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A network pharmacology approach to investigate the anti-inflammatory mechanism of effective ingredients from *Salvia miltiorrhiza*

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ABSTRACT

Salvia miltiorrhiza, known as Danshen in Chinese, has been widely used to treat cardiovascular diseases in China. Tanshinone I (Tan I) and cryptotanshinone (CST) are the lipid-soluble and effective components from *Salvia miltiorrhiza*. However, the molecular mechanism of Tan I and CST for treating inflammation is still not known. Therefore, this study was designed to use network pharmacology-based strategy to predict therapeutic targets of Tan I and CST against inflammation, and further to investigate the pharmacological molecular mechanism *in vitro*.

Inflammation targets were identified and followed by acquisition of verified targets of Tan I and CST. After constructing target-functional protein interaction network of Tan I and CST against inflammation, the core therapeutic targets of Tan I and CST against inflammation were obtained. Further, pathway enrichment analyses were performed on core therapeutic targets to evaluate key signaling pathways of Tan I and CST against inflammation. As revealed in network pharmacology analysis, 8 key hub targets for Tan I and CST against inflammation were identified, respectively: JUN, VEGFA, IL-6, TNF, MAPK8, CXCL8, and PTGS2 for Tan I, while STAT3, AKT1, CCND1, MAPK14, VEGFA, ESR1, MAPK8 and AR for CST. Pathway enrichment analysis by DAVID database indicated that Tan I and CST principally regulated the inflammation-associated pathway, such as TLR, JAK-STAT signaling pathway, focal adhesion, apoptosis, mTOR signaling pathway. *In vitro*, we found that both Tan I and CST exerts significantly effect on LPS stimulated NO secretion and iNOS expression in macrophages. Taken together, our data elucidate that anti-inflammatory pharmacological activities of Tan I and CST may be predominantly related to inhibition of TLR signaling pathway and regulating iNOS synthesis. These findings highlight the predicted therapeutic targets may be potential targets of Tan I and CST for anti-inflammation treatment.

1. Introduction

Salvia miltiorrhiza, also known as Danshen in Chinese, has been widely used treatment of various diseases including the cardiovascular and immune systems [1,2]. In China, various of dosages forms containing Danshen are applied in the clinical practice. Notably, Danshen dripping pills are worldwidely known for its effects in treating angina pectoris and coronary heart disease [3–6]. *Salvia miltiorrhiza's* bioactive chemical constitutes are classified into two major groups: liposoluble tanshinone compounds and water-soluble salvianolic acids. Tanshinones are the main active ingredients in *S. miltiorrhiza* [7]. Tanshinone I (Tan I), cryptotanshinone (CTS) and tanshinone IIA are major lipid-soluble components. Among them, tanshinone IIA is well studied and exerts a wide range of cardiovascular and other pharmacological effects, including anti-inflammatory, endothelial and myocardial protective, anticoagulation, vasodilation, anti-atherosclerosis and neuroprotective effects [8–10]. However, there is little known of the anti-inflammatory effect of Tan I and CST.

Inflammation is adaptive response to infection and tissue injury which involves the innate and adaptive immune system. It plays an

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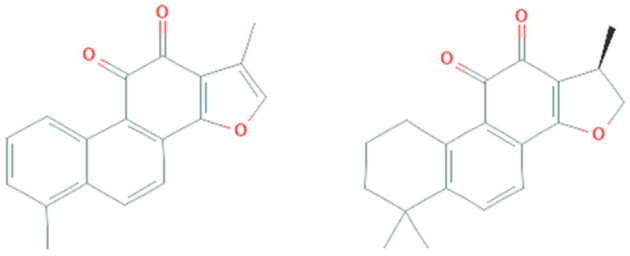

Fig. 1. Structures of Tan I and CTS (Picutures from PubChem).

important role in a variety of diseases including type 2 diabetes, neurodegeneration, cardiovascular diseases, atherosclerosis and cancers [9,11–13]. In the pathogenesis of atherosclerosis, inflammation resulting from activation of innate immunity as well as adaptive immunity plays an critical role in the initiation, progression and destabilization of atherosclerotic plaques [14]. Therefore, effective anti-inflammatory approach could lower the risk and prevent the pathogenesis of atherosclerotic cardiovascular disease. Inflammatory cells and mediators are an integral component of the inflammatory response. Macrophages, in particular, can modulate the immune system by the production of various inflammatory factors and chemokines [15], and are widely used in the research of different inflammatory diseases [16–18].

Although *Salvia miltiorrhiza* are shown to have anti-inflammatory effects in both clinical practice and research [2,19–25], due to its complex compositions, the underlying mechanism still needs further to be investigated.

The network pharmacology is an emerging subject based on the construction of multi-layer networks of disease-phenotype-gene-drug. It contributes to predict new drug targets, to decipher mode of action and to explore new drug [26]. Considering the complexity of the composition and function of traditional Chinese medicine, network pharmacology has undoubtedly provided a shortcut for identifying its target and signaling pathway. SymMap database is one of the applicable database in TCM research, which integrates traditional Chinese medicine with modern medicine at both the phenotypic and molecular levels. The databases can provide pharmaceutical scientists the ability to rank and filter promising results to guide drug discovery [27]. Therefore, the current study aimed to systematically investigate the predicted targets and biological signaling pathways of effective ingredients, Tan I and CTS (Fig. 1), and to further provide a murine inflammatory cell model to confirm the predicted result. The entire design of this study was showed in flowchart proposed (Fig. 2).

2. Materials and methods

2.1. Acquisition of Tan I and CTS anti-inflammation targets

All targets of Tan I and CTS were harvested by use of the Comparative Toxicogenomics Database [28–32] (CTD) (http://ctdbase.org/), GeneCards database (https://www.genecards.org) [33–36], STITCH (http://stitch.embl.de/) [37] and SymMap database (https://www.symmap.org/) [27]. In addition, CTD and GeneCards database was employed in detecting pathological targets for inflammation. Subsequently, Tan I and CTS-associated targets were mapped to pathologic targets of inflammation, and then therapeutic targets of Tan I and CTS against inflammation were obtained.

2.2. Construction of PPI network and topological analysis in Tan I and CTS against inflammation

Further, STRING database [38] was used to collect target and target functional proteins. Protein interactions with a confidence score > 0.7 were selected in designed setting after eliminating duplicates. Resultant data were introduced into Cytoscape (3.7.1) to establish protein–protein interaction (PPI) network of Tan I and CTS against inflammation. Cytoscape was utilized in analyzing topological parameters of mean and maximum degrees of freedom in PPI network of Tan I and CTS against inflammation. The core targets were screened according to the setting of the degree value.

2.3. Hub gene analysis

Hub gene of PPI network of Tan I and CTS against inflammation was calculated by Cytohubba plugin by MCC algorithm [39].

2.4. Pathway enrichment analyses of core targets

All proteins/genes were subjected to pathway enrichment analysis using the DAVID Bioinformatics resources 6.7 database [40], the P

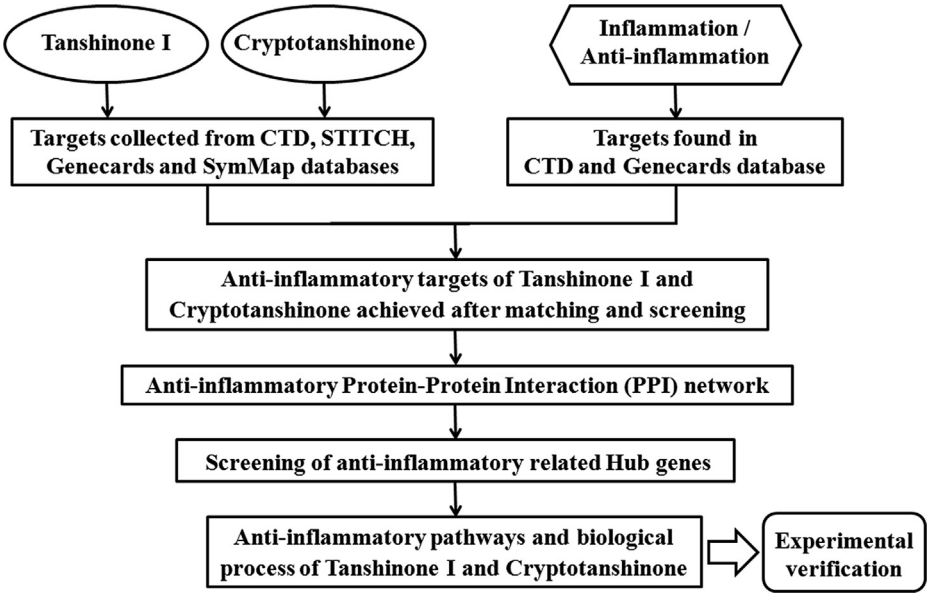

Fig. 2. Flowchart of designed analysis of Tan I and CTS against inflammation.

Table 1
Anti-inflammatory target of Tan I.

No.	Uniprot	Gene	Gene ID	Inference score	Protein
1	P01375	TNF	7124	1075.84	Tumor necrosis factor
2	P01584	IL1B	3553	965.27	Interleukin-1 beta
3	P05231	IL6	3569	937.46	Interleukin-6
4	P35354	PTGS2	5743	843.45	Prostaglandin G/H synthase 2
5	P35228	NOS2	4843	789.39	Nitric oxide synthase, inducible
6	Q04206	RELA	5970	747.44	Transcription factor p65
7	P13500	CCL2	6347	659.88	C-C motif chemokine 2
8	Q07812	BAX	581	650.83	Apoptosis regulator BAX
9	P09601	HMOX1	3162	633.26	Heme oxygenase 1
10	P10145	CXCL8	3576	611.67	Interleukin-8
11	P10415	BCL2	596	604.24	Apoptosis regulator Bcl-2
12	P05362	ICAM1	3383	594.41	Intercellular adhesion molecule 1
13	P31749	AKT1	207	531.46	RAC-alpha serine/threonine-protein kinase
14	P15692	VEGFA	7422	529.95	Vascular endothelial growth factor A
15	P05412	JUN	3725	526.44	Transcription factor AP-1
16	P01100	FOS	2353	524.9	Proto-oncogene c-Fos
17	Q16236	NFE2L2	4780	522.96	Nuclear factor erythroid 2-related factor 2
18	P38936	CDKN1A	1026	496.02	Cyclin-dependent kinase inhibitor 1
19	P19838	NFKB1	4790	488.07	Nuclear factor NF-kappa-B p105 subunit
20	P14780	MMP9	4318	486.48	Matrix metalloproteinase-9

Table 2
Anti-inflammatory target of CTS.

No.	Uniprot	Gene	Gene ID	inference score	Protein
1	P35354	PTGS2	5743	843.45	Prostaglandin G/H synthase 2
2	P35228	NOS2	4843	789.39	Nitric oxide synthase, inducible
3	P22301	IL10	3586	586.56	Interleukin-10
4	P31749	AKT1	207	531.46	RAC-alpha serine/threonine-protein kinase
5	P15692	VEGFA	7422	529.95	Vascular endothelial growth factor A
6	P24385	CCND1	595	470.33	G1/S-specific cyclin-D1
7	P04798	CYP1A1	1543	433.08	Cytochrome P450 1A1
8	P45983	MAPK8	5599	415.95	Mitogen-activated protein kinase 8
9	P29460	IL12B	3593	395.56	Interleukin-12 subunit beta
10	P40763	STAT3	6774	371.82	Signal transducer and activator of transcription 3
11	P05305	EDN1	1906	369.6	Endothelin-1
12	Q07817	BCL2L1	598	363.92	Bcl-2-like protein 1
13	Q16539	MAPK14	1432	328.5	Mitogen-activated protein kinase 14
14	P05177	CYP1A2	1544	317.98	Cytochrome P450 1A2
15	P29459	IL12A	3592	296.43	Interleukin-12 subunit alpha
16	P23219	PTGS1	5742	292.82	Prostaglandin G/H synthase 1
17	P42345	MTOR	2475	282.96	Serine/threonine-protein kinase mTOR
18	P04150	NR3C1	2908	280.34	Glucocorticoid receptor
19	P08183	ABCB1	5243	272.35	Multidrug resistance protein 1
20	P08684	CYP3A4	1576	264.42	Cytochrome P450 3A4

value was tested and corrected by the FDR error control method (FDR $<$ 0.05). The threshold value P $<$ 0.05 was finally set. The main pathways were obtained, and signaling pathways associated with of Tan I and CTS against inflammation mapped using Microsoft EXCEL software.

2.5. Cell culture

Murine macrophage cell line RAW264.7 was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The cells were cultured in DMEM supplemented with 10% FBS at 37 ˚C in a 5%CO_2 atmosphere.

2.6. Drugs and reagents

Tan I and CTS were from Selleck Chemicals (USA). 50 mM and 10 mM stock solution was prepared in DMSO, respectively. Dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS), $NaNO_2$, sulfanilamide, naphthylethylene diamine dihydrochloride and phosphoric acid were from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's Medium (DMEM) and trypsin were purchased from Gibco (USA). The CCK-8 assay kit, protease inhibitor cocktail and phosphatase inhibitor cocktail were from Bimake (Shanghai, China). BCA assay kit was from Beyotime Biotechnology (Shanghai, China). Antibodies (iNOS、GAPDH) derived from mice were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.7. WST-8 assay

WST-8 assays were performed as reported previously [41]. RAW267.4 macrophages were seeded into 96-well cell culture plates at 5×10^5 cells/mL in DMEM containing the indicated concentrations of Tan I or CTS for 24 h and 48 h 10 μL WST-8 per well were added, and the plate was placed on a plate shaker for 1 min to ensure optimal mixing. After incubation for 30 min, the optical density (OD) at 450 nm was determined using a microtiter plate reader (BioRad, Hercules, CA). The mean and standard deviation of three replicates was calculated.

2.8. Nitric oxide assay

The NO concentrations in cell culture supernatants were determined by measuring the accumulation of nitrite (NO_2^-) [42]. Briefly, 100 μL of

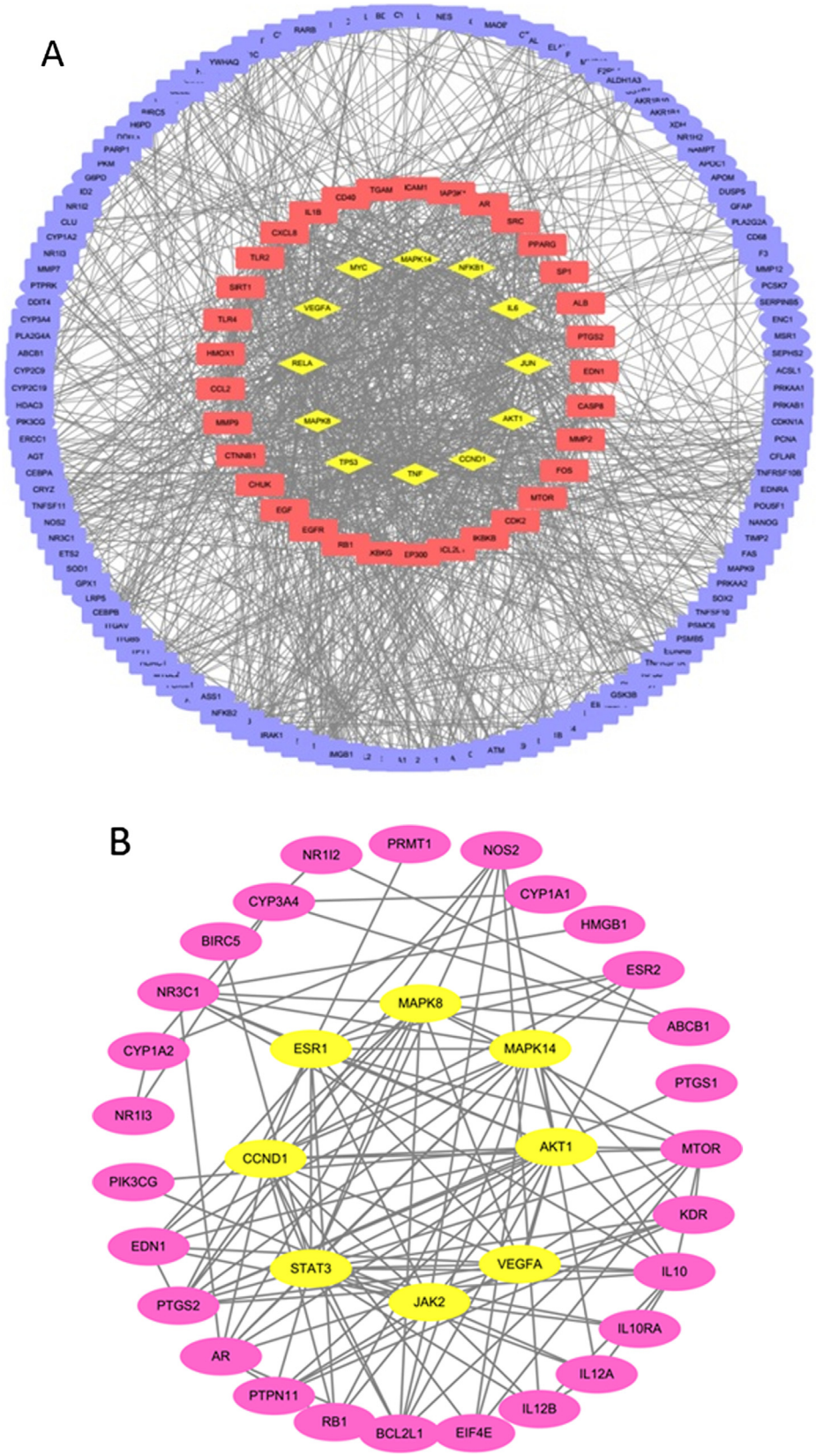

Fig. 3. (A) Anti-inflammatory target network of Tan I, the target gene are sorted according to degree. The degree of the outermost circle is 0–20 (purple), the middle circle 21–40 (red), smallest circle 41–100 (yellow) in which loop network had 104 nodes that were interconnected and associated by 1414 edges. (B) Anti-inflammatory target network of CTS, two circles in total, the degree of the large circle is 0–10, the small circle 11-21in which loop network had 35 nodes that were interconnected and associated by 122 edges.

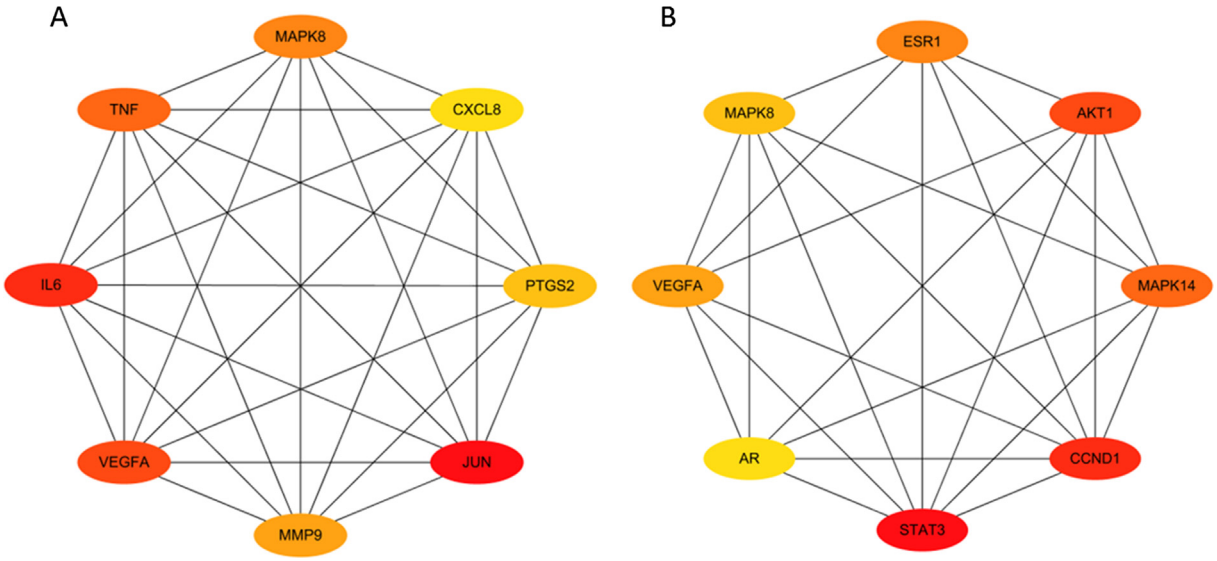

Fig. 4. (A) Analysis of the top 8hub gene network of anti-inflammatory effects of Tan I by MCC algorithm, in which red and yellow color represents the importance in the network; (B) Top 8 hub targets of CTS by MCC algorithm, in which red and yellow color represents the importance in the network.

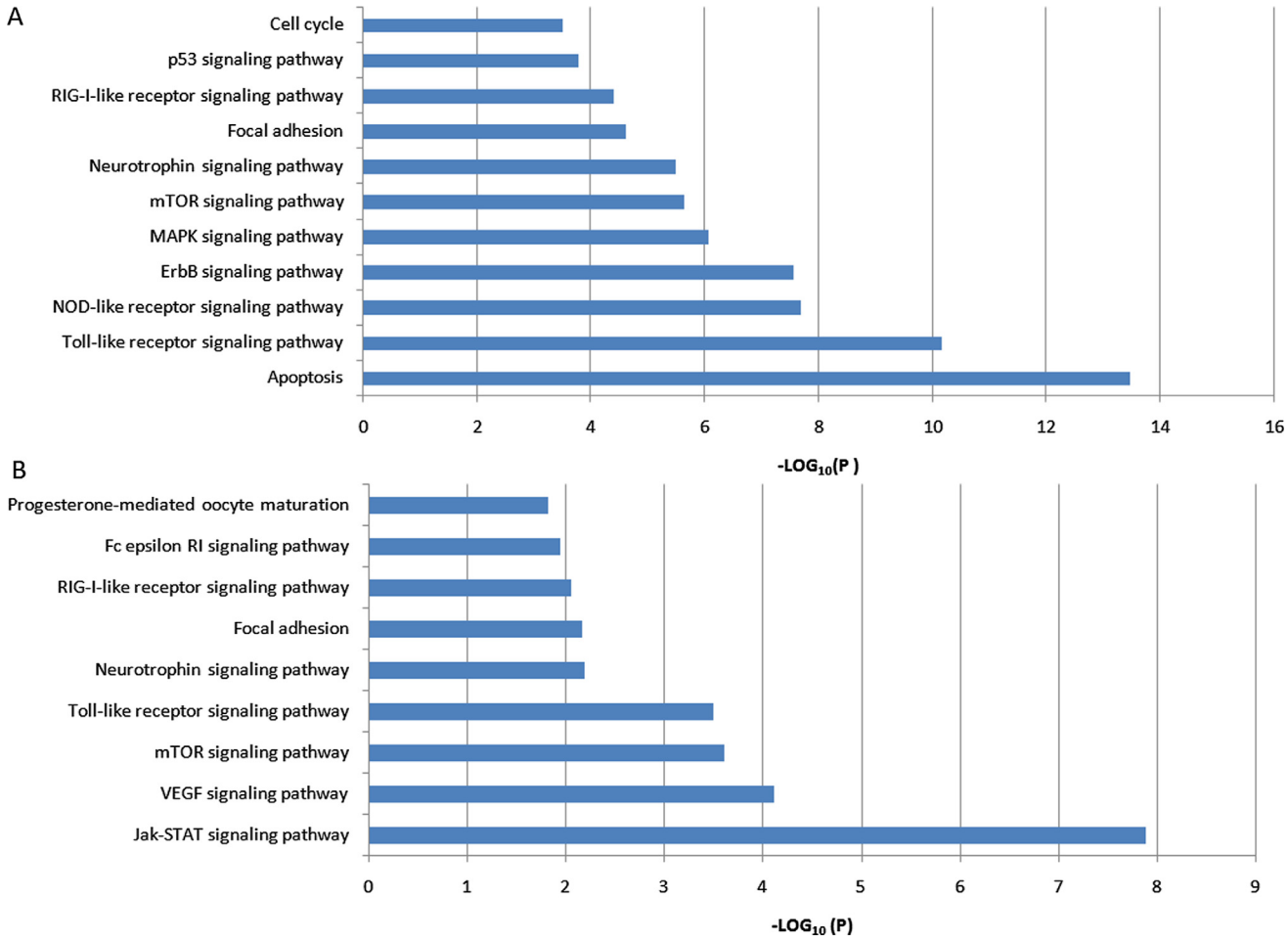

Fig. 5. KEGG pathway analyses from bioinformatics data for the molecular signal pathway of Tan I (A) and CTS (B) against inflammation.

Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diaminedihydrochloride, and 2.5% phosphoric acid) were mixed with an equal volume of culture supernatant in 96-well flat-bottomed microplates and incubated at room temperature for 10 min. The OD at 540 nm was read using a microtiter plate reader. Nitrite concentrations were determined from a standard curve established using serial dilutions of $NaNO_2$.

2.9. Western blotting

Western blotting was performed as reported [41–43]. Cells were

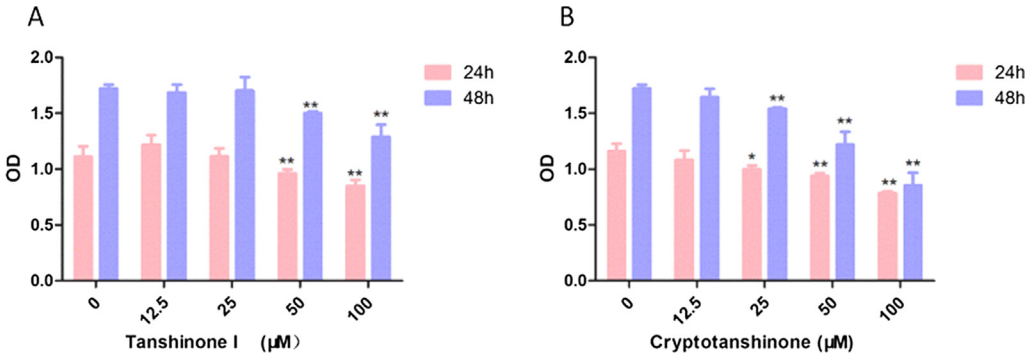

Fig. 6. Effect of Tan I (A) and CTS (B) on the proliferation of macrophage for 24 and 48 h treatment. Data are means of three replicates; * $p < 0.05$, ** $p < 0.01$ compared with the control.

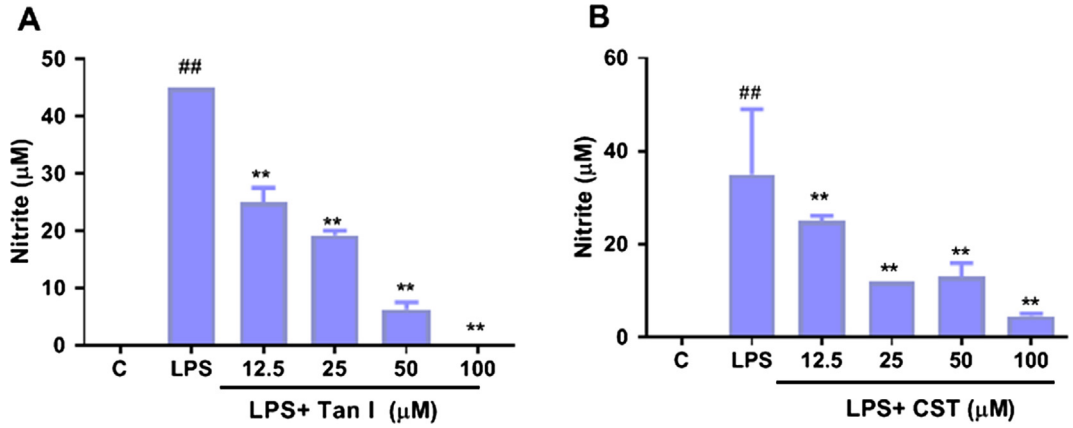

Fig. 7. Effect of Tan I (A) and CTS (B) on the NO release of LPS stimulated macrophage. C: untreated macrophage control; Data are means of three replicates; ##P < 0.01, compared with untreated macrophage control. **P < 0.01, compared with LPS + DMSO control treatment.

pretreated with or without LPS (1 μg/mL), DMSO control and indicated concentration of Tan I or CTS (12.5–100 μM) for 24 h. Cells were washed with ice-cold PBS, scraped and stored at −20 °C. Cell lysates were prepared in ice-cold lysis buffer and clarified by centrifugation. Protein concentrations were quantified by BCA assay. Proteins (20 μg) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were probed with the appropriate primary antibodies (iNOS, GAPDH) (1:1000 dilution) followed by a HRP-conjugated secondary antibody (1:3000 dilution); bands were detected by enhanced chemiluminescence.

2.10. Statistical analysis

All the experimental data are presented as $\bar{\chi} \pm SD$. The *t*-test was used for comparison between the two groups by statistical software SPSS19.0. $P < 0.05$ was considered as significant difference.

3. Results

3.1. Identify the assayed targets

6419 genes related to inflammation were collected from CTD and GeneCards database. 241 and 45 verified targets of Tan I and CTS were screened. Further, 205 and 38 anti-inflammatory targets of Tan I and CTS were selected, respectively. The top 20 target gene of Tan I and CTS against inflammation according to the inference score genes are listed in Tables 1 and 2.

3.2. Anti-inflammatory targets of Tan I and CTS and function-related protein interaction network

The anti-inflammatory targets of Tan I and CTS were then input into String10.0 to predict the interaction of the target and construct protein-protein interaction network (PPI network). Results with confidence score higher than 0.7 was selected and then imported into Cytoscape 3.7.1 to construct an anti-inflammatory protein–protein interaction network (PPI) (Figs. 3 and 4A and B).

3.3. Screening of Hub gene

Based on Cytohubba, the plug-in of Cytoscape, the Hub gene was screened in the interaction network. We used MCC algorithm to find out the top 8 Hub genes of Tan I (Fig. 4A) and CTS (Fig. 4B), and the Hub gene network diagram was constructed. Among them, Tan I contains JUN, VEGFA, IL-6, TNF, MAPK8, CXCL8, and PTGS2 while CTS contains STAT3, AKT1, CCND1, MAPK14, VEGFA, ESR1, MAPK8 and AR. Interesting, VEGFA and MAPK8 are the shared targets of Tan I and CTS.

3.4. KEGG pathway analysis

A total of 205 and 38 candidate targets of anti-inflammation of Tan I and CTS were validated in the Uniprot database (http://www.uniprot.org/)[44]. After inputting these targets to Database for Annotation, Visualization and Integrated Discovery (DAVID) database, Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathways and results were shown in Fig. 5. The molecular signal pathway of Tan I (A) and CTS (B) against inflammation were closely related to inflammation-based pathways, such as regulation of Toll-like receptor signaling pathway, Jak-STAT signaling pathway and apoptosis signaling pathway

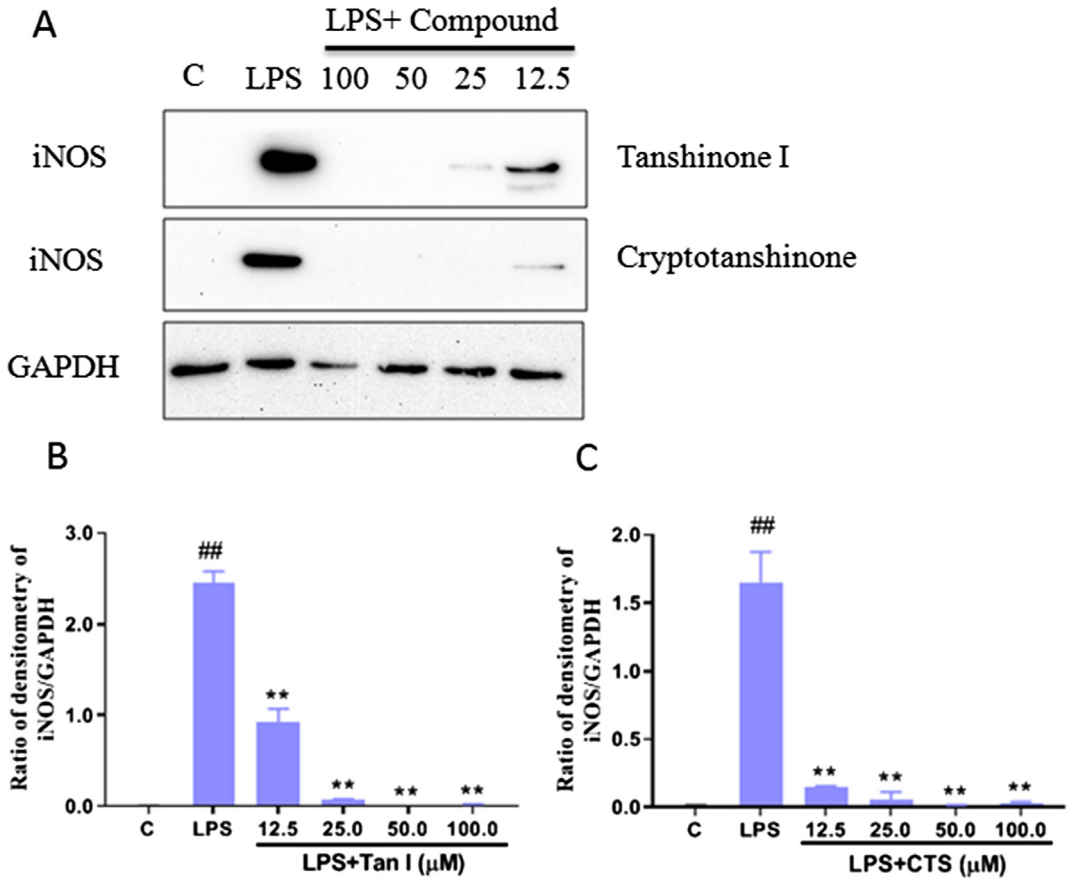

Fig. 8. Effect of Tan I and CTS on the expression of iNOS protein in RAW264.7 stimulated by LPS with Tan I and CTS. C: untreated macrophage control ; Integrated band intensities as determined using Image J software; Data are means of three replicates;## P < 0.01, compared with untreated macrophage control. **P < 0.01, compared with LPS + DMSO control treatment.

etc.

3.5. Effects of Tan I and CTS on the proliferation of RAW264.7

The effects of Tan I and CTS on the proliferation of RAW264.7 after 24 and 48 h treatment was detected by CCK-8. Compared with the DMSO control group, 50 and 100 μM of Tan I obviously inhibits the proliferation of macrophages after 24 and 48 h (Fig. 6) ($p < 0.01$). 25.0–100.0 μM of CTS significantly inhibits the macrophage proliferation after 24 and 48 h ($p < 0.01$). Moreover, the results showed that CTS exerts a stronger effect than Tan I.

3.6. Effects of Tan I and CTS on the NO secretion of RAW264.7 induced by LPS

Effects of Tan I and CTS on the NO secretion of RAW264.7 induced by LPS was detected by Griess reagent. Without LPS stimulation, the macrophages did not produce NO production. After 24 h of LPS treatment, the secretion of NO strongly increased. Both Tan I and CTS (12.5–100.0 μM) significantly inhibited LPS-induced NO secretion of RAW264.7 cells. Notably, this effects is not due to the cell toxicity of Tanshinone and CTS (Fig. 7).

3.7. Effects of Tan I and CTS on the expression of iNOS in LPS-induced macrophage RAW264.7

We further tested the Tan I and CTS on iNOS expression in LPS-induced macrophage. As can be seen from Fig. 8, without LPS stimulation, the macrophages (Control) hardly expressed iNOS. However, iNOS was dramatically expressed after LPS stimulation for 24 h. Moreover, when the cells were given 12.5 μM to 100.0 μM Tan I and CTS, the expression of iNOS in RAW264.7 induced by LPS was significantly inhibited.

4. Discussion

Inflammation is protective response against infection and injury. Recently, increasing evidence has shown the close correlation between inflammatory conditions and a variety of chronic or malignant diseases such as type 2 diabetes, atherosclerosis and cancer [12]. The anti-inflammatory therapy gained more and more attention both from basic researchers and clinical doctors. However, due to the progression of inflammation mediated diseases, it is cautious to use the long term steroidal anti-inflammatory drugs or NSAIDs to protect against inflammation. Therefore, novel therapeutic strategies are urgently needed to be applied.

Both Tan I and CTS are the monomers extracted from the root of Danshen. Previous studies have confirmed the anti-inflammatory mechanisms of *Salvia miltiorrhiza*, due to the complex components, most studies are mainly focus on the Tan IIA, such as modulation of endotoxin response and reduction of inflammatory cytokine excretion, anti-inflammatory and immunomodulatory for atherosclerosis, protection of intestinal injury [45–48]. Only limited studies have investigated the anti-inflammatory activity of Tan I and CTS [49,50], the molecular mechanism by which Tan I and CTS inhibits the macrophage-associated inflammation induced by LPS remains unclear. Therefore, our study was designed to firstly based on network pharmacology approach to predict the anti-inflammatory targets of Tan I and CTS,

As an important immune cell, macrophages participate in the development of various autoimmune, infectious and inflammatory

diseases [51], which can produce inflammatory intermediates such as nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6). Lipopolysaccharide (LPS) is a component of the cell wall of Gram-negative bacteria. It is a well studied endotoxin that induces inflammatory responses in macrophages by initiating the TLR4-NF-kB signaling cascades. LPS has been widely used as stimulus in the inflammatory model [52]. From our study, LPS induced macrophages produce a large amount of NO and induced significant expression of iNOS protein, which was consistent with the results in the literature [42].

In order to screen the key targets of Tan I and CTS against inflammation, network pharmacology was used to identify 241 (Tan I) and 45 (CTS) verified targets and 6419 inflammation-related targets. As results, 205 (Tan I) and 38 (CTS) targets against inflammation were obtained. Furthermore, 8 hub gene were screened, showing JUN, VEGFA, IL-6, TNF, MAPK8, CXCL8, and PTGS2 for Tan I, while STAT3, AKT1, CCND1, MAPK14, VEGFA, ESR1, MAPK8 and AR for CTS. Based on the analyses of Kyoto encyclopedia of genes and genomes (KEGG) pathway in target proteins, the data uncovered that a majority of the enrichment pathways was associated with inflammation such as Toll like receptor pathway, JAK-STAT pathway, focal adhesion pathways. Therefore, anti-inflammation of pharmacological activities in Tan I and CTS may be benefited through regulating apoptosis, focal adhesion and inflammation-related pathways in macrophage cells. Modulation of TNF, IL6, MAPK8, STAT3 and PTGS2 expressions maybe key pharmacological mechanism of Tan I and CTS against inflammation.

As previously report, Tan I selectively inhibited the expression of pro-inflammatory factors in activated microglia and CTS can inhibit NF-κB and MAPK signal pathway on osteoarthritis [8,53]. To verify the anti-inflammatory effect of Tan I and CTS, we further confirmed their effects in LPS induced murine inflammatory model and focus on TLR4-MyD88–NF-κB-iNOS signaling pathway. Our results suggest that both Tan I and CTS exert the anti-inflammatory effects by significantly inhibiting the secretion of NO and the expression of iNOS protein induced by LPS in macrophages. Interestingly, CTS showed a stronger effect compared with Tan I for the iNOS expression. This phenomenon could be explained by our network pharmacological predicted results. CTS not only targets on TLR pathway, but also JAK-STAT pathway. The combined inhibition of this two pathway may lead to a stronger effect on iNOS synthesis.

5. Conclusion

In summary, based on the network pharmacology approach, the mechanism of Tan I and CTS against inflammation was predicted. Both Tan I and CTS exert anti-inflammatory effect. The possible mechanism is due to the inhibition of key gene of JUN, IL-6, TNF, MAPK8, CXCL8, PTGS2, STAT3 and AKT1 and further regulation of inflammation-related pathways such as TLR pathway, JAK-STAT pathway and apoptosis, focal adhesion in macrophage.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.106040.

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