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ORIGINAL ARTICLE

Systematic Understanding the Mechanisms of *Tripterygium wilfordii* on Atherosclerosis and Pharmacodynamics Research in Apo E$^{-/-}$mice Model

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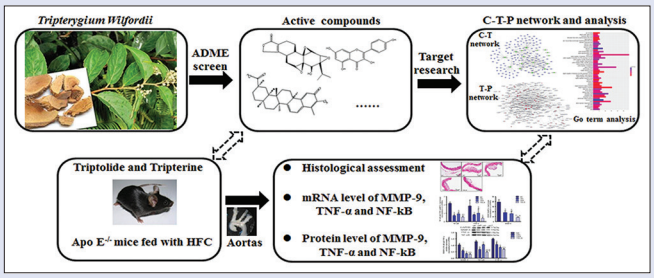
ABSTRACT

Background: Atherosclerosis (AS) is a chronic arterial disease and a major cause of vascular death, with multiple pathogenesis including chronic inflammatory. *Tripterygium wilfordii* (TGW) had a good effect on an anti-inflammatory. At present, more and more researches indicated that TGW could also regulate AS. **Objective:** The aim of this study is to clarify what the anti-atherosclerotic ingredients are in TGW and whether these ingredients improve AS synergistically. **Materials and Methods:** First, systematic pharmacology was utilized to predict the active ingredients and potential targets of TGW related to AS. Then, a bioactive compound of triptolide (TPL) and Tripterine (TPR) in TGW were evaluated if they presented the synergistically anti-atherosclerotic effects in Apo E$^{-/-}$mice fed with a high-fat/high-cholesterol diet. In the experiment, Hematoxylin and Eosin tested the plaque areas; reverse transcriptase- polymerase chain reaction and Western blot analysis detected the matrix metalloprotein 9 (MMP-9), tumor necrosis factor alpha (TNF-α), and NF-κB levels in the aortas. **Results:** The results shown that there are 17 bioactive compounds with 76 therapeutic proteins were identified. Moreover, TGW exhibits a protective effect on treatment AS likely through regulating multiple pathways including immune response, inflammatory response, and vascular structure improving. Further verified that TPL combined with TPR in TGW had synergistic effect on treatment AS by reducing levels of MMP-9, TNF-α, and NF-κB, might be the important pathway. **Conclusion:** TGW, synergistic effect of different compounds, could regulate AS by multiple pathways, especially improving immune response, inflammatory response, and vascular structure. The major compounds of Tripterine and Triptolide in TGW had a synergistic effect on anti-AS by suppressing matrix metalloprotein 9, TNF-α, and NF-κB.

Keywords: Atherosclerosis, inflammation, systems pharmacology, tripterine, *Tripterygium wilfordii*, triptolide

SUMMARY

- The major compounds of Tripterine and Triptolide in *Tripterygium wilfordii* had synergistic effect on anti-atherosclerosis.



Abbreviations used: TGW: *Tripterygium wilfordii*, TRL: Triptolide, TRR: Tripterine, TRLR: TRL plus TRR, NC: Normal control, MC: Model control, MMP-9: Matrix metalloprotein 9; NF-kB, Nuclear factor-kappa B; TNF-a, Tumor necrosis factor alpha, AS: Atherosclerosis, H and E: Hematoxylin and Eosin, ox-LDL: Oxidized low-density lipoprotein, ICAM-1: intercellular adhesion molecule-1, VCAM-1: vascular cell adhesion molecule 1, HIF-1: Hypoxia inducible factor-1, IL-2: Interleukin-2, IFN-γ: Interferon-γ, MCP-1: Monocyte chemotactic protein 1, TCMSP: Traditional chinese medicine systems pharmacology, TCM: Traditional chinese medicine, PerOB: Predict oral bioavailability, PerDL: Predict drug-likeness, HL: Half-life, HFC: High-fat/high-cholesterol diet, T-P: Target-Pathway, KEGG: Kyoto Encyclopedia of Genes and Genomes, DAVID: Database for Annotation, Visualization and Integrated Discovery, ADME: Absorption, distribution, metabolism, excretion, TBST: tris-buffered saline, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, DMSO: Dimethyl sulfoxide, HPLC: High Performance Liquid Chromatography.

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INTRODUCTION

Atherosclerosis (AS), chronic arterial disease and a major cause of vascular death, is a chronic inflammatory disease[1] promoted by hyperlipidemia.[2] Fatty streaks in arterial walls gradually develop into atheroma and characteristic plaques. The acute rupture of these atheromatous plaques causes local thrombosis, leading to partial or total occlusion of the affected artery.[3] Its major clinical manifestations include as follows: coronary heart disease,[4] cerebral infarction,[5] and peripheral vascular disease,[6] which is the leading cause of death and

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major health-care burden in worldwide regardless of different ethnicities. Lipid metabolism disorder and lipid accumulation are the foundation of atherosclerotic lesions.[7] It is thought that AS is not only a simple lipid sedimentary in a blood vessel but also a process of chronic low-grade inflammation.[8,9] Inflammation is accompanied by the occurrence and development process of AS, a lot of inflammatory factors aggravate AS by triggering inflammation. At early stage AS, ox-LDL elicits vascular endothelial releasing monocyte chemotactic protein 1,[10] which could promote the mononuclear cell into macro phagocyte entering into endangium. At this phase, NF-κB is activated and participated in the expression of inflammatory mediators.[11] Pro-inflammation cytokines, such as Interleukin-1 (IL-1) β, IL-6, and tumor necrosis factor alpha (TNF-α),[12] are stimulated and released, which would speed up the development of AS. Inner surface of blood vessels would form plaque with AS, while matrix metalloprotein (MMPs), such as MMP-9, could degrade the fibrous cap and reduce the stability of plaque,[13] further to induce thrombus. The pathomechanism is very complex and hence, the treatment of AS should consider as regulating multiple pathways.

Tripterygium wilfordii (TGW) is a famous traditional Chinese medicine (TCM). A growing number of evidence had proved that TGW had beneficial effects on various cancers,[14] immune function,[15,16] rheumatoid arthritis,[17,18] and hyperlipemia. In our previous researches, we found that extract of TGW could improve AS in mice model. For this sake, we would want to know what compounds in TGW and how these compounds could play the role of anti-AS.

Recent years, systems pharmacology[19,20] has made a notable contribution to explore and predict the molecular mechanisms of TCM through pharmacokinetics evaluation (such as ADME), compounds-targets network or target-pathway network. Here, we make use of this method to find the active compounds in TGW, correlated targets and pathways, which were strongly related to AS. To further developing the TGW on anti-AS, we chosen active ingredients of triptolide (TPL) and Tripterine (TPR) in TGW and performed pharmacodynamics research in Apo $E^{-/-}$mice AS model for further study.

MATERIALS AND METHODS

Date preparation and active compounds screening

All chemical compounds in TGW were found out from database of traditional Chinese medicine systems pharmacology (TCMSP)[21] (http://lsp.nwu.edu.cn/index.php). Three *in silico* ADME models including predict oral bioavailability (PerOB), predict drug-likeness (PerDL) and Half-life (HL) were used as filter parameter to obtain the active ingredients of TGW. The threshold values for the three screening models are set to PerOB ≥30%, PerDL ≥0.18, and HL ≥4, respectively. The screened out active compound are considered as candidate ingredients.

Collection the target proteins of selected compounds and gene data related to atherosclerosis

We used the selected compounds as baits to find out mostly likely protein targets from the TCMSP database. Genes associated with AS were collected from the database of Genecards[22] (http://www. genecards. org/). Candidate protein targets related to AS for TGM were picked conforming to both baited from TCMSP database and Genecards.

Target fishing and set up compound-target and target-pathway network

Input these candidate molecules targeted proteins into the Uniprot[23] (http://www.uniprot.org/) for further mapped to find the corresponding gene name. For the sake of clarifying the interrelation of bioactive ingredients, targets and AS, Cytoscape 3.2.1 software (University of California and Institute for Systems Biology, etc., USA; http://www. cytoscape. org/download.php) was used to establish a visualized network of the compound-target network (C-T network). Searching KEGG database[24] and found the pathway related to targets. Then establish a visualized network of Target-Pathway network (T-P network).

Geno ontology enrichment analysis

We utilized intersectional targeted genes of AS and active compound related as bits to fish the corresponding function from DAVID database[25] (https://david.ncifcrf.gov/), a comprehensive set of functional annotation tools for understanding the biological meaning behind large lists of genes, to get the gene ontology analysis. Enriched geno ontology (GO) terms was defined as significantly with adjust $P < 0.05$. The picture of GO terms was generated with ggplot2 and R software into visualization.

Pharmacodynamics of validation

TPL and TPR were important compounds in TGW. From the C-T-D network we had found that both TPL and TPR were related to the AS, we evaluated the synergistic effects of TPL and TPR on Apo $E^{-/-}$mice fed with a high-fat/high-cholesterol diet.

Modeling and treatment

Twenty-four male 8-week-old Apo $E^{-/-}$mice (18–20 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), all the animals were hosted at room temperature of 25°C and at 45%–55% relative humidity with a 12-h light-dark cycle. The mice were fed with high-fat/high-cholesterol diet (HFC, Trophic Animal Feed High-the Co., Ltd., China; 10% fat, 1.25% cholesterol and 0.5% bile salt.) and standard water, which were randomly divided into four groups (n = 6 per group), following as HFC model control group (MC), HFC plus TPL group (TRL), HFC plus TPR group (TRR), and HFC plus TPL and TPR group (TRLR). At first, the mice were fed with HFC-diet for 4 weeks. Then, the drugs or vehicle were daily administrated to mice fed with HFC-diet sustaining for 8 weeks, respectively. The drugs or vehicle administration scheme as follows: the mice in MC group were fed with HFC and received dimethyl sulfoxide (DMSO; batch number SHBC2572V; SIGMA, USA) by intraperitoneal injection. The mice in TRL, TRR, and TRLR groups were all fed with HFC and respectively received 0.2 mg/kg of TRL (99.9% detecting by HPLC analysis; batch number B20709; YuanYe Chemical Co., Ltd; Shanghai, China), 0.6 mg/kg of TRR (99.9% detecting by HPLC analysis; batch number B20707; YuanYe Chemical Co., Ltd; Shanghai, China), or 0.2 mg/kg of TRL plus 0.6 mg/kg of TRR by intraperitoneal injection. The TRL, TRR, and TRLR were all dissolved with DMSO, and the final concentration of DMSO was 1%. Six male 8-week-old C57BL/6J mice were fed the normal diet only as a normal group (NC).

Histological assessment

After the blood was taken, the aortas nearly the heart were put into 10% formalin solution and embedded in paraffin. The wax chunks with aortas were cut into four-micron-thick sections staining with Hematoxylin and Eosin. The plaque areas and endometrial thickness were using image analysis program (Image-Pro Plus 5.0).

Aortas sample collection and reverse transcriptase-polymerase chain reaction analyses

After the blood was taken, partial aortas were cut into serial pieces and extracted total RNA. Then, total RNA was reversed transcription to cDNA samples. Then, the experiments of real-time fluorescence quantification

polymerase chain reaction (PCR) were performed according to 2 × SG Fast qPCR Master Mix (Sangon Biotech; Shanghai, China) and the reaction system was total to 20 μL. The reaction condition was simply described as: 94°C predegeneration for 10 min, 94°C predegeneration for 30s; 55°C annealing 30s, 72°C extend for 30s, total 40 cycles. The primer sequence is displayed in Table 1.

Aortas sample collection and Western blot analyses

After the blood taken, partial aortas were cut into pieces. The samples were mix with buffer solution. The loading quantities of 50 μg total protein was added to 12% SDS-polyacrylamide gel electrophoresis and was electrophoresed at 60V for 30 min and 80V for 2 h in buffer systems (3.01 g tris, 18.8 g glycine and 1 g 0.5% SDS were dissolved by water to 1000 ml). After this, protein transferred to NC membranes at 80V for 2 h and half in transfer buffer systems (2.9 g tris, 5.8 g glycine and 0.37 g 0.5% SDS were dissolved by water to 800 ml, then pulsing 200 mL methanol). Then, the membranes were incubated at 4°C overnight with the primary antibody of GAPDH (1:1000; sc-59540, Santa Cruz Biotechnology, USA), MMP-9 (1:1000; sc-6840, Santa Cruz Biotechnology, USA), NF-κB p65 (1:1000; sc-8008, Santa Cruz Biotechnology, USA), and TNF-α (1:1000; ab157351, abcam, UK). After that, washing the membranes by 1xTBS with 0.2% Tween 20 and incubating the membranes with corresponding secondary antibody for 2 h. Exposing and scanning the blots, the quantitative results of blots were using the image analysis program (Image-Pro Plus 5.0).

Statistical analysis

All the data were expressed as a mean ± standard deviation, and one-way ANOVA was applied to assess the statistical significance (SPSS 15, Inc., Chicago, IL, USA). The significance of differences between the control and treatment groups was determined at level $P < 0.05$ or $P < 0.01$.

RESULTS AND DISCUSSION

Active compounds screening

A total of 144 known compounds were obtained from TGW. As results, 34 candidate molecules were conformed to filter parameters of ADME properties (PerOB ≥30%, PerDL ≥0.18, and HL ≥4), and accounting for 23.6%. Although TPR had poor ADME properties of (PerOB = 17.84%), it was isolated from in TGW and exhibit the anti-AS effect.[26] Thus, TPR was used for further study. Finally, there were 35 ingredients were chosen, shown in Table S1. ADME properties of PerOB for these active compounds were ranged from 30.16% to 107.71% and PerDL from 0.2 to 0.84. According to TCMSP database, we deleted the compounds which had no targets base on the TCMSP database or had not corresponding gene name based on Uniprot database, we obtained 17 potential compounds [Table 2] in TGW.

Compounds-target network and go term analysis

A total of 148 candidate targets for 17 compounds were obtained based on TCMSP database displayed in Table S2. Then, a visual graph of C-T network was set up based on potential ingredients and target, as shown in Figure 1. There were 165 nodes (148 candidate targets plus 17 compounds) and 325 edges, the average degree of per compound was 19.11. Kaempferol (MOL000422, degree = 57), beta-sitosterol (MOL000358, degree = 36), TPL (MOL003187, degree = 33), and TPR (MOL003186, degree = 25) possess higher degree number indicated more interaction with targets. We surmised that these ingredients in herb might be the key active compounds.

It was known that pathogenesis of AS was very complex and the major processes were including lipid accumulation, inflammatory response, immunoreactions, and vascular structure change. As the research accumulating on AS, more and more related genes to it were obtained. To know which targets in the 148 candidate targets were correlated to the pathogenesis of AS, the candidate targets were further mapped to Genecards database and finally screened out 76 potential targets related to AS, as shown in Table 3.

To validate whether the 76 potential targets actually math for AS, Gene Ontology analysis was performed for the biologic processes. Generally speaking, the obtained gene name list put into DAVID database, and the related bioinformation was collected. We analyzed the results of list top 50 biologic processes as [displayed in Figure 2], most of these targets were strongly correlated to processes including immune response, inflammatory response, regulation vascular structure, which all were in connection with AS.[27-29]

T-P network and analysis

Searching KEGG and got pathways related to candidate protein targets. Target-Pathway network (T-P network) is displayed in Figure 3, and 72 targets were mapped to 186 pathways, and an average degree of per target was 13.13, and an average pathway was 2.58. However, 4 of 76 targets had not been mapped into pathway. In the T-P network, we found that several targets could map into multiple pathways (83/176, $n \geq 5$), might be the crucial factors for AS. Serial pathways, such as TNF signaling pathway (hsa04668, degree = 11) and NF-kappa B signaling pathway (hsa04064, degree = 8) were strongly correction to pathogenesis of AS of anti-inflammatory categories, meanwhile TNF signaling pathway was including remodeling extracellular matrix of MMP-9, which was close to vulnerable plaque stability in AS; Metabolic pathways (hsa01100, degree = 13) might be related to lipid accumulation. T-cell receptor signaling pathway (hsa04660, degree = 7) could influence the inflammatory factors of IL-2, TNF-α, and IFN-γ. In addition, some targets involved in the function of vascular structure, such as HIF-1 signaling pathway (hsahsa04066, degree = 12) was involved in regulating vascular tone and angiogenesis.

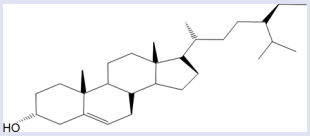
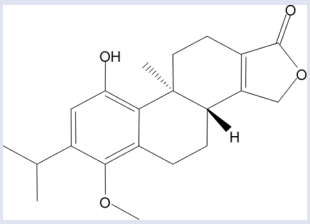
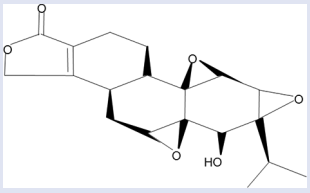
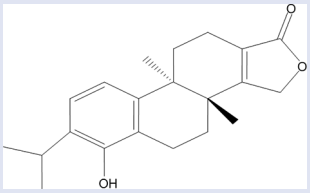
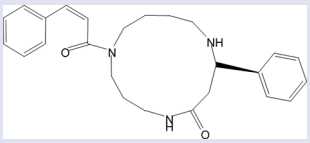
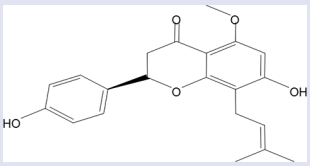
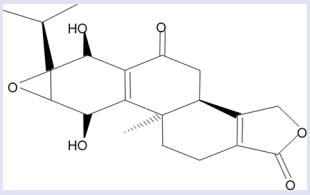
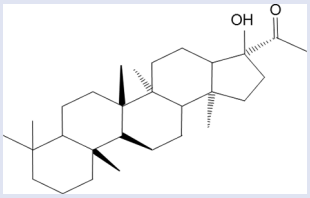
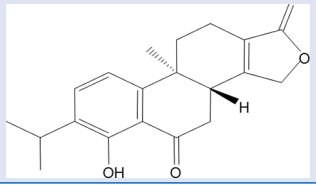
The AS is a cardiovascular disease, characterized by the combination of chronic inflammation and lipid accumulation in vascular. Its pathomechanism is very complex and had no perfect drugs to cure. We had noticed that extract of TGW could improve AS and regulate blood lipids, but the certain mechanism was still unknown. We speculated that its efficacy might be related to multi-ingredient and multi-targets, and hence, the systems pharmacology method was used to clarify our ideal. As results, we found that 17 bioactive compounds with 76 therapeutic proteins might be the key points, Compounds of Kaempferol and

Table 1: Target genes and their primer sequences

Primer name	Fwd sequence (5'to 3')	Rev sequence (5'to 3')
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
NF-κB	CCAGGCGGACATCTACAA	CAAGGCCAAATGAAAGGA
TNF-α	CTGTGAAGGGAATGGGTGTT	CAGGGAAGAATCTGGAAAGGTC
MMP-9	GACCAAGAGGGTTTTCTTCT	TACTGGAAGATGTCGTGTGA

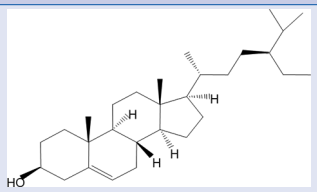
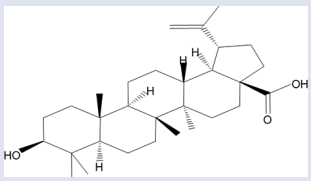
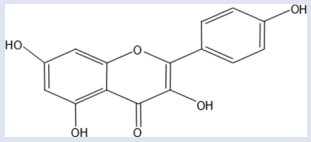
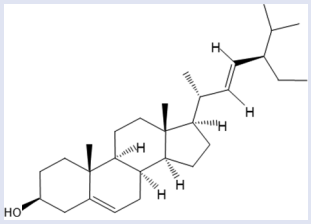
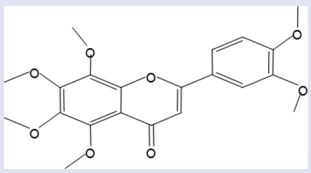
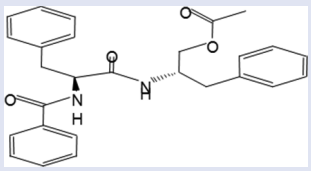
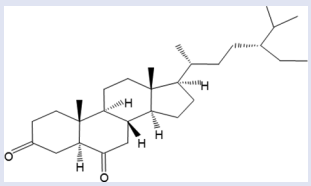
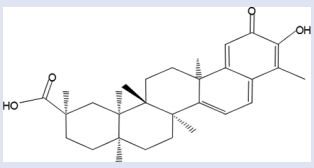
NF-κB: Nuclear factor-kappa B; TNF-a: Tumor necrosis factor alpha; MMP-9: Matrix metalloprotein 9

Table 2: Chemical information of 17 active compounds in *Tripterygium wilfordii*

Mol ID	Molecule name	Structure	MW	OB (%)	DL	HL
MOL000296	Hederagenin		414.79	36.91	0.75	5.35
MOL003184	81827-74-9		342.47	45.42	0.53	5.58
MOL003187	Triptolide		360.44	51.29	0.68	4.14
MOL003196	Tryptophenolide		312.44	48.5	0.44	4.42
MOL003209	Celallocinnine		405.59	83.47	0.59	10
MOL003217	Isoxanthohumol		354.43	56.81	0.39	17.98
MOL003224	Tripdiotolnide		360.44	56.4	0.67	4.91
MOL003266	21-Hydroxy-30-norhopan-22-one		428.77	34.11	0.77	6.66
MOL003280	Triptonolide		326.42	49.51	0.49	17.94

Contd...

Table 2: Contd...

Mol ID	Molecule name	Structure	MW	OB (%)	DL	HL
MOL000358	Beta-Sitosterol		414.79	36.91	0.75	5.36
MOL000211	Mairin		456.78	55.38	0.78	8.87
MOL000422	Kaempferol		286.25	41.88	0.24	14.74
MOL000449	Stigmasterol		412.77	43.83	0.76	5.57
MOL005828	Nobiletin		402.43	61.67	0.52	16.2
MOL007415	[(2S)-2-[[(2S)-2-(benzoylamino) -3-phenylpropanoyl] amino]- 3-phenylpropyl] acetate		444.57	58.02	0.52	6.03
MOL007535	(5S,8S,9S,10R,13R,14S,17R) -17-[(1R,4R) -4-ethyl-1,5-dimethylhexyl]- 10,13-dimethyl- 2,4,5,7,8,9,11,12,14,15,16,17- dodecahydro-1H-cyclopenta[a] phenanthrene-3,6-dione		428.77	33.12	0.79	6.56
MOL003186	Tripterine		450.67	17.84	0.78	——

MW: Molecular mass; OB: Absorption; DL: Drug-likeness; HL: Half-life

Beta-sitosterol, for the sake of higher degree and TPL and TPR were main active ingredients and process of high degree, which indicated the four ingredients in TGW might be the most important for treatment AS. Through the analysis of KEGG Pathway, TGW influenced the pathways of TNF signaling pathway (hsa04668), NF-kappa B signaling pathway (hsa04064) and metabolic pathways (hsa01100), which were strongly correction to the pathogenesis of AS, might be primary mechanisms on the anti-AS.

Effect of triptolide combined with tripterine on histological assessment in treated mice

By analysis targets protein of active compounds, we found that TPL and TPR could influence on the pathogenesis of AS from points of anti-inflammatory, vulnerable plaque stability, and vascular structure, to further developing the TGW on anti-AS, we take TPL combined with TPR as whole to research the effect on the treatment of AS.

Table 3: Information of gene targets of *Tripterygium wilfordii* related to atherosclerosis

ID	Uniprot	Protein name	Gene names	Relevance score
T01	P28223	5-hydroxytryptamine 2A receptor	HTR2A	0.67
T02	P08253	72 kDa type IV collagenase	MMP2	2.5
T03	P00325	Alcohol dehydrogenase 1B	ADH1B	0.95
T04	P00326	Alcohol dehydrogenase 1C	ADH1C	0.67
T05	P15121	Aldose reductase	AKR1B1	1.34
T06	P21397	Amine oxidase (flavin-containing) A	MAOA	0.67
T07	P27338	Amine oxidase (flavin-containing) B	MAOB	0.67
T08	P10275	Androgen receptor	AR	1.16
T09	Q07812	Antileukoproteinase	BAX	0.95
T10	P10415	Apoptosis regulator Bcl-2	BCL2	0.95
T11	P09917	Arachidonate 5-lipoxygenase	ALOX5	11.82
T12	P35869	Aryl hydrocarbon receptor	AHR	0.95
T13	P08588	Beta-1 adrenergic receptor	ADRB1	2.91
T14	P07550	Beta-2 adrenergic receptor	ADRB2	1.49
T15	P42574	Caspase-3	CASP3	0.95
T16	P32248	C-C chemokine receptor type 7	CCR7	0.95
T17	P04637	Cellular tumor antigen p53	TP53	3.98
T18	P08709	Coagulation factor VII	F7	4.07
T19	P01024	Complement C3	C3	1.77
T20	P61073	C-X-C chemokine receptor type 4	CXCR4	0.67
T21	P38936	Cyclin-dependent kinase inhibitor 1	CDKN1A	0.95
T22	P04798	Cytochrome P450 1A1	CYP1A1	3.85
T23	P05177	Cytochrome P450 1A2	CYP1A2	2.91
T24	P08684	Cytochrome P450 3A4	CYP3A4	2.91
T25	P27487	Dipeptidyl peptidase IV	DPP4	1.77
T26	P29323	Ephrin type-B receptor 2	EPHB2	0.67
T27	P16581	E-selectin	SELE	20.67
T28	P03372	Estrogen receptor	ESR1	7.71
T29	P24385	G1/S-specific cyclin-D1	CCND1	0.67
T30	P04150	Glucocorticoid receptor	NR3C1	1.64
T31	P09488	Glutathione S-transferase Mu 1	GSTM1	2
T32	P28161	Glutathione S-transferase Mu 2	GSTM2	0.67
T33	P09211	Glutathione S-transferase P	GSTP1	1.34
T34	P09601	Heme oxygenase 1	HMOX1	5.12
T35	Q9Y6K9	Inhibitor of nuclear factor kappa-B kinase subunit beta	IKBKG	0.67
T36	P06213	Insulin receptor	INSR	3.85
T37	P05362	Intercellular adhesion molecule 1	ICAM1	11.73
T38	P01579	Interferon gamma	IFNG	1.77
T39	P60568	Interleukin-2	IL2	1.34
T40	Q9NPF7	Interleukin-23 subunit alpha	IL23A	0.67
T41	P24394	Interleukin-4	IL4R	1.64
T42	P10145	Interleukin-8	CXCL8	1.89
T43	P03956	Interstitial collagenase	MMP1	4.54
T44	P09960	Leukotriene A-4 hydrolase	LTA4H	1.16
T45	P61626	Lysozyme	LYZ	0.67
T46	P14780	Matrix metalloproteinase-9	MMP9	15.23
T47	P01033	Metalloproteinase inhibitor 1	TIMP1	4.91
T48	P16035	Microtubule-associated protein 2	TIMP2	1.49
T49	P08235	Mineralocorticoid receptor	NR3C2	0.67
T50	P45983	Mitogen-activated protein kinase 8	MAPK8	0.95
T51	P08571	Monocyte differentiation antigen CD14	CD14	2.5
T52	P36544	Neuronal acetylcholine receptor protein, alpha-7 chain	CHRNA7	0.67
T53	P35228	Nitric oxide synthase, inducible	NOS2	4.54
T54	P29474	Nitric-oxide synthase, endothelial	NOS3	14.5
T55	Q15596	Nuclear receptor coactivator 2	NCOA2	0.95
T56	Q12809	Potassium voltage-gated channel subfamily H member 2	KCNH2	1.09
T57	Q9NZQ7	Programmed cell death 1 ligand 1	CD274	0.67
T58	P01100	Proto-oncogene c-Fos	FOS	2.04
T59	P31749	RAC-alpha serine/threonine-protein kinase	AKT1	2.61
T60	Q86VB7	Scavenger receptor cysteine-rich type 1 protein M130	CD163	5.61
T61	P42224	Signal transducer and activator of transcription 1-alpha/beta	STAT1	0.67
T62	P40763	Signal transducer and activator of transcription 3	STAT3	1.64
T63	Q14524	Sodium channel protein type 5 subunit alpha	SCN5A	2.21
T64	P14672	Solute carrier family 2, facilitated glucose transporter member 4	SLC2A4	0.67

Contd...

Table 3: Contd...

ID	Uniprot	Protein name	Gene names	Relevance score
T65	P06126	T-cell surface glycoprotein CD1a	CD1A	0.67
T66	P33681	T-lymphocyte activation antigen CD80	CD80	0.67
T67	P42081	T-lymphocyte activation antigen CD86	CD86	0.67
T68	P05412	Transcription factor AP-1	JUN	1.49
T69	P01137	Transforming growth factor beta-1	TGFB1	12.79
T70	P01375	Tumor necrosis factor	TNF	11.57
T71	P25942	Tumor necrosis factor receptor superfamily member 5	CD40	2.22
T72	P19320	Vascular cell adhesion protein 1	VCAM1	13.34
T73	P15692	Vascular endothelial growth factor A	VEGFA	9.84
T74	P17948	Vascular endothelial growth factor receptor 1	FLT1	1.64
T75	P35968	Vascular endothelial growth factor receptor 2	KDR	0.95
T76	P47989	Xanthine dehydrogenase/oxidase	XDH	4.24

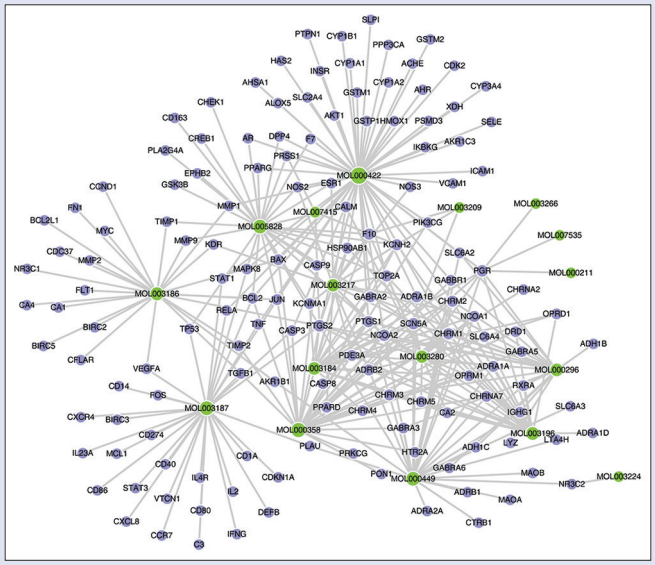


Figure 1: Compounds-Target network. Green circles represented as compounds and lavender circles represented as targets

From the H and E results, we found that large plaque was informed in model Apo E$^{-/-}$mice, compared with NC group. TRL and TRR could decrease the developing of plaque. When observed the composite group, an interesting thing was happened that treatment with TRLR could significantly suppress the developing of plaque. The results are shown in Figure 4.

Based on the TSCMP database, there were 266 kinds of herbs containing Kaempferol, such as Rubi Fructus, Chrysanthemi Flos *et al.* In addition, there were 499 kinds of herbs containing beta-sitosterol. Although Kaempferol and Beta-sitosterol were mapped to much targets, they were not the special compounds in TGW. TPL and TPR were the biomarker active ingredients in TGW. TPR is a quinone methide triterpenoid isolated from the TCM TGW. Recent studies showed that TPR (2 mg/kg/d, i. p.) inhibits atherogenesis in TPR -treated ApoE$^{-/-}$mice fed an atherogenic diet by inhibiting inflammation in the arterial wall and other researches show that TPR (1 mg/kg/d, i. g.) effectively reduced the plaque ratio.[30,31] TPL, a diterpenoid triepoxide purified from the TGW, was tested for its antitumor properties and anti-inflammatory. It had reported that TPL could suppress the release of TNF-α,[32] which is strongly related to the pathogenesis of AS. Thus, TPL and TPR may be the main compositions in TGW to research their synergistic effect on suppressing the progress of AS. The present study revealed that TPL (TRL, 0.2 mg/kg/d) and TPR (TRR, 0.6 mg/kg/d) could decrease the developing of plaque in aortas of Apo E$^{-/-}$mice, which is consistent with previous reports.[30,31,33] An interesting thing was happened that treatment with TRLR (TRL, 0.2 mg/kg/d plus TRR, 0.6 mg/kg/d) could more significantly suppress the developing of plaque, which suggested that TRL and TRR may have a synergistic effect on anti-AS.

Expression of matrix metalloprotein 9, tumor necrosis factor alpha and Nuclear factor-kappa B mRNA in aortas of Apo E$^{-/-}$mice by reverse transcriptase- polymerase chain reaction

Reverse transcriptase-PCR (RT-PCR) analyses were performed to determine the effect of TRL, TRR, and TRLR on MMP-9, TNF-α, and NF-κB in Apo E$^{-/-}$mice. The results are shown in Figure 5. The mRNA expression of MMP-9, TNF-α and NF-κB were significantly decrease in TRL group and TRR group ($P < 0.01$). Moreover, the similar trend was appeared in TRLR treated group after administrated for 8 weeks ($P < 0.01$) with more notable decrease. What is more, TRLR effect on down-regulating of mRNA MMP-9 and NF-κB expression were more significant than TRL or TRR alone ($P < 0.01$, 0.05).

Effect of triptolide combined with tripterine on matrix metalloprotein 9, tumor necrosis factor alpha and Nuclear factor-kappa B in aortas of Apo E$^{-/-}$mice by Western-blot

As we had noticed that TRL and TRR could influence the inflammatory reaction and vulnerable plaque stability indicator such as MMP-9. Western blot was used to observe the protein expression of TNF-α, NF-κB, and MMP-9 in aortas after drugs intervened.

The inflammatory factors, such as TNF-α and NF-κB were important factors in forming plaques. MMP-9, playing a significant role in the occurrence, development, and rupture of the AS plaque with inflammatory factors were applicable for investigating the anti-atherosclerotic effect. Compared to MC group, TRL, TRR, and TRLR could significantly decrease the expression of TNF-α, NF-κB, and MMP-9 ($P < 0.01$), and TRLR could reduce these indicators by a bigger margin, which indicated that TRLR had a synergistic effect on improving AS [Figure 6].

It was well known that ox-LDL could entice NF-κB activation,[34] NF-κB activated would participate in the expression of many inflammatory mediators, such as TNF-α. On the other hand, NF-κB activated by ox-LDL could regulate up expression of adherence factors, such as VCAM-1 and[35] ICAM-1[36]. In the present study, the C-T network and T-P network analysis had also found that TRL and TRR could target TNF-α directly or participate in the NF-kappa B signaling pathway through TNF-α, CD14, or CD40. Our pharmacodynamic experiment

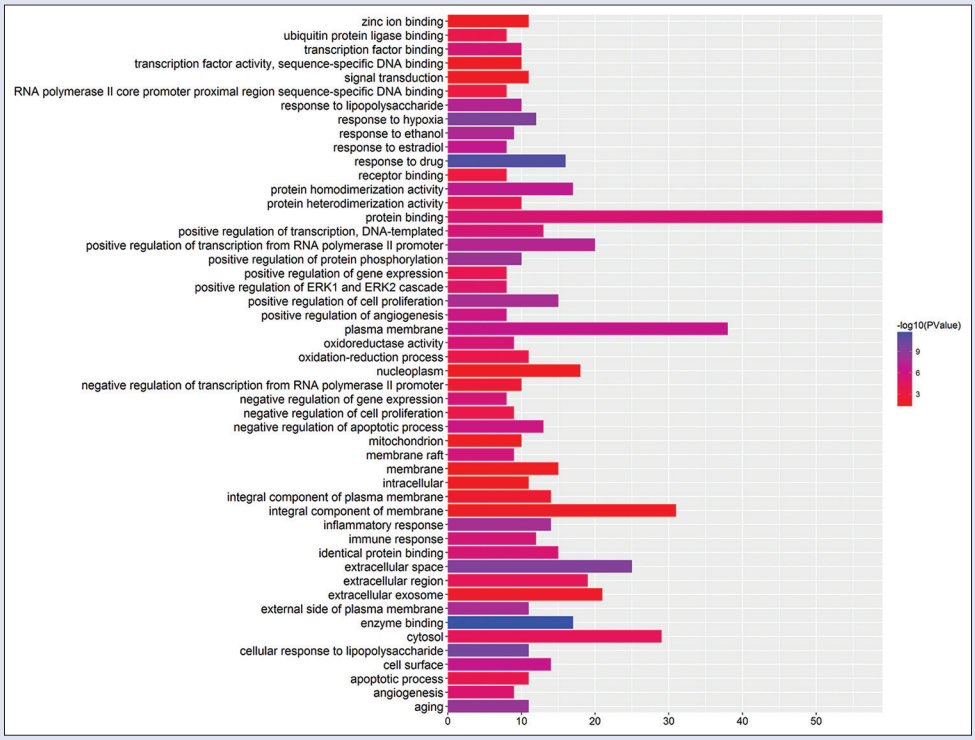


Figure 2: Geno ontology analysis of list top 50 biological processes for candidate targets X-axis represented as biological process and Y-axis represented as number of genes

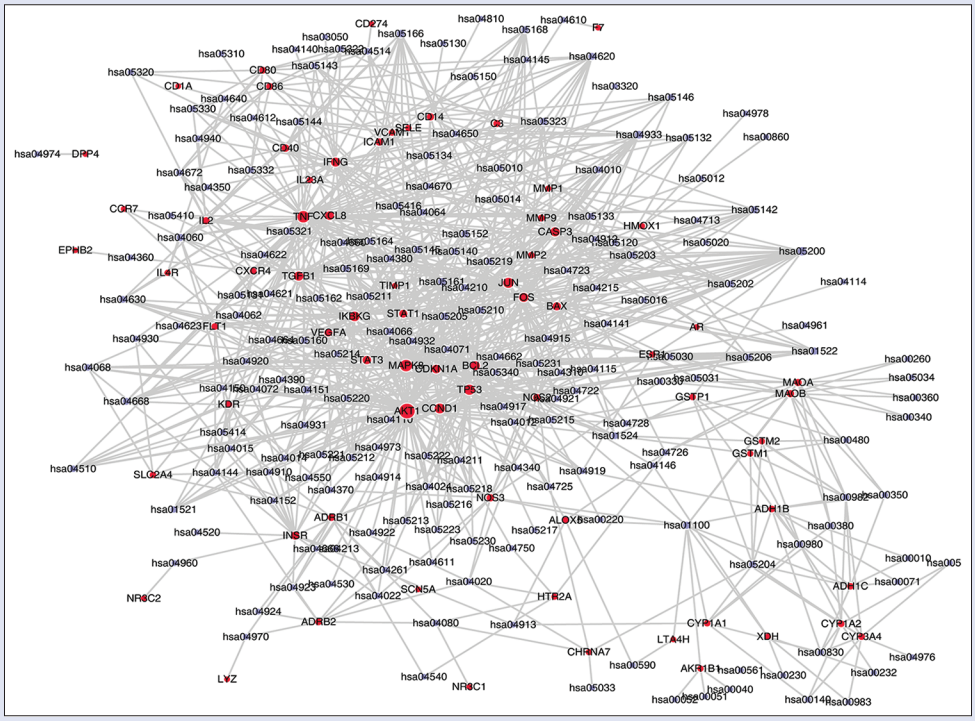


Figure 3: Target-Pathway network The lavender circles represented as targets and red circles represented as pathway. The edges were represented as connection

further verified that TRL, TRR, and TRLR could all significantly alleviate NF-κB and TNF-α in aortas of Apo $E^{-/-}$mice by RT-PCR and western blot, and the effect of TRLR was more notable.

MMP-9, which is an important biological marker of inflammatory factors, plays a significant role in the occurrence, development, and rupture of the AS plaque.[37,38] On the other hand, it had reported that macrophage

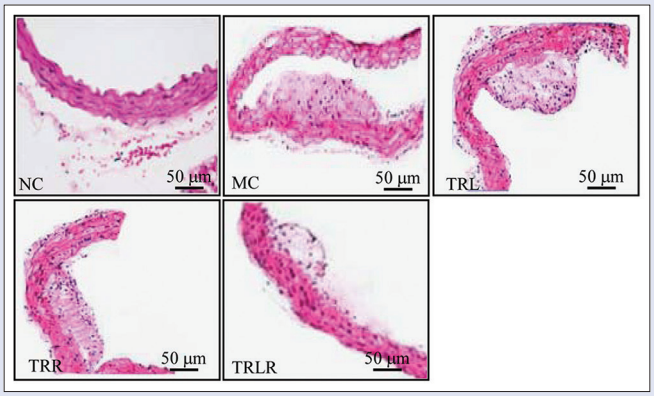


Figure 4: Representative photomicrograph of structure disturbance in the aortas with Hematoxylin and Eosin (H and E, ×100) NC, C57BL/6J mice were fed with the normal diet only as normal group; MC, HFC model control group; TRL, HFC plus Triptolide group; TRR, HFC plus Tripterine group; TRLR, HFC plus triptolide and tripterine group

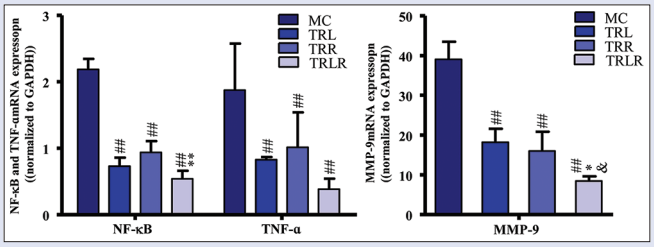


Figure 5: Triptolide and Tripterine Effect on mRNA expression of MMP-9, TNF-a and NF-kB gene in Apo E$^{-/-}$ mice.MMP-9, Matrix metalloprotein 9; NF-kB, Nuclear factor-kappa B; TNF-a, Tumor Necrosis Factor Alpha; Data are mean ± SD ($n = 6$). $^{\#\#}P < 0.01$ versus MC group; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus TRL group; $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ versus tripterine group

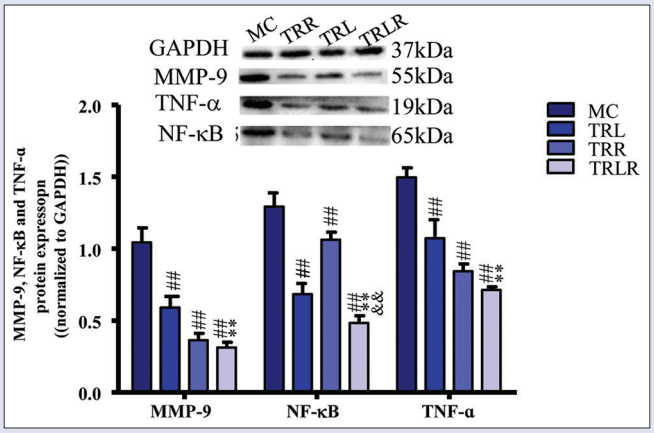


Figure 6: Triptolide and Tripterine Effect on protein expression of MMP-9, TNF-a and NF-kB in Apo E$^{-/-}$ Mice. MMP-9, Matrix metalloprotein 9; NF-kB, Nuclear factor-kappa B; TNF-a, Tumor Necrosis Factor Alpha; Data are mean ± SD ($n = 6$). $^{\#\#}P < 0.01$ versus MC group; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus TRL group; $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ versus tripterine group

and smooth muscle cell isolated from AS plaque cultured *in vitro* could secrete lots of MMP-9, which also could be pointing the damage of vascular in AS. Systems Pharmacology analysis had pointed that TRL and TRR could effect on MMP-9. In our pharmacodynamic experiment study, supplementation with TPL and TPR alone or combination of TPL and TPR to Apo E$^{-/-}$ for 8 weeks could decrease the expression of MMP-9 in aortas, with reducing atherosclerotic plaque size.

CONCLUSION

To sum up, TGW could regulate AS by multiple pathways, especially immune response, inflammatory response, vascular structure improving, had synergistic effect by interactive effect of different compounds. What's more, the major compounds of TPR and TPL in TGW had synergistic effect on anti-AS by suppressing MMP-9, TNF-α, and NF-κB.

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Conflicts of interest

There are no conflicts of interest.

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