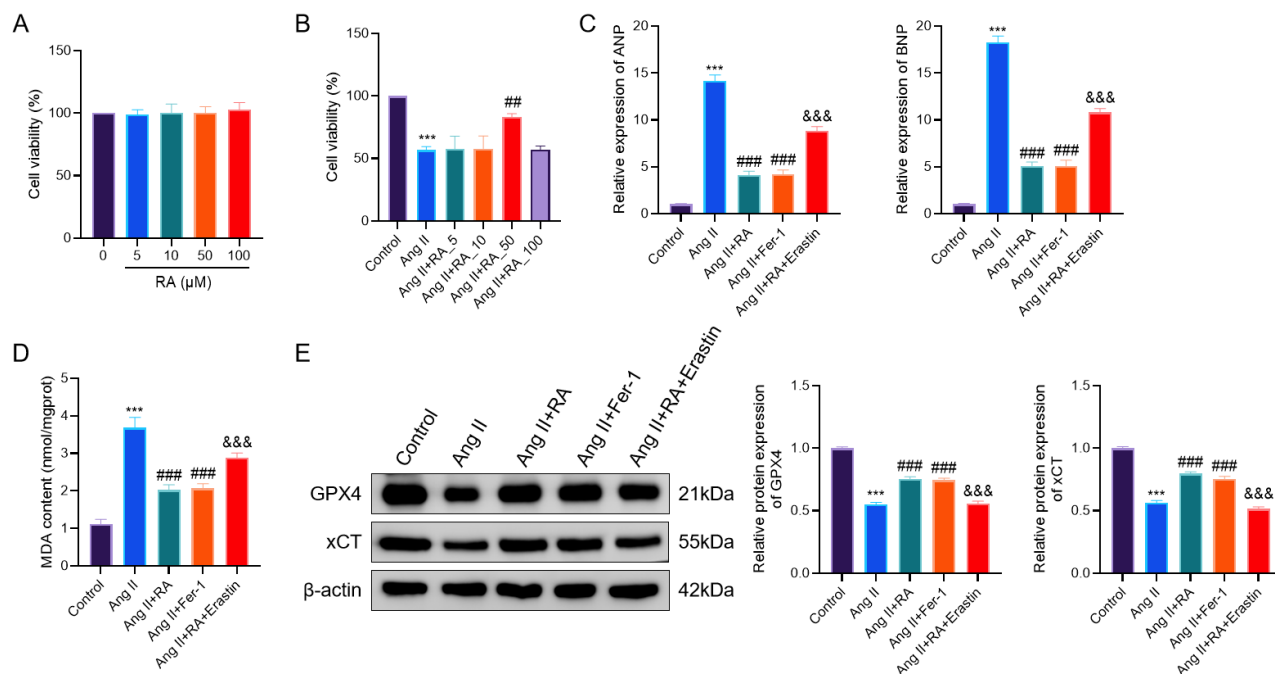


Fig. 5: Effects of RA on Ang II-induced cardiomyocyte injury and ferroptosis-related markers.

(A) The CCK-8 assay was used to detect the effects of different concentrations of RA (0, 5, 10, 50, 100 μM) on cardiomyocyte viability; (B) The CCK-8 assay was used to detect the protective effect of RA on Ang II-induced cardiomyocyte viability, with Fer-1 serving as the positive control for ferroptosis inhibitor; (C) RT-qPCR was performed to detect the relative mRNA expression levels of ANP and BNP in each group; (D) MDA content in cardiomyocytes from each group was measured using assay kits; (E) GPX4 and xCT protein expression in each group were evaluated by Western blot as ferroptosis defense-related markers. Densitometric quantification was performed by normalizing GPX4 and xCT band intensities to β-actin. Data are presented as mean ± SD from three independent biological replicates. ***P < 0.001, Compared with the control group; ##P < 0.01, ###P < 0.001, compared with the Ang II group; andandand P < 0.001, compared with the Ang II+RA group.

The erastin rescue experiment partially reversed the protective effects of RA, as reflected by increased ANP and BNP mRNA expression compared with the Ang II+RA group (both P < 0.001), suggesting that ferroptosis-related pathways may contribute to RA-mediated protection against Ang II-induced cardiomyocyte injury.

MDA detection results illustrated (Fig. 5D) that MDA content was significantly elevated in the Ang II arm compared with the control group (P < 0.001) and RA pretreatment prominently lowered MDA content (P < 0.001 vs. Ang II); erastin partially reversed the antioxidant effect of RA (P < 0.001 vs. Ang II+RA). Western blot results illustrated (Fig. 5E) that GPX4 and xCT protein expression was pronouncedly declined in the Ang II arm compared with the control group (both P < 0.001) and RA pretreatment considerably enhanced their expression (both P < 0.001 vs. Ang II); erastin significantly reversed the upregulatory effects of RA on GPX4 and xCT (both P < 0.001 vs. Ang II+RA), supporting the involvement of GPX4/xCT-associated ferroptosis defense responses rather than definitively proving direct inhibition of ferroptosis.

DISCUSSION

This study explored the potential mechanism by which RA ameliorates Ang II-induced cardiomyocyte injury through the modulation of ferroptosis-related pathways. Cellular experiments demonstrate that 50 μM RA significantly increased the expression of the ferroptosis defense-related proteins GPX4 and xCT, reduced the level of the lipid peroxidation end product MDA and attenuated Ang II-induced decreases in cell viability and upregulation of the hypertrophy-related markers ANP and BNP. The erastin rescue experiment partially reversed the protective effects of RA, suggesting that ferroptosis-related regulation contributes to RA-mediated cardioprotection. However, because this reversal was incomplete and because ferroptosis-specific assays, such as lipid ROS measurements or mitochondrial morphological assessments, were not performed, these findings should be interpreted as evidence of ferroptosis-related oxidative injury rather than as definitive proof that RA directly inhibits ferroptosis. These findings are consistent with studies reporting that active components of traditional Chinese medicine can regulate cardiac ferroptosis-related

injury via the SLC7A11/GPX4 axis, thereby improving chronic heart failure (Wei *et al.*, 2025).

Network pharmacological analysis identified 9 potential RA-related targets involved in HF-associated ferroptosis, including PTGS2, PPARG, CASP3, CDKN1A, MAPK1, AR, SNCA, CCL2 and CCL3. GO and KEGG enrichment analyses indicated that these targets were associated with oxidative stress, iron ion response, apoptotic signaling, inflammatory pathways, lipid and atherosclerosis, efferocytosis and AGE-RAGE signaling. Molecular docking further suggested favorable predicted interactions between RA and PPARG, PTGS2, MAPK1, CDKN1A and CASP3. These computationally predicted targets were not directly examined in the cellular experiments and were distinct from the experimentally measured downstream markers GPX4, xCT and MDA; therefore, they should be interpreted as putative upstream regulatory nodes rather than experimentally validated targets. In this framework, RA may first modulate inflammation-, apoptosis-, lipid metabolism- and oxidative stress-related targets or pathways, which could subsequently influence downstream ferroptosis-related events, including lipid peroxidation and GPX4/xCT-associated antioxidant defense responses. Specifically, inflammatory and stress-related pathways, such as TNF, IL-17, and AGE-RAGE signaling, may enhance ROS generation and lipid peroxidation, thereby increasing the requirement for GPX4/xCT-mediated antioxidant defense. Lipid metabolism-related pathways may further influence membrane lipid susceptibility to peroxidation, providing a potential upstream link between the predicted KEGG pathways and the experimentally observed changes in MDA, GPX4 and xCT. Accordingly, network pharmacology and molecular docking analyses provide mechanistic hypotheses at the upstream level, whereas cellular experiments provide preliminary downstream phenotypic evidence supporting the involvement of ferroptosis-related injury.

From the TCM perspective, heart failure falls under "xīn shuāi" and "xīn bì". Ferroptosis features lipid peroxidation and disturbances in iron metabolism. This aligns with the TCM theory of "toxin damaging heart collaterals" (Han *et al.*, 2024). Recently, research on TCM active components regulating ferroptosis has advanced notably. Calycosin effectively treats doxorubicin-induced cardiac injury. Its mechanism likely involves activation of the Nrf2/SLC7A11/GPX4 pathway and suppression of ferroptosis in cardiomyocytes (Han *et al.*, 2024). Similarly, lycopene improves doxorubicin-triggered cardiac dysfunction. It reduces oxidative stress and inflammation. It inhibits ferroptosis. These effects appear linked to Nrf2 signaling (Huang *et al.*, 2025). RA is also a representative natural polyphenolic compound with antioxidant and anti-lipid peroxidation properties. Previous studies have reported its cardioprotective potential in different cardiac

injury contexts (Fan *et al.*, 2025; Li *et al.*, 2020). Compared with single-target ferroptosis intervention strategies, RA possesses both free radical-scavenging and ferroptosis-related regulatory properties, which may provide broader cytoprotective potential (Wu *et al.*, 2025). Studies on RA liposomes have confirmed that nano-formulations can enhance targeted delivery efficiency (Jia *et al.*, 2024), offering new pharmaceutical insights for targeted therapy of HF. Ferroptosis is receiving widespread attention as a therapeutic target for cardiovascular diseases, with research confirming its critical role in diabetic cardiomyopathy and myocardial ischemia-reperfusion injury (Wu *et al.*, 2021); therefore, RA may represent a potential natural compound for modulating ferroptosis-related cardiomyocyte injury, although its direct targets and *in-vivo* efficacy require further validation.

This study has the following limitations: network pharmacology relies on existing databases, which may miss emerging or context-specific targets; molecular docking does not consider *in-vivo* pharmacokinetics, target engagement, or dynamic biological environments. Although PPARG, PTGS2, MAPK1, CDKN1A and CASP3 were identified as candidate targets by network analysis and molecular docking, these predicted targets were not experimentally validated in the cellular model. In addition, GPX4 and xCT were selected as canonical downstream markers of ferroptosis defense, but additional ferroptosis-specific assays, such as lipid ROS detection, would further strengthen the mechanistic evidence. ANP and BNP were measured only at the mRNA level, without protein-level or morphological assessment of cardiomyocyte hypertrophy; therefore, the present findings support that RA reduced hypertrophy-related gene expression rather than conclusively demonstrating reversal of cardiomyocyte hypertrophy. Future studies need to validate its *in-vivo* efficacy in animal models and further clarify the molecular mechanisms linking the predicted upstream targets to GPX4/xCT-associated ferroptosis defense. Additionally, this study employed only the H9c2 cell line for *in vitro* experiments, lacking validation in primary cardiomyocytes and *in vivo* models of RA's tissue distribution, metabolic transformation, and cardiac function-improvement effects.

CONCLUSION

In this study, network pharmacology, molecular docking and cellular experiments were combined to show that RA may attenuate Ang II-induced cardiomyocyte injury by modulating ferroptosis-related oxidative injury and GPX4/xCT-associated defense responses. Network pharmacology and molecular docking suggested that PPARG, PTGS2, MAPK1, CDKN1A and CASP3 may be candidate targets involved in the multi-target regulation of RA in HF-associated ferroptosis. These findings provide preliminary experimental and computational evidence for

RA as a potential cardioprotective compound, although further mechanistic and in vivo validation is required.

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None.

Author's contributions

YLY and JHL: Conceptualization, methodology, formal analysis, data curation, writing-original draft, writing-review and editing, project administration, investigation and supervision; XLW: Formal analysis, data curation, writing-review and editing, software and visualization, validation.

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Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

Ethics approval

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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