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Decoding multi-target effects of Sichuan Shai vinegar on metabolic syndrome with network analysis-molecular simulations and in vitro bioactivity

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ABSTRACT: This study presents a comprehensive profiling of 23 bioactive compounds in Sichuan *Shai* vinegar (SSV) utilizing UPLC-MS/MS. The findings elucidate its multi-target mechanism of action against metabolic syndrome (MetS) through the integration of multi-component interaction network analysis and molecular simulations. Computational analyses identified 304 MetS-associated genes modulated via eight core targets (HSP90AA1, EGFR, EP300, TP53, NCOR2, CTNNB1, ESR1, HDAC1), with molecular docking and 100 ns dynamics simulations supporting stable binding of (S)-Coriolic acid and α -eleostearic acid ($\Delta G < -5.0$ kcal/mol) that concurrently suggesting possible involvement nuclear factor kappa B (NF- κ B) while may be associated with phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathways. *In vitro* digestion demonstrated SSV's preserved inhibition of α -glucosidase, α -amylase and pancreatic lipase (IC₅₀ (17.76-97.65) μ L/mL), with a bile-salt-micelle-associated enhancement observed for BNV and ZJV, whereas SSV showed only a non-significant IC₅₀ shift, suggesting that its intestinal-phase inhibition is mainly driven by hydrophilic inhibitors. These findings provide mechanistic hypothesise SSV's potential relevance through coordinated regulation of inflammatory, oxidative and metabolic networks, providing novel insights for fermented food-based strategies in MetS management.

Keywords: Sichuan *Shai* vinegar; Metabolic syndrome; Network analysis; Molecular docking; Molecular dynamics simulation; Digestive stability

1. Introduction

Chinese vinegar, a traditional acidic condiment with a history dating back to the Zhou Dynasty, is produced via solid-state fermentation using grains and bran ^[1]. Sichuan Shai vinegar (SSV), a representative variety, undergoes traditional solid-state fermentation followed by solar-aged maturation, yielding unique flavor and bioactive constituents ^[2]. The bioactive constituents present in vinegar, such as acetic acid, caffeic acid, ferulic acid, chlorogenic acid, and gallic acid, among others, are recognized for their potential benefits to the metabolic and circulatory systems ^[3]. With polyphenol extracts from Zhenjiang vinegar, for example, alleviating alcohol-induced liver injury in mice by modulating gut microbiota and immune factors ^[4]. Chinese

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vinegar is esteemed not only for its distinctive flavor and substantial nutritional value but also for its potential health benefits, which have garnered significant attention.

Metabolic syndrome (MetS), characterized by obesity, insulin resistance, hypertension, and dyslipidemia, elevates risks of cardiovascular disease and type 2 diabetes^[5, 6]. Its global prevalence is rising, driven by urbanization and lifestyle changes. Current pharmacological interventions for MetS often have toxic side effects and focus on single targets, whereas natural products like curcumin and resveratrol exert multi-faceted effects via antioxidation, anti-inflammation, and metabolic regulation^[7]. Vinegar, rich in organic acids, polyphenols, and polysaccharides, exhibits hypoglycemic, hypolipidemic, and antioxidant properties, primarily attributed to polyphenols and organic acids neutralizing free radicals^[8]. However, SSV's unique fermentation process implies distinct chemical compositions, yet its active constituents and MetS-modulating mechanisms remain underexplored.

Vinegar contains diverse fermentation-derived constituents, yet existing studies often focus on individual components or single targets, leaving its overall bioactive basis and mechanism poorly understood. Modern analytical and computational approaches offer pathways to address this gap. Ultra-high-performance liquid chromatography-mass spectrometry (UPLC-MS/MS) enables comprehensive profiling of complex mixtures, providing a chemical foundation for mechanistic studies^[9]. Systems biology methods, such as constructing compound-target-pathway networks, facilitate the analysis of multi-component, multi-target interactions-an approach well-suited for studying complex mixtures like vinegar and traditional herbal formulations^[10]. Furthermore, molecular docking can predict compound-protein binding affinities and prioritize key interactions^[11]. While molecular dynamics simulations assess complex stability. Integrating these tools-from compositional analysis to systems-level modeling and molecular validation provides a robust framework for elucidating the bioactivity of multicomponent systems beyond single-factor investigations^[12]. Despite evidence that vinegar constituents may improve glucose and lipid homeostasis, the key bioactive constituents of SSV and their target-level relevance to MetS remain unclear. Moreover, the digestive stability of these effects is underexplored, as bioactivity observed in vitro may differ after gastrointestinal digestion. To address these gaps, this study aims to: (1) identify the major bioactive constituents in SSV using UPLC-MS/MS; (2) map MetS-relevant targets and pathways through multi-component network analysis; (3) validate representative target-compound interactions via molecular docking and dynamics simulations; and (4) assess the digestion-stage-dependent inhibition of carbohydrate and lipid-digesting enzymes in vitro. By integrating compositional, computational, and experimental approaches, this work seeks to systematically clarify the multi-component synergistic mechanisms of SSV and provide a scientific basis for its potential use in MetS management.

2. Materials and methods

2.1. Reagents and chemicals

Methanol; Acetonitrile; Isopropyl alcohol purchased from CNW Technologies (Shanghai, China), Acetic acid purchased from SIGMA-ALDRICH (Shanghai, China), α -amylase from porcine pancreas (S31302; EC

3.2.1.1); α -glucosidase from *Saccharomyces cerevisiae* (S10050; EC3.2.1.20); p-Nitrophenyl- α -D-glucopyranoside(PNPG); 200mM, pH7.0 PBS phosphate buffer solution (R27577); lipase from porcine pancreas type II (S59680; EC3.1.1.3); orlistat (S69030); Acarbose (B20003) purchased from Shanghai Yuanye Biotechnology Co., Ltd. Potassium sodium tartrate tetrahydrate; Soluble starch; KCl; KH₂PO₄; NaHCO₃; CaCl₂; NaCl; Pepsin (64007137); Pancreatin (604467) purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 3-5, dinitrosalicylic acid purchased from Macklin (Shanghai, China). Triton-100; Tris purchased from Solarbio (Beijing, China). P-nitrophenyl palmitate purchased from Rhawn (Shanghai, China). Rabbit Gastric Extract (REG,15-1G) purchased from Lipolytech (Shanghai, China). Bile salts purchased from Merck (Hangzhou, China).

2.2. Vinegar samples

Cupei samples were collected from Sichuan Yuanjingda Food Co., Ltd. (Luzhou, China). Samples of Baoning vinegar (BNV), Zhenjiang vinegar (ZJV), and Shanxi aged vinegar (SXV) were procured from Supermarket (Yibin, China).

2.3. Sample preparation and extraction

100 μ L of sample was taken, mixed with 400 μ L of extraction solution (MeOH: ACN, 1:1 (v/v)), the extraction solution contained deuterated internal standards, and mixed solution was vortexed for 30 s, sonicated for 10 min in 4 °C water bath, and incubated for 1 h at -40 °C to precipitate proteins. Then the samples were centrifuged at 16000 $\times$ g for 15 min at 4 °C. The supernatant was transferred to a fresh glass vial for analysis. The quality control (QC) sample was prepared by mixing an equal aliquot of the supernatant of samples.

2.4. LC-MS/MS Analysis

LC-MS/MS analyses were performed using a UHPLC system (Vanquish, Thermo Fisher Scientific) with a Phenomenex Kinetex C18 (2.1 mm $\times$ 50 mm, 2.6 μ m) coupled to Orbitrap Exploris 120 mass spectrometer (Orbitrap MS, Thermo). The mobile phase A:0.01% acetic acid in water; mobile phase B:IPA: ACN (1: 1, v/v). The auto-sampler temperature was 4 °C, and the injection volume was 2 μ L. The Orbitrap Exploris 120 mass spectrometer was used for its ability to acquire MS/MS spectra on information-dependent acquisition (IDA) mode in the control of the acquisition software (Xcalibur, Thermo). In this mode, the acquisition software continuously evaluates the full scan MS spectrum. The ESI source conditions were set as follows: sheath gas flow rate as 50 Arb, Aux gas flow rate as 15 Arb, capillary temperature 320 °C, full MS resolution as 60000, MS/MS resolution as 15000, collision energy: SNCE 20/30/40, spray voltage as 3.8 kV (positive) or -3.4 kV (negative), respectively.

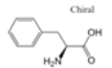
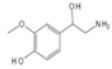
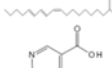
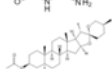
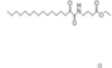
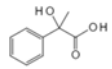
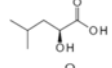
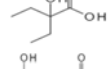
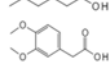
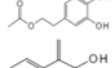
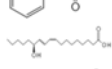
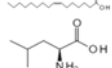
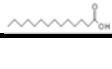
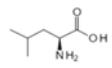
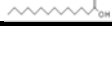
2.5. Multi-component interaction network elucidation

2.5.1. Prediction of SSV targets

In accordance with Lipinski's Rule of Five, candidate compounds were first screened using the following criteria: molecular weight $\leq$ 500 Da, hydrogen bond donors $\leq$ 5, hydrogen bond acceptors $\leq$ 10, LogP (octanol–

water partition coefficient) ≤ 5 , and rotatable bonds ≤ 10 . Compounds were semi-quantitatively estimated using an internal standard, and the relative concentration (ppm) was calculated based on the peak-area ratio to the internal standard. Compounds were ranked by the estimated relative ppm, and the top-ranked features were retained for subsequent analyses, a total of 23 compounds with favorable pharmacokinetic properties were finally selected (Table 1). The compounds subjected to screening were cross-referenced using the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) and subsequently saved in SDF format. These files were then imported into the Swiss Target Prediction database to facilitate target prediction. Throughout the screening process, the target attribute was designated as “Homo sapiens”, and all target proteins exhibiting a prediction probability greater than zero were cataloged [13].

Table 1 Active components of SSV.

Number	Ionization (ESI/ESI ⁺)	Identified compounds	CAS number	ppm	Class	Chemical structure
1	[M+H] ⁺	N,N-Diethylglycine	1606-01-5	24.10	Carboxylic acids and derivatives	
2	[M+H] ⁺	Oleamide	301-02-0	16.87	Fatty acyls	
3	[M+H] ⁺	Laurocapram	59227-89-3	16.86	Lactams	
4	[M+H] ⁺	Phenylalanine	63-91-2	15.03	Small peptides	
5	[M+H] ⁺	Normetanephrine	97-31-4	12.52	Phenols	
6	[M+H] ⁺	alpha-Eleostearic acid	506-23-0	9.27	Fatty Acids and Conjugates	
7	[M+H] ⁺	Cytosine-5-carboxylic acid	3650-93-9	9.56	Diazines	
8	[M+H] ⁺	Neotigogenin acetate	4948-43-0	8.42	Steroids	
9	[M+H] ⁺	AX-048	873079-69-7	7.57	Carboxylic acids and derivatives	
10	[M+H] ⁻	gamma-Linolenic acid	506-26-3	151.37	Fatty Acids and Conjugates	
11	[M+H] ⁻	Linolenic Acid	463-40-1	145.97	Fatty Acids and Conjugates	
12	[M+H] ⁻	9-Oxo-octadecanoic acid	4114-74-3	136.23	Octadecanoids	
13	[M+H] ⁻	Atrolactic acid	515-30-0	111.94	Benzene and substituted derivatives	
14	[M+H] ⁻	L-Leucic acid	13748-90-8	78.39	Fatty Acids and Conjugates	
15	[M+H] ⁻	2-Ethyl-2-hydroxybutyric acid	3639-21-2	53.14	Fatty Acyls	
16	[M+H] ⁻	5-Hydroxyhexanoic acid	44843-89-2	52.27	Fatty Acyls	
17	[M+H] ⁻	Homoveratric acid	93-40-3	35.98	Phenolic acids	
18	[M+H] ⁻	Hydroxytyrosol Acetate	69039-02-7	35.13	Phenylethanoids	
19	[M+H] ⁻	Atropic acid	492-38-6	33.23	Benzene and substituted derivatives	
20	[M+H] ⁻	(S)-Coriolic acid	29623-28-7	27.14	Octadecanoids	
21	[M+H] ⁻	(Z)-7-Hexadecenoic acid	2416-19-5	12.77	Fatty Acids and Conjugates	
22	[M+H] ⁻	Leucine	61-90-5	10.75	Small peptides	
23	[M+H] ⁻	Myristic acid	544-63-8	9.59	Fatty Acids and Conjugates	

2.5.2. Prediction of MetS targets

Targets closely associated with MetS were identified using the GeneCards database (<https://www.genecards.org>), using “metabolic syndrome” as the search keyword, targets exhibiting a correlation score exceeding 9 were selected as potential candidates for disease association ^[14]. The Uniprot website (<https://www.uniprot.org/>) was used to standardize the Uniprot IDs of all potential disease targets ^[15].

2.5.3. Build Protein-Protein Interaction (PPI) Network

The Venny platform (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>) was utilized to identify the intersection between the active components of SSV and the potential targets associated with MetS. Subsequently, the interactions among the common targets were analyzed using STRING (<https://cn.string-db.org/>), leading to the construction of a protein-protein interaction (PPI) network ^[16]. Ultimately, Cytoscape version 3.10.3 was utilized to generate a visualization of the network diagram ^[17].

2.5.4 Enrichment analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were conducted on the intersecting targets utilizing the DAVID database (<https://davidbioinformatics.nih.gov/>) to investigate associated biological functions and signaling pathways, set P -value < 0.05 ^[18]. Subsequently, the results of these analyses were visualized by the ggplot2 package in the R programming language ^[19].

2.6 Active ingredient-target molecular docking

AutoDock is a widely utilized molecular docking software that autonomously identifies potential binding sites, determining their central coordinates and dimensions, thereby establishing a foundation for the subsequent docking process ^[20]. The identification of binding sites constitutes a critical step in the process of protein-ligand docking. CB-Dock, a curvature-based cavity detection methodology, facilitates the automatic prediction of protein binding sites, while also determining the center and dimensions of these sites. This approach demonstrates superior performance in benchmark assessments of blind docking tools, exhibiting a notably high success rate ^[21]. Top 10 active compounds (by node size/connectivity) and 8 hub genes (from PPI) were selected. Compounds (SDF format, PubChem) and proteins (PDB format, UniProt) were docked using AutoDock Tools 1.5.7. CB-Dock predicted binding sites, and EDock performed blind docking. Binding free energy heat maps were generated via R, and conformations visualized with PyMOL. ^[22]

2.7. Molecular dynamics (MD) simulations

100 ns MD simulations of complexes were performed using Gromacs2022. CHARMM36 (protein) and GAFF2 (ligand) force fields were used ^[23], with TIP3P water models in a 1.2 nm periodic box ^[24]. Particle Mesh Ewald and Verlet algorithms handled electrostatics. Equilibration was performed in two stages: NVT for 100 ps (100,000 steps; coupling constant 0.1 ps) followed by NPT for 100 ps (100,000 steps; coupling constant 0.1 ps). Simulations ran at 310 K and 1 bar with 1.0 nm cutoffs for van der Waals/Coulomb interactions.

2.8. Inhibition of metabolic syndrome-associated enzymes

2.8.1 *In vitro static digestion simulation*

The samples underwent static *in vitro* digestion simulation in accordance with the INFOGEST 2.0 protocol [25]. The specific operation methods were shown in Appendix A.

2.8.2 *Inhibition of α -glucosidase*

The inhibitory activity of the samples was assessed using a modified version of the [26] method. Initially, α -glucosidase was dissolved in a 200 mmol/L phosphate-buffered saline (PBS) solution at pH 6.9, achieving a final enzyme activity of 1 U/mL. Subsequently, 40 μ L of the α -glucosidase solution was combined with 40 μ L of diluted samples at varying concentrations and incubated at 37 °C for 15 minutes. Following this, 40 μ L of a 200 mmol/L p-nitrophenyl- α -D-glucopyranoside (PNPG) solution was added, and the mixture was further incubated at 37 °C for an additional 15 minutes. The reaction was halted by the addition of 80 μ L of 0.2 M sodium carbonate (Na_2CO_3). The quantities of each reagent added are detailed in Table S1. The absorbance of the samples at 405 nm was measured and used in the following formula to calculate the inhibition rate:

$$\text{Inhibition rate (\%)} = \left(\frac{A_0 - A_s}{A_0} \right) \times 100$$

A_0 is the absorbance of the blank control, A_s is the sample group's absorbance.

Acarbose solution serves as the positive control in the amylase inhibition test.

2.8.3 *Inhibition of α -amylase*

Following the methodology delineated [27]. A minor modification was implemented to assess the inhibitory activity of the sample on alpha-amylase. Initially, 50 μ L of the sample was diluted to various concentrations. Subsequently, 100 μ L of 200 mmol phosphate-buffered saline (PBS) at pH 6.9 and 100 μ L of a 1 unit/mL α -amylase solution were added. The resulting mixture was incubated at 37°C for 5 minutes, followed by the addition of 200 μ L of a chromogenic reagent. The color reagent was prepared by mixing 20 mL of 96 mmol/L 3,5-dinitrosalicylic acid with 8 mL of 5.31 M sodium potassium tartrate in 2 M NaOH and 12 mL of distilled water. The mixed solution was subjected to a boiling water bath for 15 minutes. Following a cooling period, 40 μ L of the reaction solution was combined with 210 μ L of ultrapure water, and the absorbance was subsequently measured at 550 nm using a microplate reader. The quantities of each reagent added are detailed in Table S2. These results were then applied to the following formula to determine the inhibition rate:

$$\text{Inhibition rate (\%)} = \left(1 - \frac{A_s - A_1}{A_b - A_2} \right) \times 100$$

A_s and A_b denote the absorbance of the amylase and phosphate buffer solutions, and the sample and amylase solutions, respectively.

A_1 and A_2 indicate the absorbance of the phosphate buffer solution and the sample with the phosphate buffer solution, respectively.

2.8.4 *Inhibition of pancreatic lipase*

Pancreatic lipase is capable of hydrolyzing 4-nitrophenyl palmitate (pPNP) into 4-nitrophenol (PNP).

The enzymatic activity of pancreatic lipase was assessed by measuring the absorbance at 405 nm (A₄₀₅). The inhibitory activity of the sample on pancreatic lipase was determined using a specific formula, followed by the calculation of the half-maximal inhibitory concentration (IC₅₀). The assay for inhibitory activity of pancreatic lipase was slightly modified in accordance with the method [28]. The sample was diluted to the desired concentration using a 50 mmol/L Tris-HCl buffer at pH 8.0. Porcine pancreatic lipase was prepared at a concentration of 10 mg/mL in ultrapure water and subsequently hydrated in a water bath at 4 °C for 10 minutes, followed by filtration through a 0.45 µm microporous membrane. The substrate, p-nitrophenyl palmitate, was pre-prepared in a 5 mmol/L sodium acetate buffer at pH 5.0, at a concentration of 0.08% w/v, and supplemented with 1% w/v Triton X-100. Initially, the sample was placed into a 1.5 mL centrifuge tube, followed by the sequential addition of the Tris-HCl buffer solution and the enzyme solution. After mixing thoroughly, the sample was incubated at 37 °C for 10 minutes. Subsequently, upon completion of the incubation, the pPNP solution was introduced. The sample was again thoroughly mixed and incubated at 37 °C for an additional 20 minutes. Then the sample was centrifuged at 11000 × g for 5 minutes. The absorbance of the sample was measured at 405 nm. The quantities of each reagent added are detailed in Table S3. These results were then used in the following formula to calculate the inhibition rate:

$$\text{Inhibition rate (\%)} = \left(1 - \frac{B_1 - B_0}{A_1 - A_0}\right) \times 100$$

In the formula: A₀ denotes Blank, A₁ denotes Model, B₀ denotes Sample blank, and B₁ denotes Sample.

2.9. Data statistics and analysis

Each experiment was conducted in triplicate, and the results were presented as the mean ± standard deviation. Data processing was performed using Excel 22, while statistical analyses were conducted with OriginPro 2021. Statistical significance was assessed using analysis of variance (ANOVA), with a significance threshold set at $P < 0.05$.

3 Results and discussion

3.1 Active components of SSV

Total ion chromatograms of SSV samples were analyzed in both positive and negative ion modes (Fig. 1a, b), with compounds classified and relatively quantified via LC-MS (Fig. 1c). After excluding components lacking target genes based on relative content, 23 active substances were identified, with their chemical classifications, CAS numbers, concentrations, and structures detailed in Table 1. The primary components of SSV include carboxylic acids/derivatives, benzene/substituted derivatives, fatty acids, phenolic acids, and flavonoids (Fig. 1c), which are implicated in metabolic disorder amelioration and weight regulation. Previous studies have identified compounds in balsamic vinegar using gas chromatography-mass spectrometry (GC-MS), which included amino acids, carboxylic acids, and fatty acids [29]. Li et al. used metabolomics to investigate the alterations in metabolites within *Cupei* pre- and post-fermentation. Their research identified a total of 43 differentially expressed metabolites, comprising 18 esters, 9 acids, 2 alcohols, 5 ketones, and 3 aldehydes [30]. Carboxylic acids/derivatives (e.g., dicarboxylic acids and their diamides) enhance energy

expenditure and improve glucose/lipid metabolism^[31,32]. Phenolic acids regulate metabolism via antioxidation, anti-inflammation, and gut microbiota modulation^[33,34]. Fatty acids like n-3 LCPUFAs exert anti-obesity effects through energy expenditure pathways^[35]. Collectively, these compounds offer therapeutic potential for metabolic diseases via multi-mechanistic regulation.

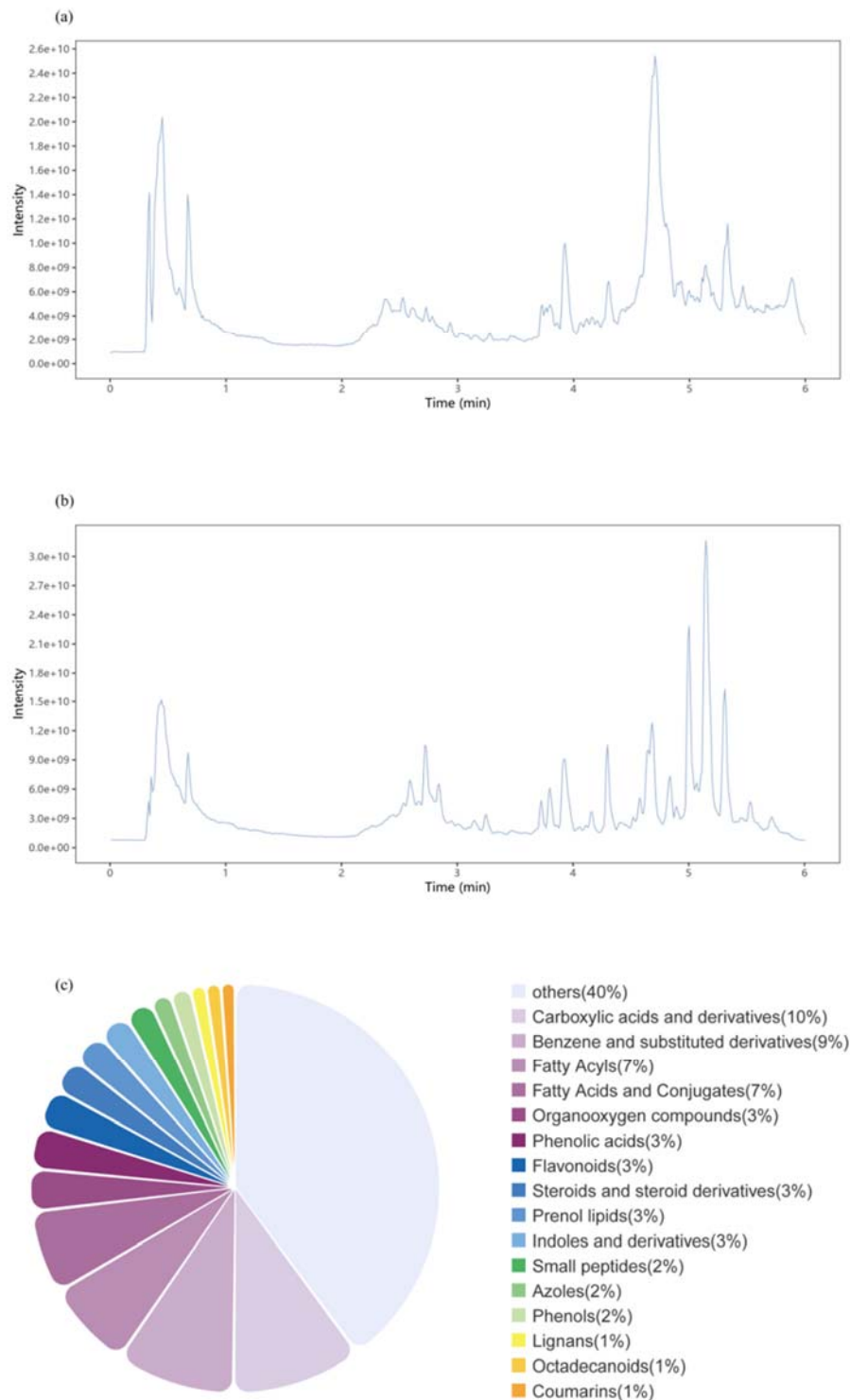


Fig.1. Extract ion chromatograms of active compounds in SSV samples using cationic (a) and negative modes (b), along with a classification diagram showing substance proportions (c).

3.2 Analysis of potential target and PPI network

We implemented a multi-component interaction network elucidation strategy to investigate the active constituents of SSV and their potential targets in the context of MetS. Through the Swiss Target Prediction database, we identified 450 unique targets associated with the components of SSV. Furthermore, we extracted 5,842 targets related to MetS from the GeneCards database. Venn analysis indicated the presence of 304 overlapping targets (Fig. 2a), highlighting critical intersections between the constituents of SSV and the pathogenesis of MetS. The global prevalence of MetS is increasing, attributed to factors such as urbanization, sedentary lifestyles, and dietary changes^[36]. MetS is strongly associated with cardiovascular disease, serving as an independent risk factor, partly due to its connection with chronic inflammation^[37]. Addressing these metabolic and immune pathways could facilitate the development of innovative preventive strategies for both MetS and cardiovascular disease^[38]. Protein-protein interaction (PPI) analysis of overlapping targets (Fig. 2b) and Cytoscape visualization (Fig. 2c) identified 10 hub genes via the cytoHubba plug-in (Fig. 2d), with the top eight—HSP90AA1, EGFR, EP300, TP53, NCOR2, CTNNB1, ESR1, and HDAC1—emerging as critical for SSV-mediated MetS intervention. HSP90AA1 regulates heat stress responses and interacts with the PI3K/Akt pathway, influencing MetS pathophysiology^[39, 40]. EGFR polymorphisms affect MetS susceptibility, with specific genotypes modifying risks under metal exposure^[41]. EP300, a key transcriptional regulator, contributes to antipsychotic-induced metabolic abnormalities and renal fibrosis in diabetic nephropathy via HIF2 α upregulation^[42, 43]. TP53 mutations drive metabolic reprogramming, impacting tumor microenvironment heterogeneity in MetS^[44]. NCOR2 (SMRT) dysfunction redirects activity to PPARs, impairing mitochondrial function and antioxidant gene expression, linked to premature aging and metabolic disease^[45]. CTNNB1 mutations associate with MetS and dysregulated bile acid metabolism in hepatocellular carcinoma^[46]. ESR1 polymorphisms correlate with MetS-related glucose abnormalities and cardiovascular autonomic neuropathy across populations^[47, 48]. HDAC1 exacerbates MetS-related liver injury by suppressing FGF21 via histone deacetylation^[49]. These eight hub genes strongly correlate with MetS pathogenesis, confirming the multi-component, multi-target nature of SSV's active constituents in modulating metabolic disorders.

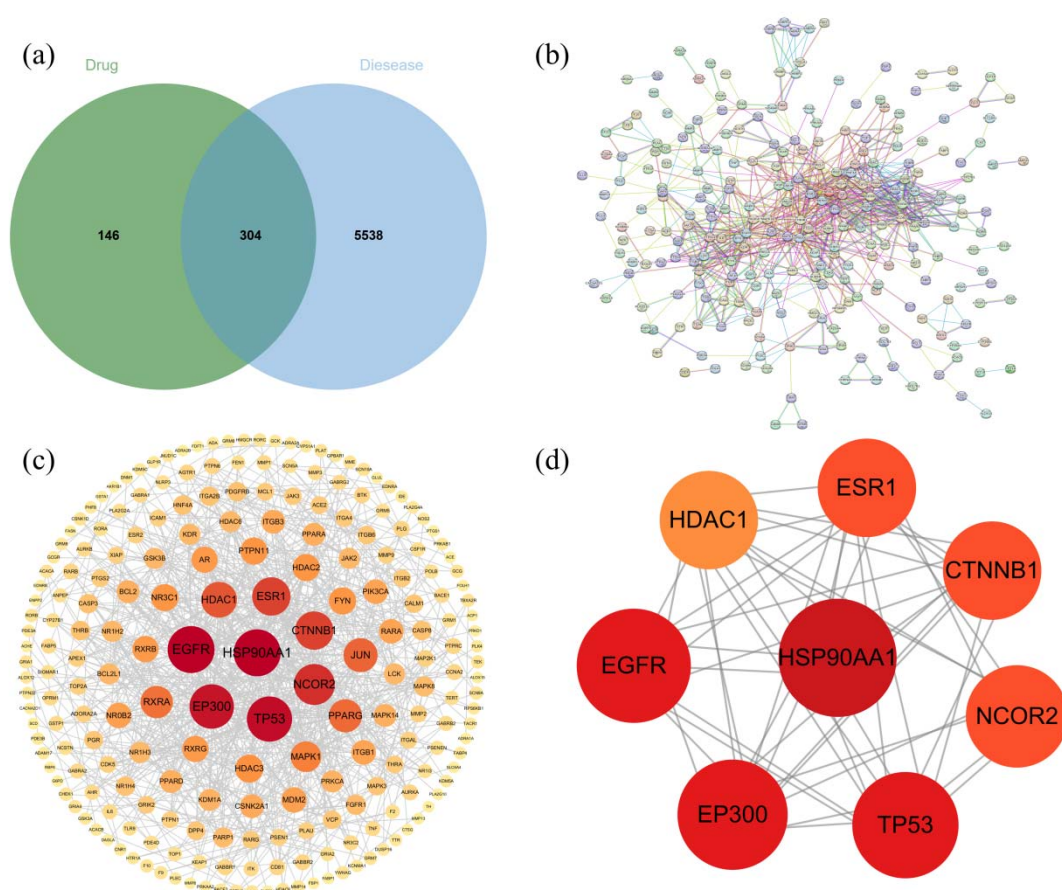


Fig.2. (a) Venn analyzed the overlap between active ingredient targets and metabolic syndrome-related targets. (b) A protein-protein interaction network was created for these overlapping targets. (c) Cytoscape visualized this network. (d) A hub gene map was generated for the intersecting targets.

3.3 Enrichment analysis of overlapping targets using GO and KEGG

The DAVID database enabled GO and KEGG enrichment analyses of overlapping targets to clarify SSV's mechanisms in MetS. A total of 1002 significant GO entries ($P < 0.05$) were identified, covering biological processes (BP: xenobiotic response, hypoxia regulation, etc.), cellular components (CC: plasma membrane, cytosol, etc.), and molecular functions (MF: protein/enzyme binding, etc.), with top 10 entries visualized (Fig. 3a). KEGG analysis yielded 168 significant pathways ($P < 0.05$), with top 20 pathways including cancer, neuroactive ligand-receptor interactions, lipid metabolism, atherosclerosis, and cyclic adenosine monophosphate (cAMP) signaling (Fig. 3b). Integrated with PPI network findings, these pathways align with key MetS pathophysiology: HSP90AA1 modulates PI3K/Akt to influence cell proliferation and apoptosis relevant to insulin resistance^[40]. MetS impairs hypoxic cerebrovascular dilation and vascular remodeling via endothelin-1 regulation^[50,51]. And cAMP signaling regulates lipid metabolism through adipocyte lipolysis and fatty acid oxidation^[52]. Notably, the cancer pathway showed substantial enrichment (false discovery rate, FDR: $6.6E-13$), as MetS may promote tumor progression via metabolic reprogramming and immune microenvironment modulation^[53,54]. Indicating SSV's potential in dual disease management. Cytoscape was utilized to construct an “ingredient-target-pathway” network, comprising 335 nodes and 1264 edges (Fig. 3d), which underscores several key components: (S)-Coriolic acid, known to regulate glucocorticoids and insulin

resistance [55]. α -eleostearic acid, which inhibits lipid synthesis [56]. (Z)-7-hexadecenoic acid, associated with lipid metabolism enzymes [57]. γ -linolenic acid, which enhances hepatic fatty acid oxidation [58]. And 9-oxooctadecanoic acid, which modulates PPAR signaling [59]. These findings substantiate that SSV operates through a multi-target, multi-pathway regulation of core MetS features, with NF- κ B, mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), peroxisome proliferator-activated receptor gamma (PPAR γ), and Akt identified as critical regulatory nodes.

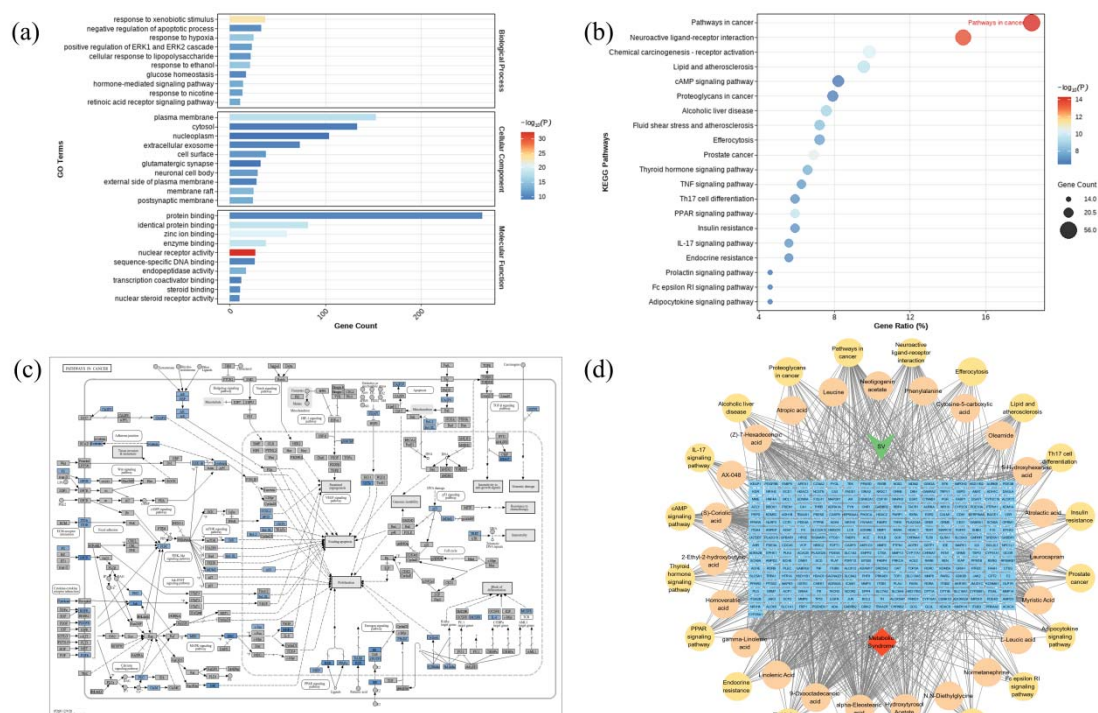


Fig.3. (a) GO enrichment bar chart of SSV components on metabolic syndrome (BP: biological process; CC: cell components; GO: Gene ontology; MF: molecular function). (b) KEGG pathway enrichment bubble diagram illustrating the impact of SSV components on metabolic syndrome (KEGG: Kyoto Encyclopedia of Genes and Genomes; cAMP: cyclic adenosine monophosphate). (c) Core targets in the cancer pathway (KEGG). (d) Network of “active ingredient-target-pathway” for SSV components in metabolic syndrome. The yellow-brown circle indicates SSV components, the blue rectangle signifies the target gene, the yellow circle shows the top 20 KEGG pathways of SSV for treating metabolic syndrome, the green inverted triangle stands for SSV, and the red diamond denotes metabolic syndrome. Grey lines illustrate their interactions.

3.4 Analysis of Molecular Docking

Molecular docking simulations were employed to investigate the interactions between the active compounds of SSV and target proteins associated with MetS. The binding affinity and stability of these interactions were assessed through binding free energy calculations, where lower values correspond to stronger interactions. Typically, a binding energy of less than -4.25 kcal/mol is indicative of a basic interaction, less than -5.0 kcal/mol signifies stable binding, and less than -7.0 kcal/mol denotes a strong affinity. In this study, the molecular binding free energies of ten SSV active compounds against eight MetS targets were computed and subsequently visualized in the form of a heat map (refer to Fig. 4a). Approximately 81% of the receptor-ligand pairs (65 out of 80) demonstrated binding energies lower than -5.0 kcal/mol, suggesting stable interactions between core components and targets. Notably, (S)-Coriolic acid displayed strong binding affinity

towards multiple targets, with the most significant binding energy observed for the EP300-(S)-Coriolic acid interaction, measured at -6.70 kcal/mol. The optimal binding configurations, characterized by the lowest energy states, were visualized for each complex using PyMOL (Fig. 4b-i). Linolenic acid exhibited hydrogen bonding interactions with ASN-51 (2.9 Å) and phe-138 (3.1 Å), in addition to hydrophobic interactions with PHE-138, VAL-186, ALA-55, and ASN-51 of HSP90AA1 (Fig. 4b). Hydroxytyrosol acetate interacted with EGFR through hydrogen bonds with MET-769 (3.1 Å) and GLN-767 (2.9 Å), seven hydrophobic interactions with leu-820, THR-776, LEU-764, LYS-721, ALA-719, VAL-720 and LEU-694, and a salt bridge of 5.4 Å with LYS-721 (Fig. 4c). (S)-Coriolic acid established three hydrogen bonds with the residues PRO-1704 (2.7 Å & 2.9 Å) and VAL-1079 (3.7 Å) of EP300, in addition to engaging in hydrophobic interactions with PHE-1075, VAL-1138, THR-1089, VAL-1079, LEU-1083, GLN-1077 (Fig. 4d). α -eleostearic acid demonstrated interactions with TP53 through hydrogen bonds with GLU-1543 (3.2/3.8 Å), hydrophobic interactions with ARG-1595, LEU-1596, ARG-1597, GLU-1598 and the formation of a 4.7 Å salt bridge with ARG-1595 (Fig. 4e). Linolenic acid bound to NCOR2 and formed four hydrogen bonds with TYR-469 (3.1 Å), TYR-479 (2.8 Å & 4.0 Å) and THR-473 (4.0 Å), alongside eleven hydrophobic interactions with PHE-444, PHE-451, LYS-449, VAL-467 and TYR-469, as well as a 4.5 Å salt bridge with ARG-441 (NCOR2–linolenic acid complex; Fig. 4f). (S)-Coriolic acid interacted with CTNNB1 through the formation of hydrogen bonds with residues TYR-254 (3.3 Å), ASN-220 (3.6 Å) and LEU-252 (3.7 Å), as well as through hydrophobic interactions with residues TYR-254, PHE-253, LEU-252, PHE-293, LYS-288 and Asp-294, and salt bridges HIS-176 (3.8 Å), LYS-180 (5.5 Å) (Fig. 4g). Hydroxytyrosol acetate formed eight hydrogen bonds with residues of ESR1 (ranging from 2.7 to 4.0 Å), hydrophobic interactions and exhibited pi/salt bridge interactions (Fig. 4h). Additionally, it bound to HDAC1 via hydrogen bonds with residues ASP-18 (2.8 Å) and TYR-23 (3.0 Å), complemented by eleven hydrophobic interactions (Fig. 4i). Collectively, the hydrogen bonding and hydrophobic interactions contributed to the stabilization of all ligand–receptor complexes. Integrating docking evidence with pathway enrichment (Section 3.3), the binding of linolenic acid to HSP90AA1 may obstruct HSP90–IKK interactions and thereby attenuate NF- κ B activation and downstream cytokine release ^[60]. α -eleostearic acid binding to TP53 may be linked to stress-response regulation (including NRF2-related processes) by influencing antioxidant and metabolic gene programs ^[61]. The hydrophobic interaction of hydroxytyrosol acetate with EGFR, particularly at residues such as LEU-764 and VAL-702, may reduce EGFR hyperphosphorylation and relieve its suppressive impact on insulin receptor substrate (IRS-1), potentially improving insulin signal transduction ^[62]. These findings indicate SSV's bioactive constituents exert multi-component, multi-pathway effects on MetS progression through stable interactions with key targets.

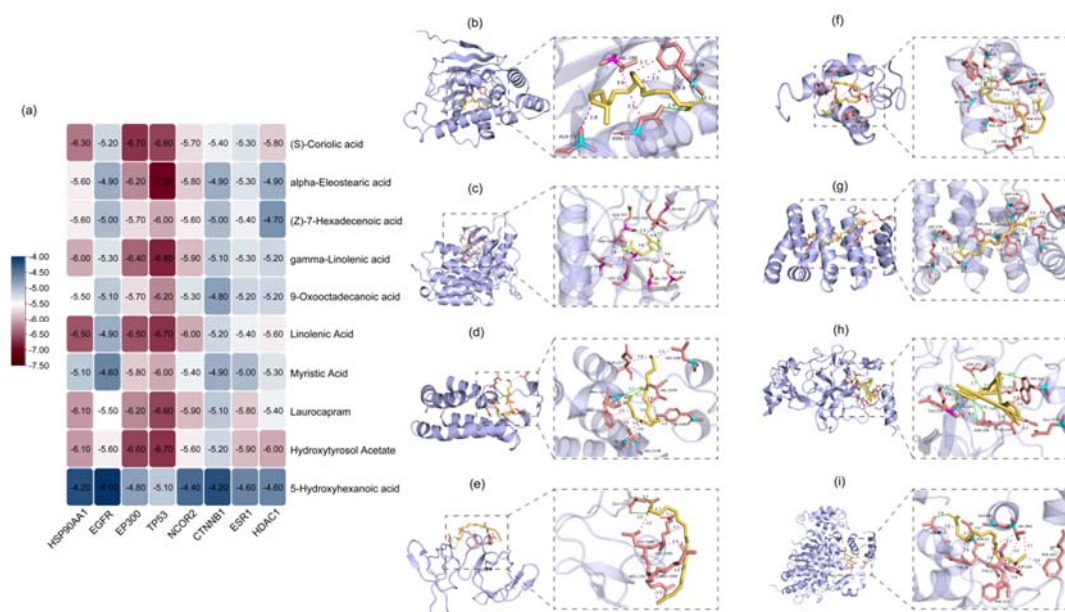


Fig.4. Visualization of the molecular docking. Heat map of free binding energy for ligand-protein docking (a). Linolenic Acid-HSP90AA1 (b). Hydroxytyrosol Acetate-EGFR (c). (S)-Coriolic acid-EP300 (d). alpha-Eleostearic acid-TP53 (e). Linolenic Acid-NCOR2 (f). (S)-Coriolic acid-CTNNB1 (g). Hydroxytyrosol Acetate-ESR1 (h). (S)-Coriolic acid-HDAC1(i).

3.5. Molecular dynamics simulation results

To further explore the interactions between the active compounds of SSV and targets associated with metabolic syndrome, molecular dynamics simulations were conducted over a duration of 100 nanoseconds on docking complexes to elucidate structural flexibility and interaction mechanisms. The primary evaluation metrics employed were: root mean square deviation (RMSD) for assessing convergence and stability of the complexes [63]. Root mean square fluctuation (RMSF) for analyzing residue mobility [64]. Solvent-accessible surface area (SASA) for examining transitions in folding states [65]. Radius of gyration (Rg) for determining molecular compactness [66]. Hydrogen bonding patterns for evaluating interaction integrity [67]. And force field energy distribution (FEL) for identifying the lowest-energy conformations [68].

The HSP90AA1-linolenic acid complex (Fig. 5a) exhibited structural stability over a duration of 100 ns, characterized by RMSD fluctuations of less than 1 nm, RMSF values below 0.8 nm (excluding terminal residues), SASA variations of less than 20 nm², and a Rg ranging from 1.65 to 1.80 nm. Beyond the initial 20 ns, FEL analysis revealed a smooth transition region and a distinct energy minimum, indicating a compact binding conformation despite the presence of a limited number of hydrogen bonds. The EGFR–hydroxytyrosol acetate complex (Fig. 5b) demonstrated significant stability, as evidenced by an RMSD of less than 0.4 nm, an RMSF of less than 0.8 nm (excluding terminal regions), a SASA variation of less than 20 nm², and an Rg ranging from 1.95 to 2.05 nm, sparse yet stable hydrogen bonds facilitated a low-energy cluster in FEL. The EP300–(S)-coriolic acid complex (Fig. 5c) exhibited high stability, characterized by an RMSD of ≤ 0.5 nm, an RMSF of less than 0.4 nm, a SASA of less than 10 nm², an Rg between 1.4 and 1.5 nm, and the formation of 1–4 stable hydrogen bonds post-30 ns; the smooth FEL with extensive low-energy regions suggested tight binding. The TP53– α -eleostearic acid complex (Fig. 5d) was highly stable, with an RMSD of ≤ 0.3 nm, an

RMSF of less than 0.3 nm, an Rg between 1.35 and 1.45 nm, a SASA of less than 15 nm², continuous hydrogen bonding, and a moderately smooth FEL with large minimum energy regions. In the case of the NCOR2–linolenic acid complex (Fig. 5e), stability was demonstrated by an RMSD of less than 1.2 nm, an RMSF of approximately 0.6 nm, a SASA of less than 15 nm², and an Rg ranging from 1.15 to 1.55 nm (after 10 ns), reduced yet stable hydrogen bonding and a minimum energy region in FEL further supported binding stability. The CTNNB1–(S)-coriolic acid complex (Fig. 5f) demonstrated stability, with an RMSD of ≤ 0.4 nm, an RMSF of less than 0.3 nm, a SASA of less than 15 nm², an Rg between 1.45 and 1.55 nm, and the formation of 1–3 hydrogen bonds; the smooth FEL indicated tight binding. The ESR1–hydroxytyrosol acetate complex (Fig. 5g) displayed stability, as indicated by an RMSD of ≤ 0.5 nm, an RMSF of less than 0.5 nm, a SASA of less than 20 nm², an Rg ranging from 1.75 to 1.85 nm, and consistent single hydrogen bonds, the smooth FEL with substantial low-energy areas confirmed stable binding. The HDAC1–(S)-coriolic acid complex (Fig. 5h) demonstrated notable stability, characterized by an RMSD of ≤ 0.6 nm, an RMSF of less than 1.0 nm, a SASA of less than 40 nm², an Rg ranging from 2.35 to 2.45 nm, and the presence of two stable hydrogen bonds, FEL showed distinct low-energy regions. In summary, the 100 ns molecular dynamics simulations confirmed the stable binding modes predicted by molecular docking for all eight complexes. These findings are consistent with the results from the elucidation of the multi-component interaction network, offering compelling evidence for the multi-target interactions of the active compounds in SSV.

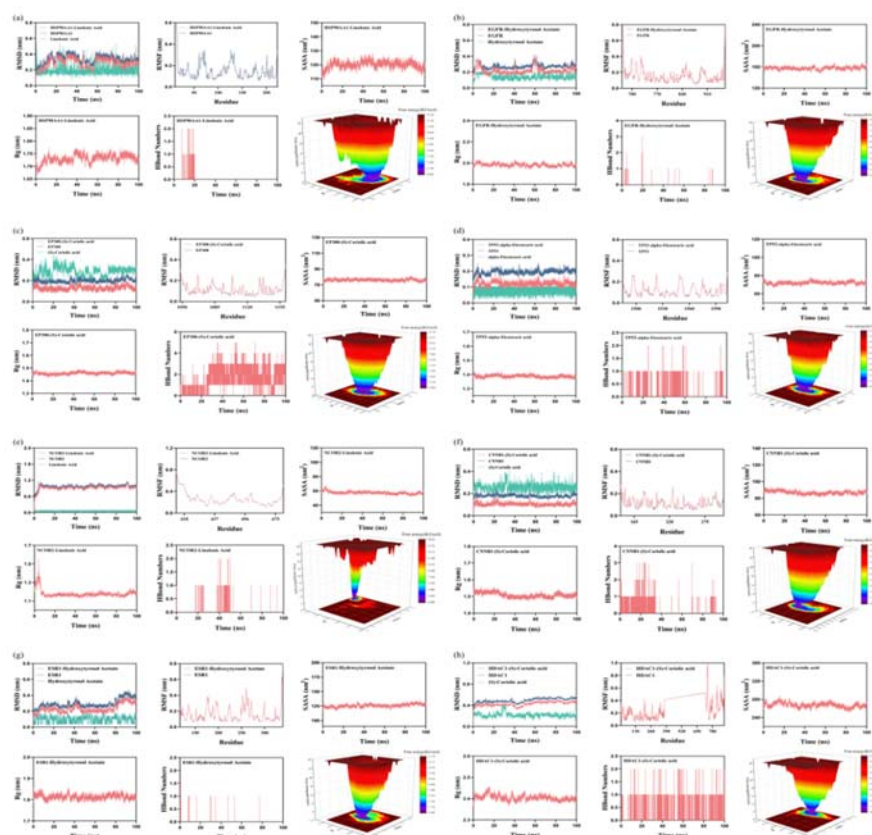


Fig. 5. Molecular dynamics simulation. Linolenic Acid-HSP90AA1(a). Hydroxytyrosol Acetate-EGFR(b). (S)-Coriolic acid-EP300(c). alpha-Eleostearic acid-TP53(d). Linolenic Acid-NCOR2(e). (S)-Coriolic acid-CTNNB1(f). Hydroxytyrosol Acetate-ESR1(g). (S)-Coriolic acid-HDAC1(h).

3.6 Enzyme inhibition research for metabolic syndrome

3.6.1 Ability to inhibit α -glucosidase

The inhibition of α -glucosidase, both prior to and following *in vitro* digestion, is affected by the degradation of polyphenols, the formation of digestive metabolites, and environmental alterations [69]. Although SSV is the primary focus of this study, BNV, ZJV, and SXV were included as representative comparative vinegar matrices to control for digestion-related matrix effects and to benchmark whether the observed digestion-stage-dependent changes are SSV-specific or generally matrix-driven. In this study, we evaluated the inhibition of α -glucosidase by vinegar samples from SSV, BNV, ZJV, and SXV across various stages of digestion, including undigested, oral, gastric, and intestinal phases. Acarbose was utilized as a positive control, demonstrating a significant concentration-dependent inhibition, with an inhibition rate of $(91.72 \pm 0.57)\%$ at a concentration of $10 \mu\text{L/mL}$ and an IC_{50} value of $2.052 \mu\text{L/mL}$ (Fig. 6a). Following digestion, all vinegar samples demonstrated a significant increase in α -glucosidase IC_{50} values (Fig. 6b). The most pronounced increase was observed in BNV, with values rising from 22.59 ± 0.16 to $97.65 \pm 3.82 \mu\text{L/mL}$. This was followed by ZJV, which increased from 20.45 ± 0.41 to $61.39 \pm 0.28 \mu\text{L/mL}$. SXV exhibited the smallest increase, with values rising from 17.76 ± 0.55 to $39.74 \pm 0.62 \mu\text{L/mL}$. This phenomenon likely involves gastric acid releasing polyphenols from protein complexes, but intestinal alkaline conditions promote polyphenol oxidation into inactive quinones and polymers [70]. Furthermore, oxygen levels affect polyphenol stability [71]. Additionally, intestinal β -glucosidase catalyzes the hydrolysis of flavonoid glycosides into aglycones, which subsequently undergo oxidation and polymerization under alkaline conditions. This process disrupts enzyme binding and diminishes inhibitory activity [72,73]. Consequently, this process results in an increased IC_{50} of the sample on α -glucosidase following intestinal digestion.

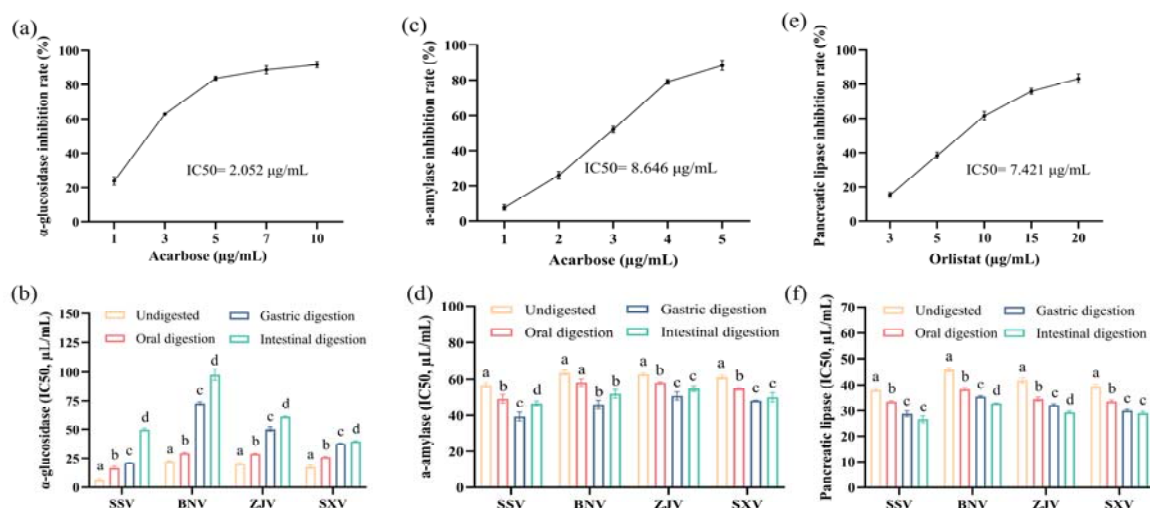


Fig. 6. Impact of various vinegar samples on enzymes linked to metabolic syndrome. The suppression of α -glucosidase activity by acarbose (a). The variations in the semi-inhibitory concentration of various samples on α -glucosidase following simulated digestion (b). The suppression of α -amylase activity by acarbose (c). The variations in the semi-inhibitory concentration of various samples on α -amylase following simulated digestion (d). The suppression of pancreatic lipase activity by orlistat (e). The variations in the semi-inhibitory concentration of various samples on pancreatic lipase following simulated digestion (f).

3.6.2 Ability of inhibit α -amylase

During *in vitro* simulated digestion, the inhibition of α -amylase is modulated by factors such as the degradation of polyphenols and structural modifications, which subsequently alter their binding affinity to the enzyme [74]. For instance, polyphenols from blueberry leaves undergo structural transformations during digestion, impacting their inhibitory effectiveness. In contrast, polyphenols from fucoidan and burdock demonstrate significant α -amylase inhibition due to their favorable molecular properties and bioavailability [75,76]. Additionally, extracts from quinoa and fenugreek exhibit enhanced inhibitory effects under intestinal conditions, attributed to the release of active components [77]. Acarbose, serving as the positive control, exhibited a notable concentration-dependent inhibitory effect on α -amylase, characterized by an IC_{50} value of 5.646 μ L/mL and an inhibition rate of $(88.40 \pm 1.11)\%$ at a concentration of 5 μ L/mL (Fig. 6c). In the case of the vinegar samples (SSV, BNV, ZJV, SXV), the IC_{50} values for α -amylase inhibition significantly decreased following oral and gastric digestion, whereas an increase was observed after intestinal digestion (Fig. 6d). This pattern can be attributed to the action of salivary amylase and pepsin, which hydrolyze starch-bound polyphenol glycosides and protein-polyphenol complexes, thereby releasing small-molecule inhibitors, such as aglycones, that exhibit a strong affinity for the active sites of α -amylase [78]. Within the acidic environment of the stomach, phenolic hydroxyl groups undergo protonation, leading to increased hydrophobicity and facilitating their insertion into enzyme active sites. Conversely, in the intestinal environment, bile salts competitively dissociate amylase-inhibitor complexes [79]. Empirical studies have demonstrated that compounds exhibiting greater hydrophobicity can manifest enhanced biological activity in certain contexts, such as more potent inhibition of NADPH oxidase and more effective suppression of cytokine release [80]. While alkaline conditions result in the deprotonation of phenolic compounds, thereby diminishing hydrophobic interactions with enzyme sites. This pH-dependent regulation is consistent with observed changes in enzymatic activity under alkaline conditions [81]. This regulatory mechanism may parallel the behavior of phenolic inhibitors in alkaline conditions, thereby elucidating the intricate nature of pH-dependent regulation of enzyme activity.

3.6.3 Ability of inhibit pancreatic lipase

Polyphenolic compounds, particularly flavonoids, are acknowledged as significant modulators of pancreatic lipase activity during simulated digestion, with their inhibitory efficacy influenced by structural characteristics such as the configuration of phenolic hydroxyl groups, the degree of polymerization, and the glycosylation status [82]. Recent studies underscore the potential of plant-derived extracts as effective lipase inhibitors. Specifically, extracts from *Bambusa multiplex* demonstrate both *in vitro* and *in vivo* inhibitory effects through mixed inhibition mechanisms [83]. Additionally, phenolic acids from mistletoe show digestion-dependent alterations in bioavailability and antioxidant capacity, which may directly influence their lipase inhibitory efficacy [84]. In this study, orlistat was utilized as the positive control, exhibiting a strong concentration-dependent inhibition of pancreatic lipase, with an IC_{50} of 7.421 μ L/mL, and achieving an inhibition rate of $(83.23 \pm 1.12)\%$ at a concentration of 20 μ L/mL (Fig. 6e). For the four fermented vinegar

samples (SSV, BNV, ZJV, SXV), the IC₅₀ values for pancreatic lipase decreased significantly following oral and gastric digestion (Fig. 6f). This reduction is attributed to the protonation of phenolic acids mediated by gastric acid, which enhances their hydrophobicity. This increased hydrophobicity facilitates their incorporation into lipid droplets and promotes targeted interactions with the lipase catalytic triad, thereby exerting competitive inhibition ^[85]. Following intestinal digestion, distinct trends were observed: SSV and SXV exhibited non-significant reductions in IC₅₀ values (from 28.80 to 26.66 μ L/mL and from 30.04 to 29.05 μ L/mL, respectively), whereas BNV and ZJV demonstrated significant decreases. This variation is likely attributable to differences in the hydrophilicity of the inhibitors. SSV and SXV primarily consist of hydrophilic inhibitors that interact directly with peripheral sites of lipase without the need for bile salt micelles. Conversely, the hydrophobic inhibitors in BNV and ZJV are effectively encapsulated by bile salt micelles, forming nanocarriers that concentrate the inhibitors near the enzyme's active sites ^[86]. This mechanism is analogous to the interaction between tea polyphenols and bile acids, which modulates cholesterol solubility, thereby enhancing inhibitory efficacy post-intestinal digestion ^[87]. Accordingly, this micelle-mediated enrichment mechanism plausibly explains the significant post-intestinal IC₅₀ decrease observed in BNV and ZJV, whereas the change in SSV was not statistically significant. This discrepancy stems from the distinct hydrophilic-hydrophobic profiles of pancreatic lipase inhibitors in different vinegars: BNV and ZJV are rich in hydrophobic components that can be efficiently encapsulated by bile salt micelles to concentrate near enzyme active sites, while SSV's predominantly hydrophilic inhibitors interact directly with lipase peripheral regions without such micelle-mediated enrichment, leading to non-significant IC₅₀ changes post-intestinal digestion.

4 Conclusion

This study profiled 23 constituents in SSV by UPLC–MS/MS and combined network-based analysis with molecular docking and 100 ns molecular dynamics simulations to propose plausible mechanisms relevant to metabolic syndrome. Eight hub targets (HSP90AA1, EGFR, EP300, TP53, NCOR2, CTNNB1, ESR1 and HDAC1) were highlighted, and representative ligand–target complexes showed stable interactions, supporting potential multi-target modulation of inflammation- and metabolism-related pathways. Simulated digestion further demonstrated digestion-stage-dependent inhibitory activities against α -glucosidase, α -amylase and pancreatic lipase, with SSV retaining measurable inhibition after intestinal digestion. Importantly, bile salt micelle-associated enhancement of lipase inhibition was evident for BNV and ZJV, whereas the intestinal IC₅₀ shift for SSV was not statistically significant, suggesting that SSV's post-intestinal inhibition is primarily attributable to hydrophilic inhibitors under the present conditions. These results connect SSV composition to mechanistic hypotheses and digestive stability of enzyme inhibition, and future work should validate key compounds and pathways in cellular and/or in vivo models and clarify dose–response relationships under realistic dietary intake scenarios.

CRedit authorship contribution statement

Zhicheng Li: Writing-original draft, conceived the research, analyzed the data. **Yuting Liao:** Conceived

the research, analyzed the data. **Jun Liu:** Supervision designed and supervised the experimental work. **Gerang Lamu:** Analyzed the data. **Jiajie Zhu** and **Xiangpeng Kong:** Data curation, analyzed the data and validation. **Suyi Zhang:** Conceived the research. **Qixu Fu:** Conceived the research. **Xiaobin Wang:** Data curation. **Ruiqi Xue** and **Xuemei Shi:** Data validation. **Li Li:** Supervision, designed and supervised experimental work, funding acquisition.

Declaration of interest statement

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

Data availability

Data will be made available on request.

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