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ORIGINAL ARTICLE

Therapeutic Targets and Biological Mechanisms of Curcumol on Atherosclerosis: A Study Based on Network Pharmacology Approach and Biological Studies

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ABSTRACT

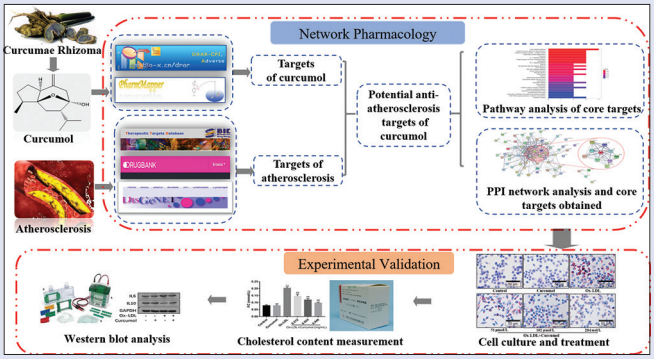
Background: Atherosclerosis is an extremely predominant condition. The morbidity and mortality of cardiovascular and cerebrovascular ailments caused by atherosclerosis have augmented significantly in recent years. The discovery and research of new drugs are therefore obligatory and will be cooperative in the clinical treatment and management of atherosclerosis. **Objective:** Curcumol extracted from the traditional Chinese medicine (TCM) E-Zhu (*Curcumae Rhizoma*), is one of the chief bioactive ingredients of Curcuma oil (a marketed drug in China). **Materials and Methods:** To classify core targets and discover the main biological mechanisms of curcumol against atherosclerosis, a network pharmacology tactic was commenced for data mining, and biological experiments were engaged for validation. **Results:** Absorption, metabolism and excretion screening using TCM Systems Pharmacology Database and Analysis Platform server, target forecast using public databases, protein-protein interaction network analysis using the STRING database and Kyoto Encyclopedia of Genes and Genomes pathway analysis were engaged to examine the potential mechanisms of curcumol on atherosclerosis. On the basis of data mining, 10 core targets and related pathways were recognized. Furthermore, the key target signaling pathways, interleukin 6 (IL-6), IL-10, and JAK2/STAT3, were authenticated using biological studies. **Conclusion:** Curcumol could improve atherosclerosis by reducing the formation of foam cells; the mechanism of action was ascribed to the downregulation of IL-6 expression, upregulation of IL-10 expression and inhibition of the JAK-STAT pathway. Therefore, this study will be a basis for further study of the biological roles of curcumol on atherosclerosis.

Key words: Atherosclerosis, curcumol, mechanism, network pharmacology

SUMMARY

- The anti-atherosclerosis activity and the mechanism of curcumol against atherosclerosis were examined in this study. The results revealed that curcumol can inhibit the atherosclerosis progress by reducing the foam cell formation, and the mechanism is to downregulate interleukin 6 (IL-6) expression, up-regulate IL-10 expression and to inhibit the JAK-STAT pathway. These outcomes would provide beneficial reference for further research on curcumol in the treatment of atherosclerosis.

Abbreviations used: TCM: Traditional Chinese medicines; ADME: Absorption, distribution, metabolism and excretion; TCMSP: Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform; TTD: Therapeutic targets database; PPI: Protein-protein interaction; KEGG: Kyoto Encyclopedia of Genes and Genomes; MW: Molecular weight; Hacc: Hydrogen bond acceptors; Hdon: Hydrogen bond donors; RBN: Rotatable bond; DL: Drug-likeness; OB: Oral bioavailability; TC: Cellular total cholesterol; FC: Free cholesterol; CE: Cholesterol-ester content.



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INTRODUCTION

Atherosclerosis is exceedingly dominant in most parts of the world, and is the fundamental cause of angina, myocardial infarction, stroke and sudden cardiac death. It suggestively contributes to morbidity, mortality and healthcare costs, which is one of the foremost causes of death worldwide.[1-3] Earlier studies have established that the pathological process of atherosclerosis is complex,[3,4] and mostly related to inflammation, infection, and immunity.[5-7] Hence, there is an urgent necessity to develop therapeutic candidate drugs for atherosclerosis that act by aiming the molecular mechanisms of the disease.

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Traditional Chinese Medicines (TCMs) have been extensively employed to treat atherosclerosis. Recently, overwhelming suggestion from research data, preclinical and clinical studies have confirmed that certain TCMs and their bioactive compounds play key roles in the prevention and management of atherosclerosis.[8-14] According to the principle of TCM, atherosclerosis is thoroughly involved with pathological products such as turbid phlegm and static blood. TCMs that progress blood circulation and reverse stasis have a curative effect on atherosclerosis. *Curcumae Rhizoma* (Ezhu in Chinese) is a well-known Chinese medicine that promotes Qi, activates blood, releases accumulation and eases pain. Curcumol [Figure 1a] is a monomeric sesquiterpenoid and the chief bioactive compound isolated from *Curcumae Rhizoma*.[15,16] Results from animal experiments have established that curcumol activates blood circulation and dissipates blood stasis.[17] Therefore, curcumol may be a latent drug for atherosclerosis treatment.

Of late, network pharmacology is evolving an essential tool in discovering novel bioactive compounds and clarifying their mechanism of action.[18-22] Contrary to the current "one target, one drug" mode, network pharmacology applies the "multi-component, multi-target" and "single-component, multi-target" approach in biological networks to construct a relation of component-target-disease, which assimilates a large number of database resources to target a biological network of compounds.[23-26] To systematically examine the pharmacological mechanisms of curcumol on atherosclerosis, the network pharmacology method was employed in our study for data mining. Besides, to confirm the role of curcumol in regulating atherosclerosis, corresponding cell-based experiments were considered to test the hypotheses of network pharmacology [Figure 1b].

MATERIALS AND METHODS

Chemicals and reagents

Curcumol (>98% purity) was procured from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China, No. C114054). Human Ox-LDL was acquired from Yiyuan Biotechnologies (Guangzhou, China, No. YB-002). Cellular total cholesterol (TC) test kit was purchased from Changchun Huili Biotech Co., Ltd. (Changchun, China, No. C048-a). Free cholesterol (FC) test kit was procured from Beijing Solarbio Technology Co., Ltd. (Beijing, China, No. BC1890). Antibodies interleukin 6 (IL-6), IL-10 and p-STAT3 were purchased from Affinity Biosciences (OH, USA, No. DF6087, DF6894, AF3293). Antibodies JAK2 and STAT3 were purchased from Wuhan Sanying Biotechnology Co., Ltd. (Wuhan, China, No. 10253-2-AP, 17670-1-AP). Antibodyp-JAK2 was procured from Beijing Bioss Biotechnology Co., Ltd. (Beijing, China, No. bs-3206R, bs-0439R). Antibody GAPDH was delivered by Hangzhou Xianzhi Biology Co., Ltd. (Hangzhou, China, No. AB-P-R 001).

Target fishing to identify corresponding targets of curcumol on atherosclerosis

Atherosclerosis-related targets were recovered from the DisGeNET database (http://www.disgenet.org/web/DisGeNET/menu/home), therapeutic targets database (TTD; http://bidd.nus.edu.sg/BIDD-Databases /TTD/TTD.asp) and Drugbank (https://www.drugbank.ca/). The potential targets of curcumol were downloaded from the PharmMapper and DRAR-CPI databases. Curcumol-related targets were mapped to the pathological targets of atherosclerosis and the intersection targets between curcumol and atherosclerosis were attained.

Protein-protein interaction network analysis and Kyoto Encyclopedia of genes and genomes pathway analysis

The putative targets for curcumol against atherosclerosis were input into the STRING database (https://string-db.org/) and the protein-protein interaction (PPI) network was achieved. Then, the network was imagined using Cytoscape 3.7.2. The node degree value and other topological parameters of the PPI network were assessed using Network Analyzer. The target pathways of curcumol in atherosclerosis were screened based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Cell culture and treatment

Murine RAW264.7 cells were obtained from the Procell Life Science and Technology Co., Ltd. (Wuhan, China) and preserved in a high glucose Dulbecco's Modified Eagle's Medium (DMEM) culture medium (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco, USA), 100 U/mL penicillin and 100 mg/mL streptomycin. All cells were incubated at 37°C in a humidified atmosphere encompassing 5% CO_2. The cells were treated with 100 μg/mL human Ox-LDL for 24 h to induce cellular differentiation into foam cells. When RAW264.7 cells reached 80% confluency, the differentiated cells were incubated for 24 h with several curcumol concentrations (51, 102 and 204 μmol/L).

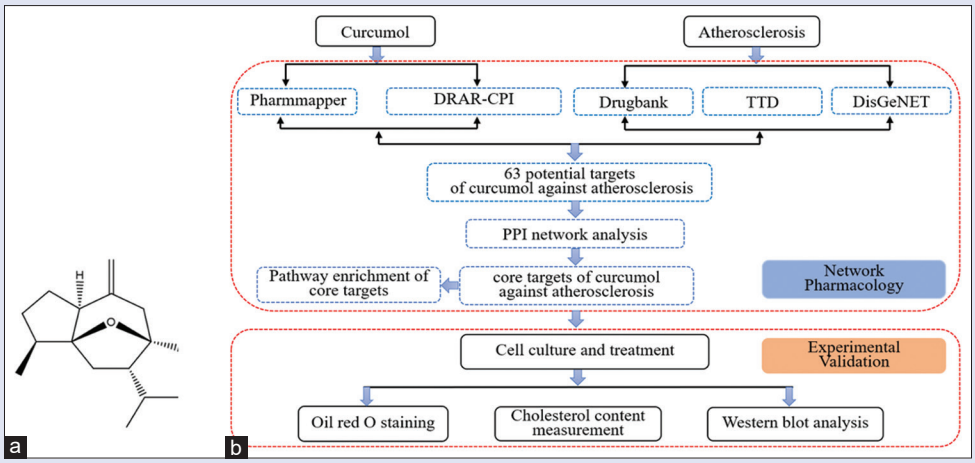

Figure 1: (a) Chemical structure of curcumol from the Chemspider database (PubChem CID: 14240392); (b) Flowchart for the investigation of curcumolin atherosclerosis

Oil red O staining

The cell slides were congregated after Ox-LDL treatment to regulate the degree of foam cell differentiation and fixed in 4% paraformaldehyde for 15 min. Next, cells were stained with 0.5% Oil Red O and 0.1% hematoxylin. The Oil Red O staining area of each sample was examined using IPP6.0 software.

Cholesterol content measurement

After treatment, the cell supernatants were collected to determine cholesterol content. The cellular TC and FC were measured using the commercial assay kits according to the manufacturer's guidelines. The cholesterol-ester content (CE) of each sample was considered by subtracting FC from TC.

Western blot analysis

The cells were lysed for 30 min on ice using RIPA lysis buffer. Then, the lysates were collected and centrifuged at 12,000 rpm for 20 min at 4°C. The protein concentration was determined using Biocinchonic Acid (BCA) assay. The aliquots (40 μg) of cell lysates were detached using 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS PAGE) and then transferred onto aPolyvinylidene difluoride (PVDF) membrane. excluding for the phosphorylated proteins that were blocked with 1% BSA, the membranes were obstructed using 5% skim milk and incubated at 4°C overnight with primary rabbit antibodies including anti-IL-6 (1:1000 dilution), anti-IL-10 (1:1000 dilution), anti-JAK2 (1:1000 dilution), anti-p-JAK2 (1:1000 dilution), anti-STAT3 (1:1000 dilution), anti-p-STAT3 (1:1000 dilution) and anti-GAPDH (1:1000 dilution). After the membranes were eroded five times using TBST, they were incubated with the secondary antibodies for 2 h. The bands were visualized using ECL and quantified using Image J software.

Statistical analysis

Statistical data were studied using SPSS 19.0 and represented as mean ± standard deviation. The significant differences were determined using ANOVA. GraphPad Prism 7.0 was used to interpret statistical results. Statistically significant and highly significant were articulated by $P < 0.05$ and $P < 0.01$, respectively.

RESULTS

Pharmacokinetics of curcumol

The information on distribution, absorption, metabolism and excretion (ADME) were projected using an *in silico* integrative ADME model from TCM Systems Pharmacology Database and Analysis Platform (TCMSP, http://tcmspw.com/tcmsp.php).[27] The pharmacokinetic characteristics are thoroughly related to its molecular mechanism, which are supportive for the screening of drug-likeness (DL). Thus, the ADME-related properties of curcumol were explored, which were in accordance with the Lipinski's rule of five.[28] Notably, the predicted oral bioavailability was ≥30% and DL was closer to 0.18 [Table 1].

Table 1: Pharmacokinetics of curcumol

Compound	Curcumol
MW	236.39
AlogP	2.79
Hdon	1
Hacc	2
OB (%)	103.55
Caco-2	1.12
BBB	1.23
DL	0.13
FASA	0.25
TPSA	29.46
RBN	1
HL	9.38

DL: Drug-likeness; RBN: Rotatable bond; MW: Molecular weight; Hacc: Hydrogen bond acceptors; Hdon: Hydrogen bond donors; OB: Oral bioavailability; HL: Half-life; FASA: Fractional negative accessible surface area; TPSA: Topological polar surface area; BBB: Blood-brain barrier

Identification of potential targets for curcumol on atherosclerosis

Disease-related targets were recovered and screened from the DisGeNET database, TTD and Drugbank; only the "*Homo sapiens*" proteins were collected. Then, 1216 targets were retrieved from the DisGeNET database, 33 targets from Drugbank and three other therapeutic targets from TTD. Compound-related targets were composed from the PharmMapper and DRAR-CPI databases. A total of 300 predicted targets were attained from the PharmMapper database and 182 targets were screened out from the DRAR-CPI database. By mapping compound-related targets to the disease-related targets, 63 potential anti-atherosclerosis targets of curcumol were nominated for further investigation [Table S1].

Protein-protein interaction analysis of potential targets

The PPI network of 63 projected targets was examined using the STRING database. As shown in Figure 2, there were 63 nodes and 283 edges in the network. To get key targets, topological parameters of the PPI network were examined. The average degree was 8.98 and the maximum was 42, in addition, the average shortest path length was 2.25 [Table 2]. The shortest path length and the higher degree value of the node characterized the core target in the PPI network. Eventually, with the average degree value of > 17 and the average shortest path length < 2.25, 10 core targets were acknowledged, showing INS, IL-6, IL-10, CASP3, MPO, HMOX1, IL-4, SOD2, ACE, and SOD1. Using the MCODE cluster, the subnetwork of the 10 core targets was recognized and there was a strong interaction with each other. Therefore, the PPI network recommended that these 10 targets might be the key objectives of curcumol on atherosclerosis.

Pathway enrichment analysis of core targets

The related pathways of the core targets of curcumol in the treatment of atherosclerosis were investigated using the KEGG. Filtered by $P \leq 0.05$, the top 30 pathways were gained [Figure 3]. The FoxO, IL-17, HIF-1 and JAK-STAT signaling pathways were principally involved. Numerous studies establish that the JAK-STAT signaling pathway plays a role in the development of atherosclerosis.[29-31] Moreover, the cytokines IL-6 and IL-10 are known to directly participate in the process of atherosclerosis.[32,33] Through activating the JAK-STAT pathway, IL-6 and IL-10 play an important role in the development of atherosclerosis.[31,34] Therefore, the core targets IL-6, IL-10 and JAK-STAT signaling pathway were hypothesized to be comprised in the curcumol-mediated regulation of atherosclerosis and were designated for succeeding validation studies.

Effect of curcumol on Ox-LDL-induced foam cell formation

To examine the effect of curcumol on foam cell formation, RAW264.7 macrophages were treated with Ox-LDL for 24 h. Then, the cells were treated in the presence and absence of curcumol (51, 102 and 204 μmol/L) for an additional 24 h. The macrophages treated with Ox-LDL exhibited lipid droplets by Oil Red O staining, which

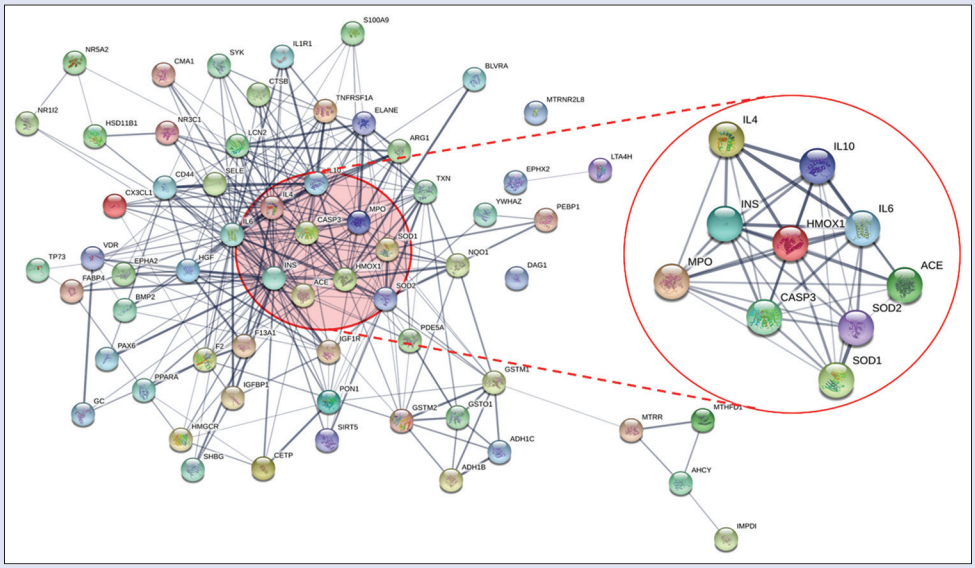

Figure 2: Protein-protein interaction network of 63 potential targets of curcumol on atherosclerosis

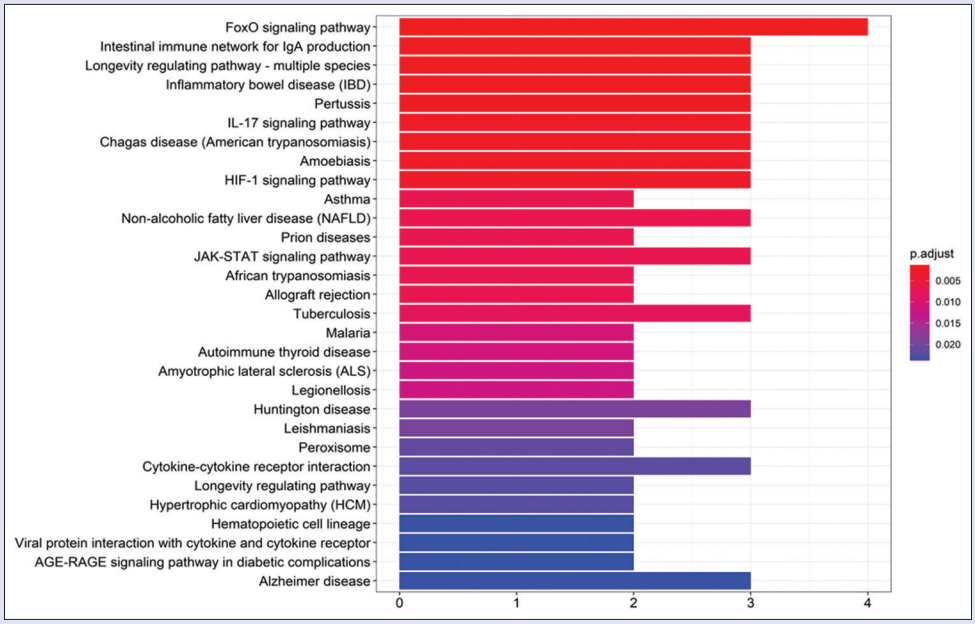

Figure 3: Kyoto Encyclopedia of Genes and Genomes pathway enrichment of the 10 core targets

specified that the foam cell model was successfully recognized [Figure 4a and b]. After treatment with curcumol (51, 102 and 204 μmol/L), the lipid droplets of foam cells decreased expressively [Figure 4a and b]. In addition, the cells treated with curcumol (204 μmol/L) and without Ox-LDL were in the same state as cells in the normal group, which designated that curcumol did not affect cell viability. Therefore, these results recommended that the formation of the lipid droplets in foam cells was reduced by curcumol treatment.

Effect of curcumol on cholesterol content

Furthermore, the cholesterol levels of each group were measured using the consistent test kits. As revealed in Figure 4c, the intracellular TC content of the Ox-LDL treatment group increased significantly compared to that of the control group ($P < 0.01$); however, the TC content of the curcumol treatment group (51, 102, and 204 μmol/L) decreased significantly compared to that of the Ox-LDL treatment group ($P < 0.01$). Likewise, the FC content and CE content were lower after curcumol treatment compared to those in the Ox-LDL treatment group [Figure 4d and e]. Together, the above results specified that curcumol played a role in reducing the lipid accumulation of macrophages *in vitro*.

Effect of curcumol on the expression of core targets and JAK-STAT signaling pathway

Curcumol affected IL-6 and IL-10 expression and also affected the phosphorylation levels of JAK2 and STAT3. As revealed in Figure 5a and b, curcumol inhibited the expression of IL-6, whereas augmented IL-10 at the dose of 204 μmol/L. The total proteins and the phosphorylation of JAK and STAT were verified using western blot method. As highlighted in

Table 2: Core targets of curcumol against atherosclerosis

PDB ID	Protein name	Target	Degree	Average shortest path length	Source
5MAM	Insulin	INS	42	1.41	DRAR-CPI
2IL6	Interleukin-6	IL6	36	1.55	PharmMapper
2ILK	Interleukin-10	IL10	27	1.72	DRAR-CPI
3H0E	Caspase-3	CASP3	24	1.76	DRAR-CPI
4C1M	Myeloperoxidase	MPO	20	1.90	PharmMapper
6EHA	Heme oxygenase 1	HMOX1	20	1.74	DRAR-CPI
3QB7	Interleukin-4	IL4	20	1.74	DRAR-CPI
1XDC	Superoxide dismutase 2	SOD2	18	1.79	PharmMapper
6H5W	Angiotensin-converting enzyme	ACE	17	1.81	DRAR-CPI
5O3Y	Superoxide dismutase 1	SOD1	17	1.90	DRAR-CPI

PDB: Protein data bank; DRAR-CPI: Drug repositioning and adverse drug reaction chemical-protein interactome

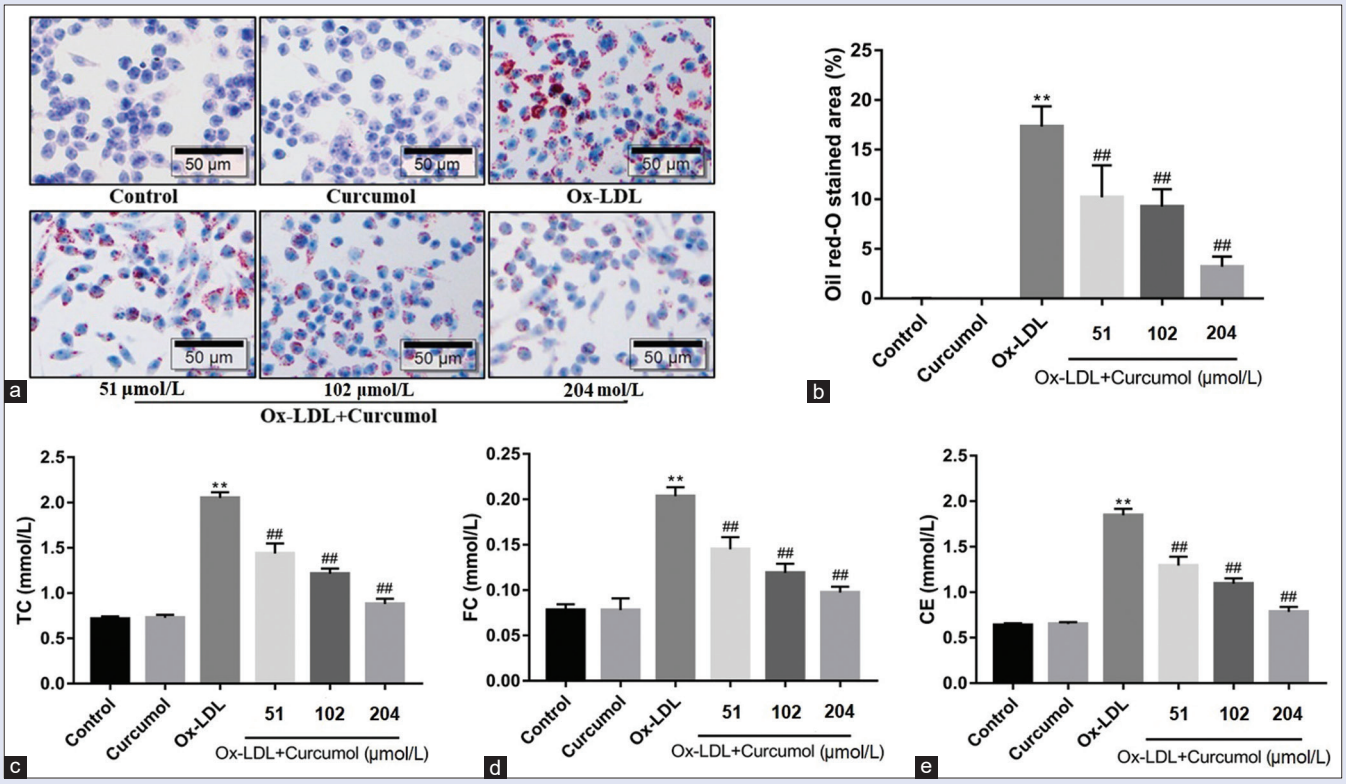

Figure 4: (a) Oil Red O staining of RAW264.7 cells incubated with Ox-LDL for 24 h and in the absence and presence of curcumol (51, 102 and 204 μmol/L) for 24 h (200 × magnification). (b) Oil Red O-stained area. (c) Intracellular total cholesterol content. (d) Freecholesterol content. (e) Cholesterol-ester. $^{**}P < 0.01$ versus control, ## $P < 0.01$ versus Ox-LDL

Figure 5c and d, curcumol meaningfully inhibited the expression of p-JAK and p-STAT when curcumol was employed at a dose of 204 μmol/L.

DISCUSSION

Recently, network pharmacology has been considered as a powerful methodology to systematically analyze drug effectiveness, particularly in the preliminary stage. In our study, network pharmacology was applied for a data mining related to the role of curcumol on atherosclerosis. As a result, 63 potential targets linking curcumol and atherosclerosis were attained and by using PPI network analysis, 10 core targets (INS, IL-6, IL-10, CASP3, IL-4, MPO, HMOX1, SOD2, SOD1 and ACE) were screened out. Furthermore, the biological pathways related to these core targets were investigated and the primary signaling pathways were achieved.

Atherosclerosis is a chronic inflammatory disease, involved characterized by endothelial cell injury, macrophage polarization, inflammation, and immune responses.[35] In early atherosclerosis, monocytes infiltrate and differentiate into macrophages. Next, these macrophages take up Ox-LDL, transform into foam cells, and contribute in the following process of atherosclerosis that succeeds. Hence, the formation of lipid-laden foam cells has been documented as a hallmark of the pathological process of atherosclerosis.[1,3] In our study, Ox-LDL-induced foam cells were employed to examine the role of curcumol in regulating atherosclerosis. Using Oil Red O staining and measuring cholesterol levels, we confirmed that curcumol could reduce cholesterol content and diminish the accumulation of intracellular cholesterol-ester.

Numerous studies specify that some cytokines are involved in the regulation of atherosclerosis.[36] For example, IL-6 is known to promote atherosclerosis,[32,37,38] while IL-10 has been exposed to inhibit atherosclerosis *in vivo*.[33,39] By activating the JAK-STAT signaling pathway, the proinflammatory cytokine IL-6 and anti-inflammatory cytokine IL-10 directly modulate the foam cells formation.[29,35]

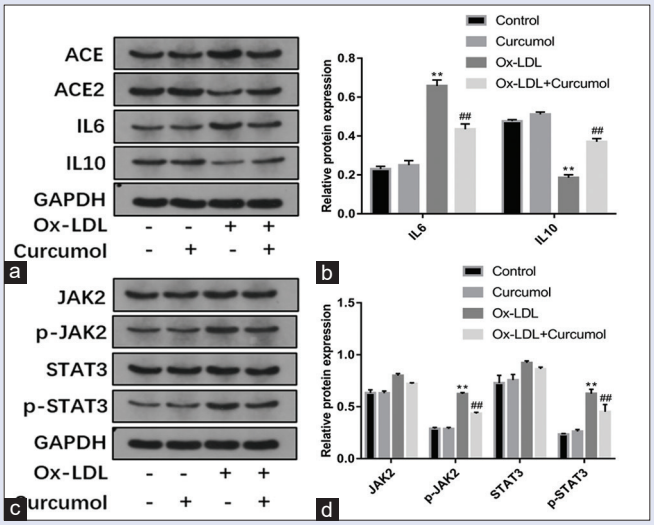

Figure 5: Effect of curcumol on the expression of interleukin-6, interleukin-10 and JAK2/STAT3 signaling pathway. (a) Western blot analysis of interleukin-6, interleukin-10. (b) Comparison of the gray value of interleukin-6, interleukin-10. (c) Western blot analysis of JAK2, p-JAK2, STAT3, p-STAT3. (d) Comparison of the Gray value of JAK2, p-JAK2, STAT3, p-STAT3. $^{**}P < 0.01$ versus control, ## $P < 0.01$ versus Ox-LDL

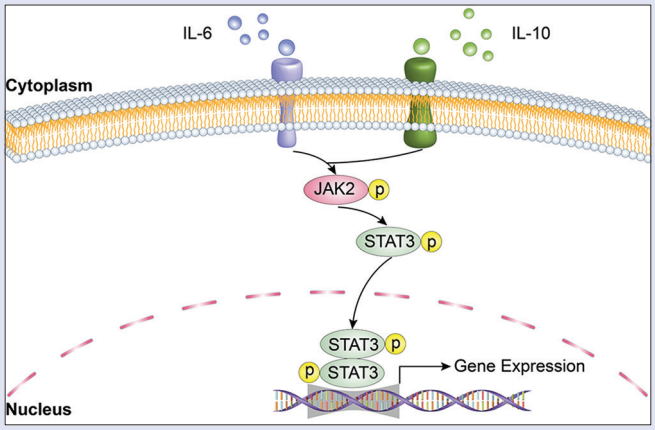

Figure 6: The core target interleukin-6, interleukin-10 and JAK2/STAT3 signaling pathways in atherosclerosis for curcumol treatment

Therefore, based on the network pharmacology estimate and literature research, the key targets IL-6, IL-10 and JAK2/STAT3 signaling pathway, were nominated to further explore the potential mechanism of curcumol in atherosclerosis. Using Western blot method, the IL-6, IL-10 and JAK2/STAT3 signaling pathway were considered. The results presented that curcumol inhibited the expression of IL-6 and promoted that of IL-10. Concurrently, curcumol blocked the phosphorylation of JAK2 and STAT3.

During the development of atherosclerosis, IL-6 and IL-10 both induce suppressor of cytokine signaling 3 (SOCS3) expression via the same JAK2/STAT3 signaling pathway. SOCS3 targets IL-6 but not the IL-10 receptor. As a result, the STAT3 activity of IL-6-driven is shorten, while that of IL-10-driven is prolonged.[40] Moreover, STAT3 participates in the differentiation of monocyte-to-macrophage. Therefore, the inhibition of the JAK2/STAT3 signaling pathway can conquer this differentiation of monocyte-to-macrophage and accordingly suppress inflammation.[41] Taken together, with the formation of Ox-LDL-induced foam cells, the JAK2/STAT3 signaling pathway was activated by cytokines IL-6 and IL-10. When foam cells were treated with curcumol, IL-6 expression was inhibited and IL-10 expression was promoted, which resulted in the suppression of the JAK2/STAT3 signaling pathway. Therefore, curcumol could impede the differentiation of monocytes into macrophages and further diminish the inflammatory response by downregulating IL-6, upregulating IL-10 and inhibiting the JAK2/STAT3 signaling pathway [Figure 6]. Eventually, the formation of foam cells was abridged and atherosclerosis was amended. Thus, curcumol may serve as a potential drug in the treatment of atherosclerosis.

CONCLUSION

In general, network pharmacology tactic was supportive for target screening and to discover the mechanisms of curcumol on atherosclerosis. Using network pharmacology analysis and experimental validation, our study specified that curcumol gained from the medicinal Chinese herb, *Curcumae Rhizoma* (E-Zhu), could decrease foam cell formation and further inhibit the progression of atherosclerosis. This study provides a basis for forthcoming research on the anti-atherosclerosis activity of curcumol and the screening of drugs for atherosclerosis.

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Conflicts of interest

The authors declare no conflicts of interest.

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