Effects of ginkgo flavone aglycone on atherosclerosis based on network pharmacology, molecular docking, and in vitro experiments

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Abstract

Background: Ginkgo flavone aglycones (GA), a Ginkgo (Ginkgo biloba) extract, has been proven to have good biological activity in atherosclerosis (AS) treatment. Moreover, its active compounds and the corresponding mechanism for the treatment of AS remain unclear. Methods: To evaluate and identify the potential pharmacological mechanisms of GA in AS treatment, the program Cytoscape was used to generate network mappings of the GA-AS-potential target gene. GO and KEGG enrichment analyses were performed to further investigate the potential mechanism of AS and the pharmacological properties of GA. A molecular docking approach was utilized to determine the GA components that interact with Akt. In vitro experiments were carried out to identify the anti-atherosclerotic effects of GA by targeting Akt. Results: Network pharmacological research determined that the active components of GA (quercetin, kaempferol, and isorhamnetin) correlated with AS target genes such as AKT1, EGFR, SRC, ESR1, PTGS2, MMP9, KDR, GSK3B, APP, and MMP2, respectively. GO enrichment and KEGG analysis showed that PI3K-Akt signaling may play an important role in GA treatment. Molecular docking experiments indicated that quercetin, kaempferol, and isorhamnetin integrate into the binding pockets of the most potentially beneficial GA-AS target protein (Akt). Consequently, cell experiments were conducted to support the anti-atherosclerotic activity of GA on AS by inhibiting the phosphorylation of AKT1 and its downstream signaling molecules, which regulated the proliferation of HASMCs. Conclusion: Our results detailed GA’s active ingredients, potential targets, and molecular basis against AS. GA may exert anti-atherosclerotic effects by suppressing Akt phosphorylation and inhibiting the proliferation of HASMCs. It also proposed a viable approach to determining the scientific foundation and therapeutic mechanism of Chinese herbal medicine extracts in disease therapy.

Keywords: network pharmacology; ginkgo flavone aglycones; atherosclerosis; molecular docking; kaempferol; quercetin; isorhamnetin

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Abbreviations

GA, Ginkgo flavone aglycones; AS, atherosclerosis; VSMCs, vascular smooth muscle cells; MMPs, matrix metalloproteinases; ox-LDL, oxidized low-density lipoprotein; HASMCs, Human aortic smooth muscle cells; AKT1, AKT Serine/Threonine Kinase 1; HPLC, high-pressure liquid chromatography.

Citation


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Background

Atherosclerosis (AS) is one of the most significant causes of death [1]. Several processes contribute to the development of AS, including vascular endothelial dysfunction, inflammatory cell infiltration, abnormal vascular smooth muscle proliferation and migration, lipid peroxide buildup, and modifications in the vascular matrix [2, 3]. The formation of atherosclerotic plaques is partly due to phenotypic switching in intimal vascular smooth muscle cells (VSMCs), including increased proliferation, loss of contractility, and elevated levels of matrix metalloproteinases (MMPs) [4]. Proliferation and inflammation of VSMCs are critical steps in atherogenesis, which causes VSMCs to move into the intima and produce intimal thickening in AS [5]. Meanwhile, VSMCs could be induced by inflammatory factors to consume oxidized low-density lipoprotein (ox-LDL) and generate foam cells [6]. MMPs, notably MMP-2 and MMP-9, play a role in VSMC migration from the media to the intima by degrading the extracellular matrix (ECM) and accelerating VSMC migration [7]. However, the specific regulatory mechanism of VSMCs in AS remains unknown.

The majority of individuals in developing countries depend on supplementary and alternative medications, especially those derived from natural sources. Natural plants are utilized as a source of treatment for fundamental health requirements since they are less harmful and have fewer adverse side effects than synthetic drugs [8, 9]. Consequently, there has been a growth in interest in natural treatments and the therapeutic use of natural products for the prevention of AS [10]. Ginkgo biloba L. is an ancient herb that has been flourishing in China for centuries. Ginkgo fruits and seeds were used in traditional Chinese medicine to treat chronic cough and enuresis [11]. Since the early 1990s, EGb761, a standardized extract of Ginkgo leaves, has been the most popular dietary supplement for treating circulatory disorders and enhancing memory [12]. In addition, it is a well-known traditional Chinese medicine with anti-atherosclerotic and anti-hyperlipidemic properties.

Ginkgo biloba leaf extract (GBE) has been commonly used to prevent and treat cardiovascular disorders due to its reported capacity to promote vasodilatation, limit the formation of atherosclerosis and inflammation, and suppress free radicals [13]. Ginkgo biloba leaf extract is mostly composed of ginkgo flavone glycosides and terpenoids. Flavone aglycones are a kind of flavonoid whose in vitro and in vivo biological functions are gaining increasing attention [14–16]. Moreover, the biological activity of flavonoid aglycones in scavenging oxygen free radicals was better than that of glycosides [17]. Ginkgo flavone aglycones are a class of active substances in Ginkgo, mainly containing three active substances: kaempferol, quercetin, and isorhamnetin, which showed a better absorption by the small intestine, probably because of the better lipid solubility and smaller molecular space structure of aglycones [18]. Our previous study also found GA was demonstrated to reduce ox-LDL-induced oxidative damage to anti-AS [19]. Nevertheless, the underlying mechanisms remain unclear.

Integrating systems biology, bioinformatics, and polypharmacology, network pharmacology-based pharmacology is an emerging, cost-effective method for drug research [20]. Network pharmacology incorporates network architectures for the discovery and evaluation of multicomponent and multitarget medicines [21]. Chinese herbal medicine extracts with potential efficacy against vascular disorders such as GA might serve as the basis for the development of multiple-component, multi-target therapy for AS. In this study, using network pharmacology assessments, molecular docking, and in vitro experimental validation, we explored the active ingredients and mechanisms of insight into the effects of GA on AS (Figure 1).

Figure 1 Workflow for network pharmacology-based prediction and validation of GA therapy on AS
Materials and methods

GA ingredient extraction and identification
As in previous articles, Ginkgo biloba extract was added to the prepared hydrolysis solution (ethanol and 4 M hydrochloric acid: 8:2), heated to 68 °C, and extracted with heat preservation and stirring for 2 h [22]. The extract was cooled to room temperature by injecting cooling water, and the extract was pumped into storage tank 1 and the pH was adjusted to neutral. Then, the extract was pooled and concentrated in a vacuum to obtain the flavonoid aglycones crude extract. The flavonoid aglycones crude extract was pulverized and sieved through sieve # 80. A diethyl ether: ethyl acetate mixture was applied for extraction. The extract was concentrated under reduced pressure and lyophilized. Ethanol was added to dissolve and samples were placed on the sample target to crystallize naturally. Finally, the reaction mixture was concentrated to dryness in vacuo.

Agilent 1100 Series high-performance liquid chromatography (HPLC) System (Agilent, USA) was used to analyze the GA ingredient. Analyses were performed on a Hypersil BDS C18 column (5 μm, 250 mm by 4.6 mm). The flow rate of the mobile phase (methanol-water-phosphoric acid = 48:52:0.3) was 0.8 mL/min. The detector, wavelength, and column temperature were 260, 360 nm, and 45 °C, respectively. The standard substances isorhamnetin, kaempferol, and quercetin were analyzed in the same conditions.

Clustering of target genes related to GA and AS
The GA-related target genes were categorized according to the canonical SMILES strings extracted from the PubChem database and submitted to the SwissTargetPrediction website (http://www.swisstargetprediction.ch/) for molecular composition target prediction. GeneCard (http://www.genecards.org) was processed for AS-related target genes. The intersection of Venny 2.1 (https://bioinfo.cnb.csic.es/tools/venny/) was used to identify potential target genes of GA treatment for AS.

Protein-Protein Interaction (PPI) network diagram of potential GA–AS target genes
PPI analysis is conducted by uploading overlapping targets of active drugs in GA and AS to the STRING database with the greatest number of species and molecules to identify the primary regulatory targets. The species was restricted to Homo sapiens, direct interactions between genes with a total confidence score of at least 0.4 were used, and independent target protein nodes were concealed. The PPI data were then generated from STRING and loaded into Cytoscape 3.8.0, which is frequently used to create and analyze networks, particularly in network pharmacology research. CytNoCA, a Cytoscape plugin for network centrality analysis, was used to determine the network’s most essential genes. Firstly, the genes with the highest centrality scores in the top 30 percent were selected for subnetwork construction. Then, the genes with the highest betweenness centrality values in the top 30 percent of the subnetwork were selected as relevant genes and formed the key network.

Construction of a GA ingredient-target interaction network
The intersection of the GA component targets and the AS-related genes was used to determine the major GA targets in the AS. Using Cytoscape 3.8.0, the herb-active ingredient-target interaction network was displayed. Using key objectives and active ingredients as nodes, Microsoft Excel was used to create the matching link, which was then imported into Cytoscape 3.8.0 to build the interaction network of GA in the treating of AS. The Cytoscape Analyze Network plug-in was then used to calculate and rank the network’s topological properties (degree).

Analysis of GO and KEGG pathway enrichment
The Bioinformatics database (http://www.bioinformatics.com.cn) was used to conduct GO enrichment analysis and KEGG pathway enrichment analysis to further investigate the functions of possible anti-atherosclerotic target genes of GA. Only concepts and routes with q values of 0.05 were deemed statistically significant and preserved.

Molecular docking determines the binding capacity of active ingredients to key target genes
The structural ID for the Akt protein data retrieved from the protein database (RCSB PDB, www.pdb.org) was 6CYY. By minimizing energy, the Molecular Operating Environment (MOE) software converted the 2D structure of GA active components (isorhamnetin, kaempferol, and quercetin) to a 3D structure. Docking adheres to the principle of "induced fit," which states that the conformation of the ligand and receptor will vary throughout the non-rigid process of molecular docking. The docking site was positioned in the middle of the original ligand within a cuboid box, and a grid map of each atom type within the box was calculated. Molecular docking of putative targets and components was simulated using the MOE program. The binding method with the smallest binding free energy was recognized as the most feasible. Image processing of docking results using MOE.

Cell culture
Human aortic smooth muscle cells (HASMCs) were purchased from ScienCell (Cat. 6110) and cultured in SMC medium (ScienCell) with 5% CO2 at 37 °C according to the manufacturer’s recommendation, which contains 2% FBS and 1% smooth muscle cell growth supplement (SAGM, Cat. 1152). As previously mentioned, ox-LDL (50 μg/mL, YB0011) was obtained from Viyuan Biotechnologies (China) and used for constructing cell models [23]. HASMCs were serum-starved 24 h before ox-LDL or GA treatments. Then, HASMCs were divided into five experimental groups for 24 h: 1) Vehicle; 2) GA (30 μg/mL); 3) ox-LDL (50 μg/mL); 4) ox-LDL (50 μg/mL) + GA (15 μg/mL); 5) ox-LDL (50 μg/mL) + GA (30 μg/mL).

Cell proliferation assay
As per the manufacturer’s instructions, the CCK8 Cell Proliferation Kit (Beyotime, C0037, China) was utilized for the CCK8 test. A specific number of cells were centrifuged after collection and then planted at a density of 200 μL or 5 × 103 cells per well in 96-well plates, where they were exposed to various interventions for 24 h and 72 h. Then, 10 μL of CCK8 reagent was added to each well, followed by a 1.5 h incubation at 37 °C. A microplate reader was used to determine the absorbance at 490 nm.

Western blotting
As previously, Western blotting was conducted [24]. Briefly, cells were trypsinized, washed in PBS, and lysed on ice for 30 minutes in RIPA buffer (Beyond time, P0013K, China) with protease inhibitor cocktail (Roche, S2697, Switzerland) and phosphatase inhibitor cocktail (Yeasen, 20109ES05, China). For nuclear and cytosolic protein extraction, cells were removed according to the manufacturer’s instructions through a nuclear protein extraction kit (Beyotime, P0027, China). Using the Bradford technique, all proteins were quantified. SDS-PAGE was used to separate an equal quantity of protein before transferring it to a PVDF membrane. PVDF membrane was blocked and incubated overnight at 4 °C with primary antibody (anti-Akt, Proteintech, 10176-2-AP, 1:2000 dilution; anti-p-Akt, Proteintech, 80455-1-RR, 1:5000 dilution). The blots were then treated with the corresponding secondary antibodies. Enhanced chemiluminescence (Tanon, China) was used to view the signals, which were captured on a Gel Doc 2000 imaging scanner (Bio-Rad, USA). Normalization of relative protein abundance versus-actin.

Quantitative polymerase chain reaction (qPCR)
Total RNAs were isolated from HASMCs with different treatments using TRIzol reagent (Invitrogen, 15596026, USA). 1 μg of total RNA was used for reverse transcription using the HiFiAirTM II 1st Strand cDNA Synthesis SuperMix (Yeasen, Shanghai, China). The cDNA samples were performed using an ABI PRISM7500 qPCR System (ABI, USA) with Hioff qPCR SYBR Green Master Mix (Low Rox Plus) (Yeasen, 11022ES08, China) to perform qPCR. The PCR conditions were 95 °C/5 min (1 cycle), 95 °C/10 sec, 55°C/20 sec and
72 °C/20 sec (35 cycles), and 72 °C/20 sec (1 cycle). The expression of MMP2, MMP9, and PCNA was normalized to GAPDH. Primer sequences are listed in Table 1.

**Statistical analysis**
Data are presented as mean ± SEM. One Way ANOVA was performed to determine statistical significance among different treated groups. All statistical calculations were performed by Graph Prism 8 software, and \( P < 0.05 \) was considered statistically significant.

**Results**

**GA active ingredient database establishment**
The HPLC analysis showed that GA mainly contained 3 compounds, namely quercetin, kaempferol, and isorhamnetin (Supplementary Figure S1). In addition, the 2D-chemical structures of quercetin, kaempferol, and isorhamnetin were drawn using ChemDraw software and are presented in Figure 2a.

**Building and evaluating the GA-AS-potential target gene network**
A scan of the GeneCard databases yielded a total of 4,710 AS potential genes. Likewise, target genes for three GA active components were collected from the PubChem database. After removing duplicates and verifying the results, 114 genes remained. The intersection of AS-associated genes and GA molecular targets using Venny 2.1 software generated 82 possible candidates (Figure 2b).

To get a GA-AS-potential target gene network, we first input the data from GA targets and AS-associated genes into Cytoscape (Figure 2c). From the GA-AS-potential target gene network, a total of 85 nodes and 219 lines were obtained. These genes include MMP9, estrogen receptor 1 (ESR1), myeloperoxidase (MPO), arachidonate 5-lipoxygenase (ALOX5), myosin light chain kinase (MYLK), coagulation factor II (F2), Prostaglandin-Endoperoxide Synthase 2 (PTGS2), and MMP2.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primers used in this study</th>
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| MMP2 (Homo sapiens)     | Forward: TACAGGATCATTGGCTACACACC  
Reverse: GGTCACATCGCTCCAGACT                                                                 |
| MMP9 (Homo sapiens)     | Forward: TGTACCGCTATGGTTACACTCG  
Reverse: GGCAAGGACAGTTGCTCTT                                                                 |
| PCNA (Homo sapiens)     | Forward: CCTGCTGAGATATTAGTCCAGCCA  
Reverse: CAGCGGTAGGTGTCAGACC                                                                 |

Figure 2 Construction of GA-AS-potential target gene network. (a) 2D molecular structure obtained from PubChem database of quercetin, kaempferol, and isorhamnetin. (b) The Venny results of potential target genes of GA therapy for AS. (c) The GA-AS-potential target gene network.
The PPI network map of GA therapy for AS and potential target genes

The PPI network map was created by importing 82 candidate genes into the STRING database (Figure 3a), with 81 nodes and 441 edges. The interaction data was then loaded into Cytoscape and optimized as shown in Figure 3b. Following a topological investigation of the network, AKT Serine/Threonine Kinase 1 (AKT1), Epidermal Growth Factor Receptor (EGFR), SRC proto-oncogene (SRC), ESR1, PTGS2, MMP9, MMP2, kinase insert domain receptor (KDR), glycogen synthase kinase 3 beta (GSK3B), and amyloid beta precursor protein (APP) were among the top ten genes in the core of the PPI network, indicating that these genes had a more extensive relationship with the others (Table 2).

![Figure 3 The PPI network map of GA and AS. (a) The PPI network map of 82 target genes. (b) The Core PPI network was constructed using Cytoscape.](image)

<table>
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<th>Targets</th>
<th>Degree</th>
<th>Closeness centrality</th>
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<td>50</td>
<td>0.71</td>
</tr>
<tr>
<td>EGFR</td>
<td>40</td>
<td>0.65</td>
</tr>
<tr>
<td>SRC</td>
<td>39</td>
<td>0.62</td>
</tr>
<tr>
<td>ESR1</td>
<td>35</td>
<td>0.61</td>
</tr>
<tr>
<td>PTGS2</td>
<td>34</td>
<td>0.62</td>
</tr>
<tr>
<td>MMP9</td>
<td>30</td>
<td>0.58</td>
</tr>
<tr>
<td>MMP2</td>
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</tr>
<tr>
<td>KDR</td>
<td>21</td>
<td>0.54</td>
</tr>
<tr>
<td>GSK3B</td>
<td>20</td>
<td>0.53</td>
</tr>
<tr>
<td>APP</td>
<td>20</td>
<td>0.54</td>
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GO and KEGG enrichment analysis

The 82 target genes were screened for enrichment analysis based on the intersecting network, and the top ten substantially enriched GO functions were determined according to their gene numbers (Figure 4a). 366 GO items were retrieved, of which 237 were for biological process (BP), 44 for cellular component (CC), and 85 for molecular function (MF), and depicted the top 10 entries. Signal transduction, plasma membrane, protein binding, protein phosphorylation, and ATP binding are the primary biological roles of these genes. These findings demonstrate that GA is engaged in the therapy of AS via several gene biochemical activities that add to the knowledge of anti-AS.

To further investigate the underlying mechanisms of GA in the treatment of AS, a KEGG pathway analysis was also undertaken. The KEGG pathway enrichment analysis revealed nine signaling pathways overall, and the ranking of the proportion of genes in the gene set was displayed. As depicted in Figure 4b, several signaling pathways are strongly linked to AS, including the Pathways in cancer, PI3K-Akt signaling pathway, Proteoglycans in cancer, Chemical Carcinogenesis-reactive oxygen species, and MicroRNAs in cancer signaling pathways. The PI3K/Akt pathway is involved in several atherosclerotic pathogenic processes, including smooth muscle cell proliferation, endothelial cell apoptosis, and macrophage migration (25–27). Hence, the PI3K/Akt pathway may be involved in the GA treatment of AS. As shown in Figure 5, the crucial target genes of GA-AS are in the PI3K-Akt signaling pathway.

Molecular docking determines the binding capacity of active ingredients to Akt

The target protein Akt (PDB ID: 6CCY) was molecularly docked with three active compounds of GA (quercetin, kaempferol, andisorhamnetin; corresponding 2D-chemical structures are shown in Figure 2a), and the outcomes were envisioned using MOE (2D and 3D) as shown in Figure 6. As shown in Figure 6a–6c, the findings demonstrated that Akt1 interacted with quercetin, kaempferol, andisorhamnetin, respectively. As demonstrated in Figure 6d,isorhamnetin may form a hydrogen bond with Asp292 and one pi-H bond with Val164 in Akt. As seen in Figure 6e,kaempferol's structure may create two pi-H bonds with Val164 and one pi-H bond with Thr291, respectively, in Akt. As demonstrated in Figure 6f, quercetin's structure might interact with Asp292 and Val164 through two pi-H bonds, respectively. These compounds may bind well to the active sites of protein targets, as evidenced by the docking binding energy data in Table 3. Among these, the binding energy between Akt and kaempferol was the highest (−5.93 kcal/mol), however, the binding energy between Akt and quercetin orisorhamnetin was −6.1 kcal/mol, which indicates high binding activity between all three active ingredients and Akt. Together, three main components of GA (quercetin, kaempferol, andisorhamnetin) may bind well with the primary target of AS (Akt), suggesting that they might all play a critical role in the therapy of AS.

GA inhibited Akt phosphorylation in ox-LDL induced HASMgs

According to the bioinformatics study, Akt has been the most significant target, and the PI3K/Akt signaling pathway is one of the most critical pathways in the therapy of GA for AS. In addition, molecular docking demonstrated that the three components of GA (quercetin, kaempferol, andisorhamnetin) could interact well with Akt. In vitro tests were conducted to further confirm the impact of GA on Akt in the treatment of AS.

![Figure 4 Analysis of GO and KEGG pathway enrichment. (a) The enriched GO analysis for the biological function of GA-AS potential targets genes. (b) The bubble diagram of enriched KEGG analysis for the signaling pathway of GA-AS potential target genes.](image-url)
Figure 5 KEGG pathway for PI3K-Akt signaling pathway. Nodes in red represent GA-AS potential target genes.

Figure 6 The docking model of Akt with GA ingredients. (a) The 3D binding mode of Akt1 with isorhamnetin. (b) The 3D binding mode of Akt1 with kaempferol. (c) The 3D binding mode of Akt1 with quercetin. (d) The 2D binding pattern of Akt1 with isorhamnetin. (e) The 2D binding pattern of Akt1 with kaempferol. (f) The 2D binding pattern of Akt1 with quercetin.

Table 3 The docking binding energy results of GA to Akt

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Best binding score (kcal/mol)</th>
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<tr>
<td>isorhamnetin</td>
<td>$-6.1223$</td>
</tr>
<tr>
<td>kaempferol</td>
<td>$-5.9248$</td>
</tr>
<tr>
<td>quercetin</td>
<td>$-6.1254$</td>
</tr>
</tbody>
</table>
Akt modulates cellular survival, growth, proliferation, inflammation, and migration [28, 29]. Given the crucial roles of Akt in ox-LDL-induced proliferation and migration in SMCs, we tested the corresponding phenotype in HASMCS. As shown in Figure 7a, the CCX8 assay was used to investigate cell proliferation. The results demonstrated that GA inhibited the growth of ox-LDL-induced HASMCS. Next, we tested the expression of Akt and p-Akt in HASMCS treated with or without GA. The results demonstrated that ox-LDL generated an increase in Akt phosphorylation while total Akt remained unchanged. However, treatment with various doses of GA dramatically reduced the expression of the ox-LDL-driven rise in p-Akt (Figure 7b).

**GA inhibited the expression of downstream molecules of Akt in ox-LDL-induced HASMCS**

The activation of Akt leads to the phosphorylation of a variety of downstream, for example, the transcription factors FoxO1 and NF-κB that control the expression of proliferative genes to modulate the smooth muscle phenotype. Hence, we tested the expression of downstream molecules of the Akt pathway to assess the inhibition of Akt by GA. As shown in Figure 7c, the expression of MMP2, MMP9, and PCNA in HASMCS treated with or without GA were tested at mRNA level. The results showed GA inhibited ox-LDL-induced Akt downstream molecules (MMP2, MMP9, and PCNA) expression, which may further inhibit the proliferation of smooth muscle cells.

**Discussion**

*Ginkgo biloba* has beneficial pharmacological activity and therapeutic effects in the current clinical treatment of AS. In streptozotocin-induced diabetic mice, *Ginkgo biloba* leaf extract reduced plasma cholesterol homeostasis, blood glucose, and inflammatory cytokines. Flavonoids are employed as active ingredients in a variety of pharmaceuticals and have biological effects such as antioxidants, anti-cancer, anti-aging, cardiovascular protection, and immune system enhancement.

The three active ingredients of GA have been extensively studied and proven, and it has been demonstrated that they exert anti-atherosclerotic benefits. In a previous study, Jiying et al. (2017) determined that flavonoids were the most active compounds present in *Ginkgo biloba* [30]. They acquired 10 active ingredients from *Ginkgo biloba* using high-pressure liquid chromatography (HPLC) with electrospray ionization mass spectrometry (ESI-MS), with quercetin, kaempferol, and isorhamnetin accounting for 96.71 percent of the total flavonol. Other investigations have had comparable results [31-33]. In addition, it was discovered that quercetin, kaempferol, and isorhamnetin greatly decreased smooth muscle proliferation and inflammation [34-37].

Network pharmacology is used to find potential targets for ginkgo extracts in the treatment of various diseases such as non-alcoholic fatty liver, cerebral atherosclerosis, and hypertension [38-40]. As shown in the GA-AS-potential target gene network (Figure 3), various GA chemicals potentially cover a large number of target genes. Included among these genes are MMP9, ESRI, MPO, ALOX5, MLK4, F2, PTGS2, and MMP2. The results suggest that the biological properties of GA treatment for AS are multi-component and multitargeted. In addition, the PPI data indicate that the 82 target proteins are not distinct from the other, but rather are interconnected and interact [41]. Through the control of numerous proteins, our findings further suggest that GA may contribute to the relief and treatment of AS. As demonstrated in Table 2, the PPI essential target genes were AKT1, EGFR, SRC, ESRI, and PTGS2.

Analysis of GO and KEGG enrichment findings for the 82 target genes indicated that 366 biological functions and 9 signaling pathways are associated with the development of AS, indicating that these gene biological functions and signaling pathways may be the process by which GA might treat AS. For a discussion on the mechanism of GA therapy for AS, the pathway with the highest association has been chosen. The PI3K/Akt pathway is essential for the survival and proliferation of smooth muscle cells, which may influence the development of AS. Hong Zhi et al. found that the inhibition of the Akt-mediated signaling pathway negatively regulates smooth muscle cell proliferation, dedifferentiation, and migration [42]. In addition, pharmacological inhibition of Akt activation effectively mitigates AS [43-45].

Based on network pharmacology results, we identified prospective GA targets. Consequently, molecular docking was performed to validate the conclusion of the network pharmacology analysis. The findings indicated that quercetin, kaempferol, and isorhamnetin all interact effectively with Akt, and the binding energy of GA compounds was between −5.9 to −6.12 kcal/mol, indicating that Akt was the most likely binding target of GA. To further investigate the effect of GA on Akt in treating AS *in vitro* tests were conducted. The experiments confirmed that GA inhibited the downstream molecules of Akt and had an anti-atherosclerotic effect in ox-LDL-induced HASMCS, as several studies found that the expression of p-Akt was lower in treatment groups compared to AS groups [46, 47]. These results verified the network pharmacology approach's prediction.

**Figure 7** GA inhibited ox-LDL-induced Akt pathway activated and downstream molecules. HASMCS were treated with ox-LDL with or without GA. (a) Cell proliferation was assayed by CCX8. (b) Western blot showed Ox-LDL-induced Akt phosphorylation was inhibited by GA. (c) qPCR was performed to test the expression of MMP2, MMP9, and PCNA in mRNA level. \(^*P < 0.05, n = 3.\)
Nonetheless, the current research has several limitations. First, the online database was based on verified and forecasted data; hence, we would not include unverified or unrecorded chemicals or targets in our research. Second, because of the poor water solubility, short elimination half-life, and fast in vivo elimination of GA, the absorption routes, effective portions, and metabolism in AS should be investigated. Thirdly, it is widely acknowledged that a binding energy less than −4.25 kcal/mol, −5.0 kcal/mol, or −7.0 kcal/mol implies that the ligand and receptor have a probable, moderate, or strong binding affinity [48]. The stronger the affinity of the receptor and the ligand, the lower the binding energy, and the more stable the confirmation. The binding energy of the three GA components to Akt, however, was only −5.9 to −6.1 kcal/mol, indicating a moderate binding action. Finally, although we have demonstrated in previous studies that GA inhibits the formation of atherosclerosis in high-fat fed rabbits, we did not perform in vivo animal experiments in this study, hence, further animal models and clinical trials are required to corroborate this [49].

Conclusion

In this study, we used network pharmacology to determine that Akt was the most important target in the anti-atherosclerotic actions of GA. In addition, the binding energy of GA compounds was between −5.9 to −6.12 kcal/mol, indicating that Akt was the most likely binding target of GA. In vitro studies verified GA could reduce the expression of Akt downstream molecules (MMP2, MMP, and PCNA), and inhibit ox-LDL induced proliferation of smooth muscle cells. The current research, based on a multidisciplinary approach, offers evidence for the therapeutic effect of GA in AS, as well as possible mechanisms and main target genes in GA.

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