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Study on the Mechanism of the Danggui−Chuanxiong Herb Pair on Treating Thrombus through Network Pharmacology and Zebrafish Models

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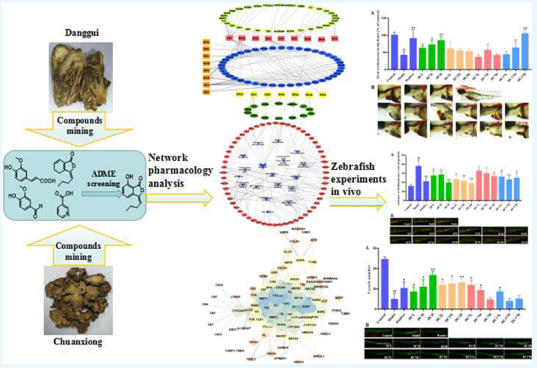
ABSTRACT: Danggui−Chuanxiong (DC) is a commonly used nourishing and activating blood medicine pair in many gynecological prescriptions and modern Chinese medicine. However, its activating blood mechanism has not been clearly elucidated. Our research aimed at investigating the activating blood mechanisms of DC using network pharmacology and zebrafish experiments. Network pharmacology was used to excavate the potential targets and mechanisms of DC in treating thrombus. The antithrombotic, anti-inflammatory, antioxidant, and vasculogenesis activities of DC and the main components of DC, ferulic acid (DC2), ligustilide (DC7), and levistilide A (DC17), were evaluated by zebrafish models in vivo. A total of 24 compounds were selected as the active ingredients with favorable pharmacological parameters for this herb pair. A total of 89 targets and 18 pathways related to the thrombus process were gathered for active compounds. The genes, TNF, CXCR4, IL2, ESR1, FGF2, HIF1A, CXCL8, AR, FOS, MMP2, MMP9, STAT3, and RHOA, might be the main targets for this herb pair to exert cardiovascular activity from the analysis of protein−protein interaction and KEGG pathway results, which were mainly related to inflammation, vasculogenesis, immunity, hormones, and so forth. The zebrafish experiment results showed that DC had antithrombotic, anti-inflammatory, antioxidant, and vasculogenesis activities. The main compounds had different effects of zebrafish activities. Especially, the antithrombotic activity of the DC17H group, anti-inflammatory activities of DCH and DC2H groups, antioxidant activities of DCM, DCH, DC2, DC7, and DC17 groups, and vasculogenesis activities of DCM, DCH, and DC2 groups were stronger than those of the positive group. The integrated method coupled zebrafish models with network pharmacology provided the insights into the mechanisms of DC in treating thrombus.



1. INTRODUCTION

Thrombotic diseases are a group of diseases that can affect tissues and organs throughout the body and seriously endanger human health and life. After thrombosis, blood flow is blocked, or the downstream blood flow is interrupted by the embolism, which will cause ischemia and necrosis of tissues and organs and further induce various serious cardiovascular and cerebrovascular diseases.[1]

Traditional Chinese medicine (TCM) is an effective method to prevent and treat thrombosis due to its synergistic effect and few side effects.[2] Danggui (the radix of *Angelica sinensi* (Oliv.) Diels) and Chuanxiong (the radix of *Ligusticum chuanxiong* Hort.) were both first recorded in the traditional Chinese medicine classics of Shennong Bencao Jing (200−300 A.D., Han Dynasty). Danggui and Chuanxiong have basically the same effect. They are blood-activating drugs and are usually used for the treatment of cardiovascular diseases. Danggui still has a certain effect of nourishing blood in the application.[3] Paired use of these two drugs can enhance the effects of promoting blood circulation, removing blood stasis, and nourishing blood.[4] High-dose extracts of the Danggui−Chuanxiong herb pair (DC, 80 mg/kg) had an antagonistic effect on the formation of thrombus in experimental rats.[5] In addition, DC showed certain anti-platelet aggregation and elimination of blood stasis effects.[6,7]

Network pharmacology has become a novel and effective method to observe the effects of drugs at the organ and organism levels and to explore the pharmacological mechanism of TCMs. Network pharmacology based on database information of genes, proteins, diseases, and drugs systemati-

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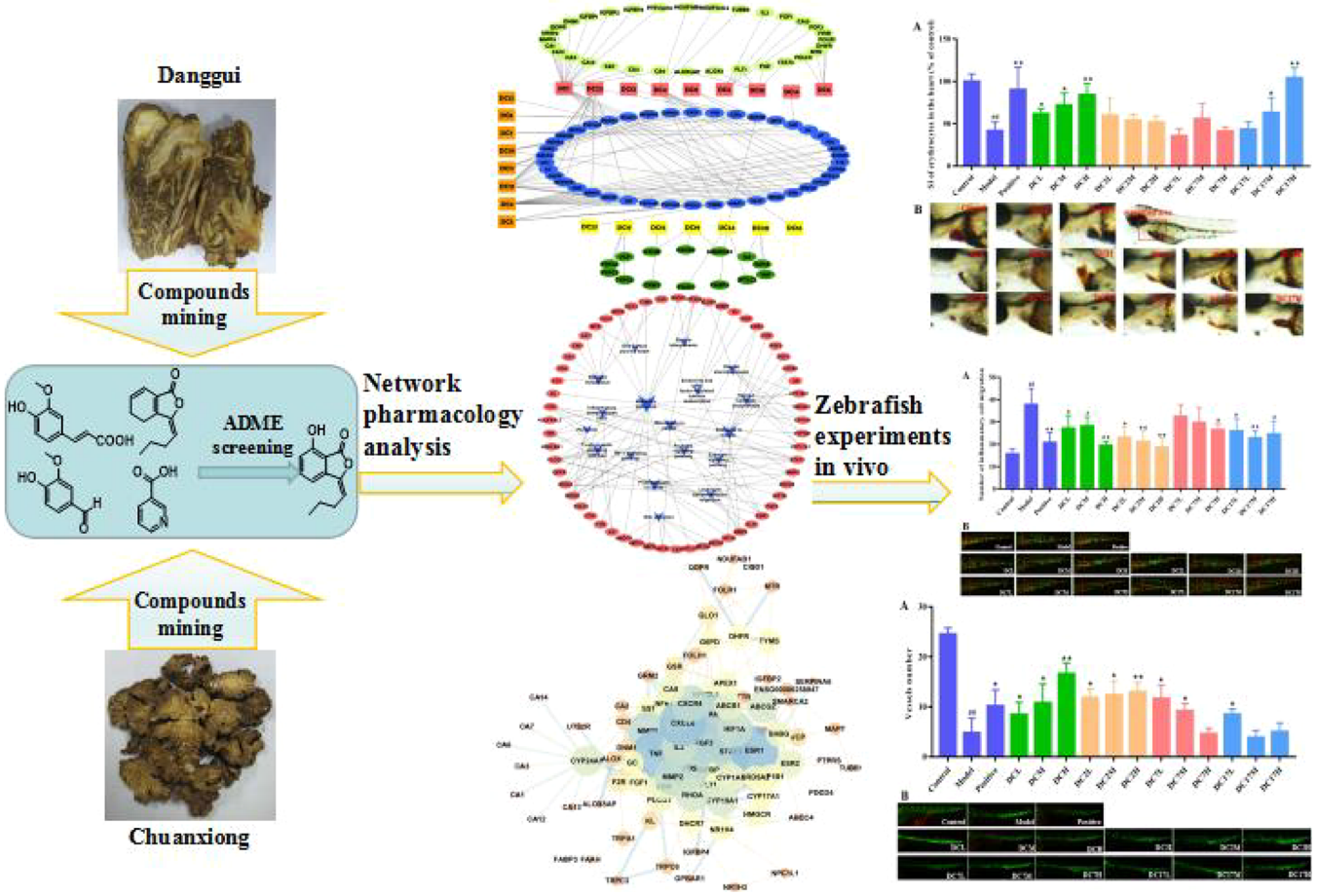

Figure 1. Conceptual framework of this study.

cally observes the intervention and influence of drugs on the disease, revealing the synergy features of multimolecule drugs.[8,9] The mechanisms of the herb pair Danggui−Honghua for treating blood stasis syndrome were predicted by the network pharmacology approach.[10] The effect of Shexiang Baoxin Pill on regulating disease by gene interactions was uncovered by network pharmacology.[11]

Zebrafish is a small tropical freshwater fish that can be used as a vertebrate model for human disease research.[12] Compared with other common in vivo models such as mice, zebrafish has many advantages,[13] such as low cost, short life cycle, easy experimental operation, low demand weight of test compounds, and high-throughput screening.[14] The zebrafish model has a thrombosis process similar to that of mammals.[15] Using zebrafish as a model to study the effects of drugs on thrombosis has certain advantages.

In this study, we used network pharmacology and zebrafish models to analyze the mechanism of DC in the treatment of thrombus (Figure 1). It might provide a research foundation for better application of DC in treating thrombus.

2. RESULTS AND DISCUSSION

2.1. Active Ingredients. The constituents in Danggui and Chuanxiong were gathered from TcmSP and manually replenished through extensive text mining methods. The major components of Danggui and Chuanxiong are both phthalides and organic acids.[16,17] Moreover, Chuanxiong is also rich in alkaloids.[17] A total of 288 (mutual 27) constituents were gathered from Danggui (125) and Chuanxiong (190) (Table S1).

Two key ADME (absorption, distribution, metabolism, and excretion) parameters, oral bioavailability (OB) and druglikeness (DL), were used to screen the active compounds in DC. In addition, some compounds with high content and biological activity were also selected, even though they did not meet the two criteria. The ultrahigh-performance liquid chromatography Q-Exactive HF mass spectrometry (UHPLC-Q-Exactive HF/MS) method was used to confirm that the selected active compound was included in the DC extract (Figure S1). A total of 24 compounds were selected as the active ingredients for this herb pair (Table 1).

The main active ingredients were organic acids (DC1−DC6) and phthalides (DC7−DC18). DC2 is the quality-control component of Danggui and Chuanxiong in the Chinese Pharmacopoeia.[18] It can significantly improve blood flow, inhibit platelet aggregation, and reduce blood lipids to prevent thrombosis.[19] DC17 is the quality-control component of Chuanxiong in the Chinese Pharmacopoeia.[18] DC4, the ester of ferulic acid, is abundant in Danggui and also has vasodilating and antioxidant effects.[20,21] DC1 has antioxidant, anti-inflammatory, and anti-cancer effects.[22] Moreover, DC1 and its ester, DC3, are reported to have antioxidant and cardio-protective properties.[23] Although phthalides have low DL values, they have significant effects on the cardiovascular and cerebrovascular systems.[17] DC7 and DC9 possess anti-platelet aggregation and anti-thrombosis effects and regulate heart function.[24,25] DC12 and DC13 could induce heme oxygenase-

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Table 1. Active Compounds and Their ADME Parameters of DC[a]

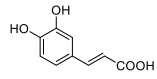
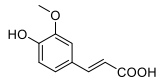
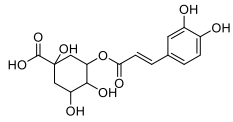
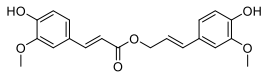
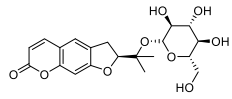
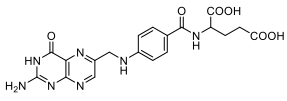
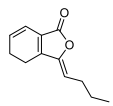
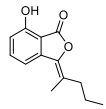
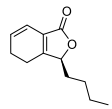
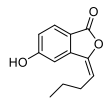
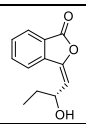
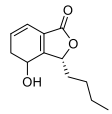
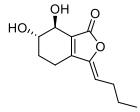
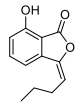
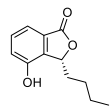
No.	Name	Structure	OB (%)	DL	Herbs
DC1	caffeic acid		54.97	0.19	AS
DC2	ferulic acid		54.97	0.06	LC and AS
DC3	chlorogenic acid		11.93	0.33	LC and AS
DC4	coniferyl ferulate		4.54	0.39	AS
DC5	nodakenin		57.12	0.69	AS
DC6	folic acid		68.96	0.71	AS
DC7	cis-ligustilide		51.3	0.07	LC and AS
DC8	3-butylidene-7-hydroxyphthalide		62.68	0.08	LC and AS
DC9	senkyunolide A		68.28	0.07	AS
DC10	senkyunolide C		46.8	0.08	LC and AS
DC11	senkyunolide E		34.4	0.08	LC and AS
DC12	senkyunolide K		61.75	0.08	LC and AS
DC13	senkyunolide I		46.8	0.08	AS
DC14	(3E)-3-butylidene-7-hydroxy-2-benzofuran-1-one		42.17	0.08	AS
DC15	4-hydroxy-3-butylphthalide		70.31	0.08	LC

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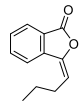
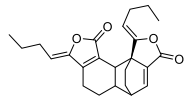
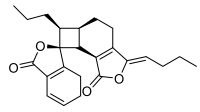
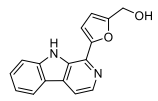
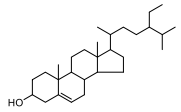
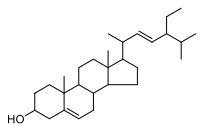
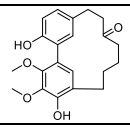
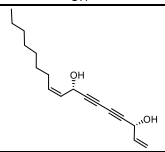
Table 1. continued

No.	Name	Structure	OB (%)	DL	Herbs
DC16	butylidenephthalide		42.44	0.07	LC
DC17	levistolid A		2.15	0.82	LC
DC18	riligustilide		9.83	0.7	AS
DC19	perlolyrine		65.95	0.27	LC
DC20	tetramethylpyrazine		20.01	0.03	LC
DC21	sitosterol		36.91	0.75	LC and AS
DC22	stigmasterol		43.83	0.76	AS
DC23	myricanone		40.60	0.51	LC
DC24	falcarindiol		39.30	0.11	LC

[a]AS stands for *A. sinensis* (Oliv.) Diels; LC stands for *L. chuanxiong* Hort.

1 expression and inhibit smooth muscle cell proliferation.[26,27] DC16 could alleviate cardiac fibrosis in aging rats after myocardial infarction.[28] DC18 can be used as a pharmaceutical composition for treating or preventing cardiovascular diseases.[29] DC20 is considered to be the effective component of Chuanxiong with anti-platelet aggregation, vasodilation, and anti-portal hypertension effects.[30]

2.2. Target Proteins of DC. A total of 89 targets related to the thrombus process for active compounds were gathered from similarity ensemble approach (SEA) and STITCH databases and filtrated with the therapeutic target database (TTD), comparative toxicogenomics database (CTD) and existing literature reports (Table 2). ABCB1, ABCC4, and ABCG2 (ABC transporters) in the heart are related to the outflow of therapeutic and cardiotoxic agents from the heart. They also affect the conduction characteristics of the heart KATP potassium channels (SUR proteins) or contribute to the lysosomal function.[31] ESR1 and ESR2 in the vascular system are relevant to the functional recovery of injured blood vessels.[32] Carbonic anhydrase (CA) expression in the ventricle may be along with ventricular hypertrophy/failure. Elevated CA can be used as a biomarker for early detection of cardiac hypertrophy and heart failure. CA inhibition can improve the function of the heart.[33]

2.3. Network Construction and Mechanism Analysis. *2.3.1. Component−Target Network.* The component−target (C−T) network was constructed using Cytoscape 3.6.1 software to visualize the corresponding relationship between compounds and targets. A total of 130 associations were generated between 24 active ingredients and 89 targets for DC (Figure 2). The average degree of the potential target per active compound from Danggui was 6.65 and that from Chuanxiong was 4.25. The compounds with high interconnections illustrated the multirelationships between compounds and targets in the C−T network. DC1 (degree = 23), DC21 (degree = 18), DC2 (degree = 13), and DC4 (degree = 13) showed higher relevance degrees, which may play important roles in treating thrombus for DC. This is consistent with the fact that phenolic acid compounds are the active ingredients of DC in the literature.[34] As shown in the C−T network (Figure 2), the efficacy of this herb pair was not only concentrated on modulating the crucial targets involved in the thrombosis (F2R, TUBB1, HIF1A, MMP2, and MMP9) but also, more essentially, focused on the regulation of the other proteins

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Table 2. Target Information of DC

no.	target	Uniprot ID	gene name
T1	multidrug resistance protein 1	P13568	ABCB1
T2	multidrug resistance-associated protein 4	O15439	ABCC4
T3	ATP-binding cassette sub-family G member 2	Q9UNQ0	ABCG2
T4	arachidonate 5-lipoxygenase	P09917	ALOX5
T5	5-lipoxygenase-activating protein	P20292	ALOX5AP
T6	DNA-(apurinic or apyrimidinic site) lyase	P27695	APEX1
T7	androgen receptor	P10275	AR
T8	CA 1	P00915	CA1
T9	CA 12	O43570	CA12
T10	CA 13	Q8N1Q1	CA13
T11	CA 14	Q9ULX7	CA14
T12	CA 2	P00918	CA2
T13	CA 3	P07451	CA3
T14	CA 6	P23280	CA6
T15	CA 7	P43166	CA7
T16	CA 9	Q16790	CA9
T17	T-cell surface glycoprotein CD4	P01730	CD4
T18	CDGSH iron−sulfur domain-containing protein 1	Q9NZ45	CISD1
T19	C-X-C chemokine receptor type 4	P61073	CXCR4
T20	steroid 17-α-hydroxylase/17,20 lyase	P05093	CYP17A1
T21	cytochrome P450 19A1	P11511	CYP19A1
T22	cytochrome P450 1A1	P00185	CYP1A1
T23	cytochrome P450 1B1	Q16678	CYP1B1
T24	1,25-dihydroxyvitamin D(3)24-hydroxylase, mitochondrial	Q07973	CYP24A1
T25	7-dehydrocholesterol reductase	Q9UBM7	DHCR7
T26	dihydrofolate reductase	P9WNX1	DHFR
T27	dynamin-1	Q05193	DNM1
T28	estrogen receptor	P03372	ESR1
T29	estrogen receptor β	Q92731	ESR2
T30	proteinase-activated receptor 1	P25116	F2R
T31	fatty acid amide hydrolase 1	O00519	FAAH
T32	fatty acid-binding protein, heart	P05413	FABP3
T33	fibroblast growth factor 1	P05230	FGF1
T34	fibroblast growth factor 2	P09038	FGF2
T35	vascular endothelial growth factor receptor 1	P17948	FLT1
T36	glutamate carboxypeptidase 2	Q04609	FOLH1
T37	folate receptor α	P15328	FOLR1
T38	proto-oncogene c-Fos	P01100	FOS
T39	glucose-6-phosphate 1-dehydrogenase	P11413	G6PD
T40	vitamin D-binding protein	P02774	GC
T41	lactoylglutathione lyase	Q04760	GLO1
T42	G-protein-coupled bile acid receptor 1	Q8TDU6	GPBAR1
T43	metabotropic glutamate receptor 2	Q14416	GRM2
T44	glutathione reductase, mitochondrial	P00390	GSR
T45	hypoxia-inducible factor 1-α	Q16665	HIF1A
T46	3-hydroxy-3-methylglutaryl-coenzyme A reductase	P04035	HMGCR
T47	insulin-like growth factor-binding protein 1	P08833	IGFBP1
T48	insulin-like growth factor-binding protein 2	P18065	IGFBP2
T49	insulin-like growth factor-binding protein 4	P22692	IGFBP4
T50	interleukin-2	P60568	IL2
T51	interleukin-8	P10145	IL8
T52	klotho	Q9UEF7	KL
T53	microtubule-associated protein tau	P10636	MAPT
T54	72 kDa type IV collagenase	P08253	MMP2
T55	matrix metalloproteinase-9	P14780	MMP9
T56	methionine synthase	P13009	MTR
T57	NADH dehydrogenase [ubiquinone] 1 α subcomplex subunit 4-like 2	Q9NRX3	NDUFA4L2
T58	acyl carrier protein, mitochondrial	O14561	NDUFAB1
T59	nuclear factor erythroid 2-related factor 2	Q14494	NFE2L2
T60	nuclear factor NF-kappa-B p105 subunit	P19838	NFKB1
T61	NPC1-like intracellular cholesterol transporter 1	Q71UR5	NPC1L1

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Table 2. continued

no.	target	Uniprot ID	gene name
T62	puromycin-sensitive aminopeptidase	P55786	NPEPPS
T63	oxysterols receptor LXR-α	Q13133	NR1H3
T64	bile acid receptor	Q96RI1	NR1H4
T65	programmed cell death protein 4	Q53EL6	PDCD4
T66	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase γ-1	P19174	PLCG1
T67	PTPsigma-(brain)	Q13332	PTPsigma
T68	dihydropteridine reductase	P09417	QDPR
T69	transforming protein RhoA	P61586	RHOA
T70	corticosteroid-binding globulin	P08185	SERPINA6
T71	sex hormone-binding globulin	P04278	SHBG
T72	NAD-dependent protein deacylase sirtuin-5, mitochondrial	Q9NXA8	SIRT5
T73	probable global transcription activator SNF2L2	P51531	SMARCA2
T74	serine palmitoyltransferase 2	O15270	SPTLC2
T75	3-oxo-5-α-steroid 4-dehydrogenase 2	P31213	SRD5A2
T76	somatostatin	P61278	SST
T77	signal transducer and activator of transcription 3	P40763	STAT3
T78	tumor necrosis factor	P01375	TNF
T79	transient receptor potential cation channel subfamily A member 1	O75762	TRPA1
T80	short transient receptor potential channel 3	Q8NET8	TRPC3
T81	short transient receptor potential channel 6	Q9H1D0	TRPC6
T82	transthyretin	P02766	TTR
T83	tubulin β-1 chain	Q9H4B7	TUBB1
T84	tubulin β-3 chain	Q13509	TUBB3
T85	thymidylate synthase	P0A884	TYMS
T86	tyrosinase	P14679	TYR
T87	urotensin-2 receptor	Q9UKP6	UTS2R
T88	transitional endoplasmic reticulum ATPase	P55072	VCP
T89	vitamin D3 receptor	P11473	VDR

mediating inflammation and vascular systems (IL2, TNF, ESR1, ESR2, MAPT, FGF1, and FGF2).

2.3.2. Target−Pathway Network. The selected target protein information was imported into the database for annotation, visualization, and integrated discovery (DAVID) to get the pathways of the target enrichment. The target−pathway (T−P) network was constructed using Cytoscape 3.6.1 software to visualize the corresponding relationship between targets and pathways (Figure 3). The main pathways in the T−P network included the metabolic pathways (degree = 19), pathways in cancer (degree = 13), proteoglycans in cancer (degree = 10), nitrogen metabolism (degree = 9), microRNAs in cancer (degree = 9), and T cell receptor signaling pathway (degree = 7). In addition to these highly relevant pathways, there were several pathways about hormones, including the steroid hormone biosynthesis, prolactin signaling pathway, ovarian steroidogenesis, estrogen signaling pathway, and steroid biosynthesis. The major targets were NFKB1 (degree = 7), PLCG1 (degree = 7), RHOA (degree = 6), STAT3 (degree = 6), MMP9 (degree = 5), FOS (degree = 5), ESR1 (degree = 4), MMP2 (degree = 4), TNF (degree = 4), and CYP17A1 (degree = 4).

The formation of thrombus is one of the important mechanisms that cause cardiovascular disease (CVD). In various cardiovascular diseases, antithrombotic therapy has played a positive role in reducing the disability and mortality.[35,36] The T−P network was constructed; the leading pathway was metabolic pathways. Hypertension, thrombosis, and other diseases are defined as metabolic syndrome and cause endothelial dysfunction, which in turn accelerates atherosclerosis.[37] In addition, CVD is a major vascular complication of metabolic disease hyperglycemia. The link between hyperglycemia and the CVD focuses on the role of mediators and pathways, containing oxidative stress, glucose metabolic pathway, and nonoxidative glucose pathways.[38] Nitrogen metabolism is another important pathway with a high degree. Nitric oxide (NO) and nitrite are the intermediate metabolites in nitrogen metabolism. Nitrite can be converted into NO under pathological conditions and therefore represents a physiologically relevant NO capacity in blood or tissue.[39] NO produced physiologically has a useful function in the cardiovascular system, including controlling the vascular tone, leukocyte adhesion, and platelet aggregation.[40,41] In addition, NO also has anti-inflammatory and antioxidant effects.[40,42] The T cell receptor signaling pathway is the vital regulated pathway because it controls the oxygen diffusion to the central part of the heart.[43]

2.3.3. Protein−Protein Interaction Network. In order to further explore the importance of the selected targets, the protein−protein interaction (PPI) of target proteins selected above was built from the STRING platform (https://string-db.org/),[44] and the PPI network was obtained through Cytoscape 3.6.1 software. There were 85 nodes and 346 edges in the PPI network, and the more the number of the edge, the stronger the interaction of the protein (Figure 4). ESR1, CXCL8, TNF, AR, FOS, FGF2, CXCR4, MMP2, IL2, HIF1A, MMP9, and STAT3 showed stronger interactions with other factors, which maybe the main targets for DC in treating thrombus. These targets are mainly regulating factors related to inflammation, vasculogenesis, immunity, hormones, and so forth.[45−47]

2.4. Zebrafish Activities of DC In Vivo. From the abovementioned results, the main genes analyzed from the PPI

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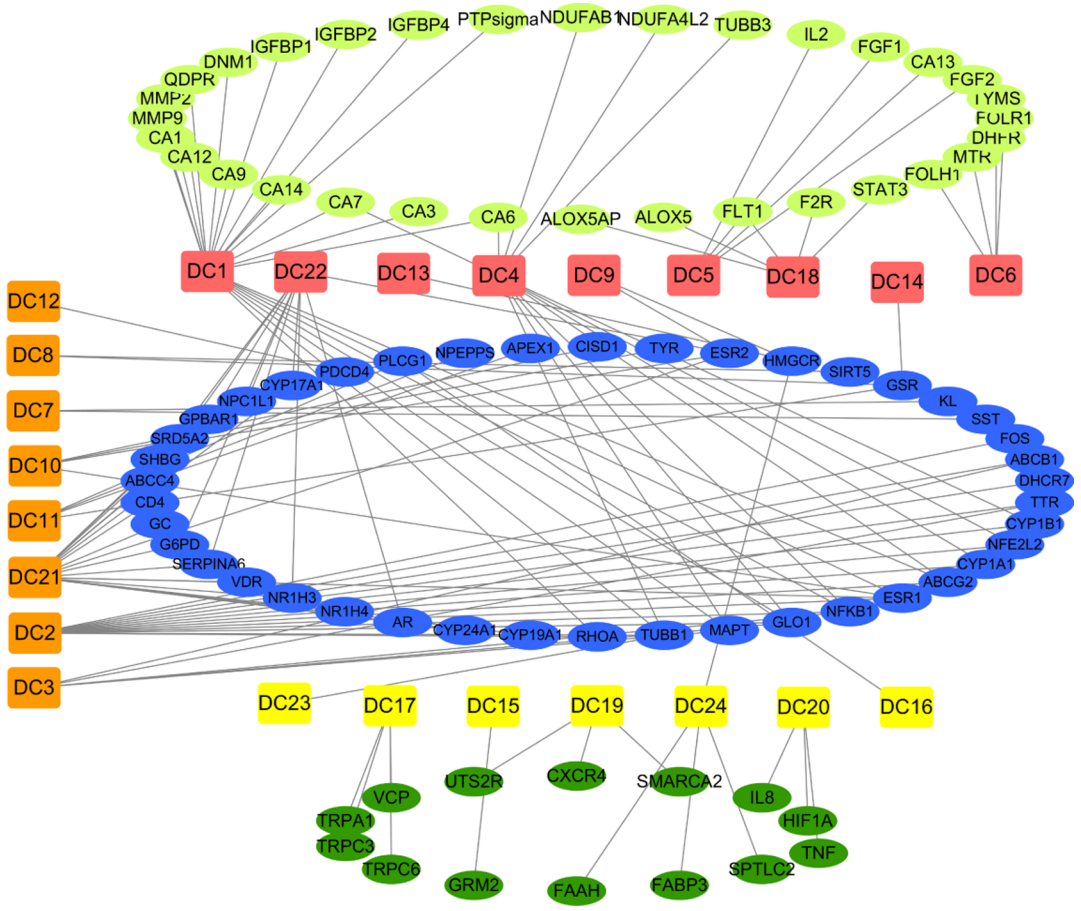

Figure 2. C−T network of DC. The squares are active compounds, and the ellipses represent targets. The red squares are active compounds from Danggui, the yellow ones represent those from Chuanxiong, and the orange ones are those shared. The chemical names of DC1−DC24 can be found in Table 1. The light-green ellipses are targets hit by the compounds of Danggui, the green ones represent those by Chuanxiong, and the blue ones are those shared.

network and KEGG pathway were mainly related to inflammation, vasculogenesis, immunity, hormones, and so forth. The pharmacological experiments on zebrafish models included anti-thrombotic, anti-inflammatory, antioxidant, and vasculogenesis activity experiments. Danggui and Chuanxiong both contain DC2, DC7, and other components. Among them, DC2 is the main component of Danggui and Chuanxiong. It is also an index component for the quality control of both Danggui and Chuanxiong in the 2020 Chinese Pharmacopoeia.[18] DC17 is the quality-control component of Chuanxiong in the Chinese Pharmacopoeia.[18] Therefore, the zebrafish activities of DC2, DC7, and DC17 components were also evaluated.

2.4.1. Antithrombotic Activity. First, the activating blood effect of DC was proven using a zebrafish thrombosis model. Zebrafish larvae have a heart → dorsal aorta and dorsal longitudinal anastomotic → caudal vein → heart blood circulation system. A thrombus formed in the zebrafish caudal vein results in the reduction of heart red blood cells (RBCs).[48] Therefore, the staining intensity of the heart RBCs was used to evaluate the degree of thrombus formation. The model of arachidonic acid (AA)-induced zebrafish thrombosis was used to evaluate the activating blood effects of DC, DC2, DC7, and DC17 in vivo. In the model control group, the stained RBC intensity declined compared with that in the normal control group (circled in the red line, $P < 0.01$) (Figure 5). The intensity of heart RBCs in DC, DC2, and DC17 groups increased compared with that in the model control group. The activities in DC and DC17 groups were concentration-dependent. Especially, the RBC intensity of the DC17H group was more than that of the positive control group. These results suggested that DC and DC17 could prevent the thrombus formation induced by AA. DC17 may be the main pharmacodynamic substance for DC to exert antithrombotic activity.

2.4.2. Anti-inflammatory Activity. Inflammatory cytokines can stimulate and regulate the coagulation system; the two interact and are closely related to thrombosis.[49] $CuSO_4$ as an inorganic salt can induce neuromast damage in the zebrafish lateral line system and can easily cause the characteristics of inflammation, namely, the infiltration of inflammatory cells.[50] In the present study, the anti-inflammatory activities of DC, DC2, DC7, and DC17 were assessed in the zebrafish inflammation model induced by $CuSO_4$. The results showed that the inflammatory cells of zebrafish migrated significantly after $CuSO_4$ treatment, namely, green fluorescent cells above the red horizontal line (Figure 6). All test groups had varying degrees of anti-inflammatory effects. Especially, the effects of DCH and DC2H groups ($P < 0.01$) were stronger than those of the positive control group. These results indicated that DC and DC2 had a much stronger effect of preventing $CuSO_4$-

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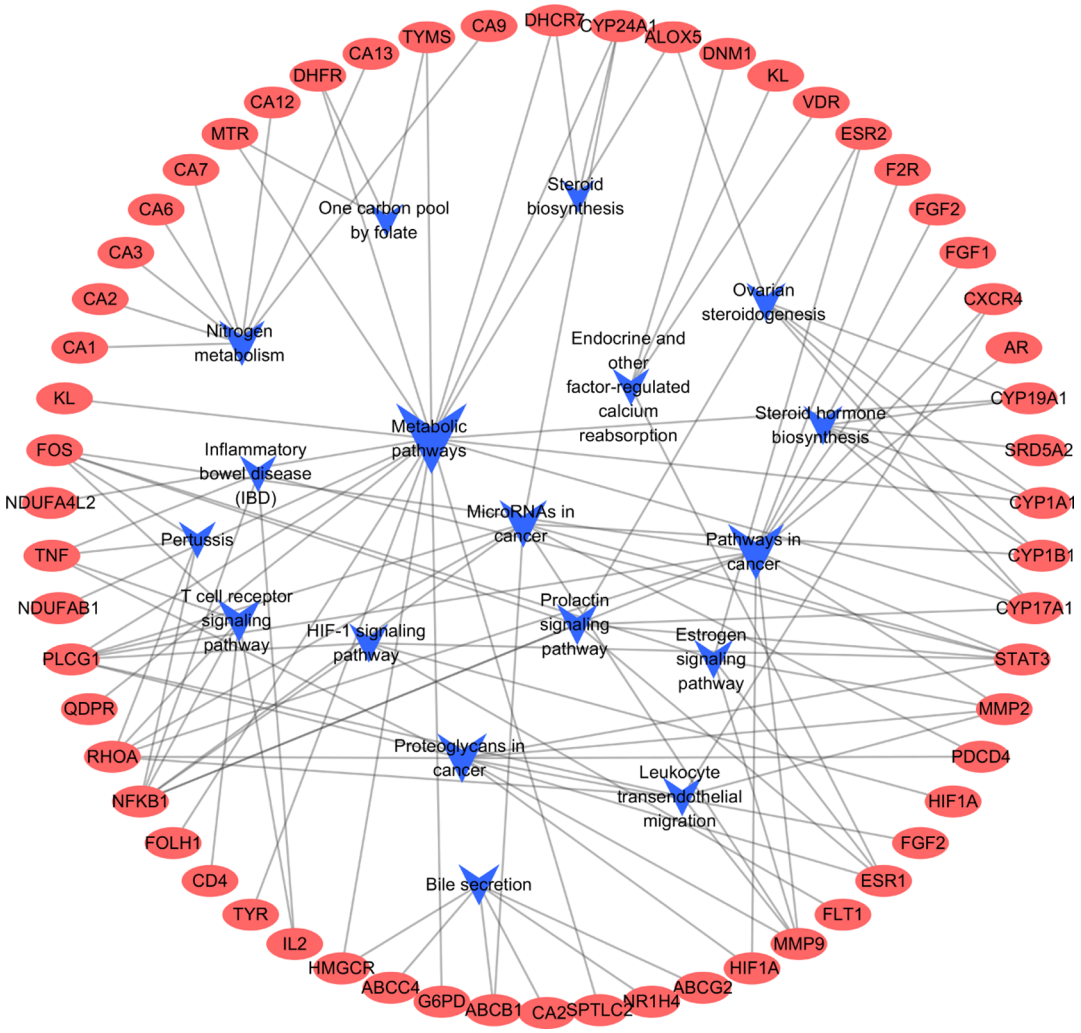

Figure 3. T−P network of DC. The ellipses are targets, and the arrows represent pathways of which the size is proportional to its degree.

induced inflammation. DC2 may be the main pharmacodynamic substance for DC to exert the anti-inflammatory activity.

2.4.3. Antioxidant Activity. In the vascular system, oxidative stress helps in regulating vasoconstriction, platelet aggregation, angiogenesis, and other physiological reactions.[51] Many studies have shown that oxidative stress is involved in the pathological process of thrombotic diseases, such as arterial atherosclerosis, cerebral apoplexy, cardiac muscle infarction, and pulmonary embolism.[52,53] For evaluating antioxidant activities of DC, DC2, DC7, and DC17, metronidazole (MTZ) was used as the modeling drug because it can result in reactive oxygen species overproduction, skin cell apoptosis, and skin fluorescent spot (FS) reduction.[54] The FS amount of the model control group was significantly less than that of the normal control group (Figure 7). The amounts of FS increased slightly when treated with DC, DC2, DC7, and DC17. These results suggested that DC, DC2, DC7, and DC17 could alleviate the oxidative stress response induced by MTZ.

2.4.4. Vasculogenesis Activity. Vascular damage, reduced blood flow, and increased clotting are the main causes of thrombosis.[55] Vatalanib (PTK787) is an inhibitor of angiogenesis.[56] In the vasculogenesis activity experiment, the intersegmental vessel (ISV) number of the model control group lessened obviously after PTK787 treatment, revealing a serious vascular injury (Figure 8). The ISV number of DC and DC2 treatment groups increased compared with that of the model control group. Especially, the ISV numbers of DCH, DC2L, DC2M, and DC2H groups were more than those of the positive control group ($P < 0.05$). The activities in DC and DC2 groups were concentration-dependent. The result indicated that DC and DC2 had preventive effects against PTK787-induced vascular injury. However, the vasculogenesis activity of the DC7 groups declined as the concentration increased. DC17 groups even had the effect of inhibiting angiogenesis. DC7 and DC17 may not be the main pharmacodynamic substances for DC to exert vasculogenesis activity.

The main genes of the DC herb pair analyzed from the network pharmacology were mainly related to inflammation, vasculogenesis, immunity, hormones, and so on. DC displayed anti-inflammatory, antioxidant, and vasculogenesis activities in zebrafish experiments. Additionally, the main compounds of DC, DC2, DC7, and DC17 showed different effects of anti-inflammatory, antioxidant, and vasculogenesis activities. The anti-thrombotic effect of DC should be through the combined effects of different phytochemical components acting on different targets. The zebrafish experiments verified the results of network pharmacology in vivo, and the combination of these two experiments conducted a preliminary exploration of the anti-thrombotic mechanism of DC. Systematic studies are

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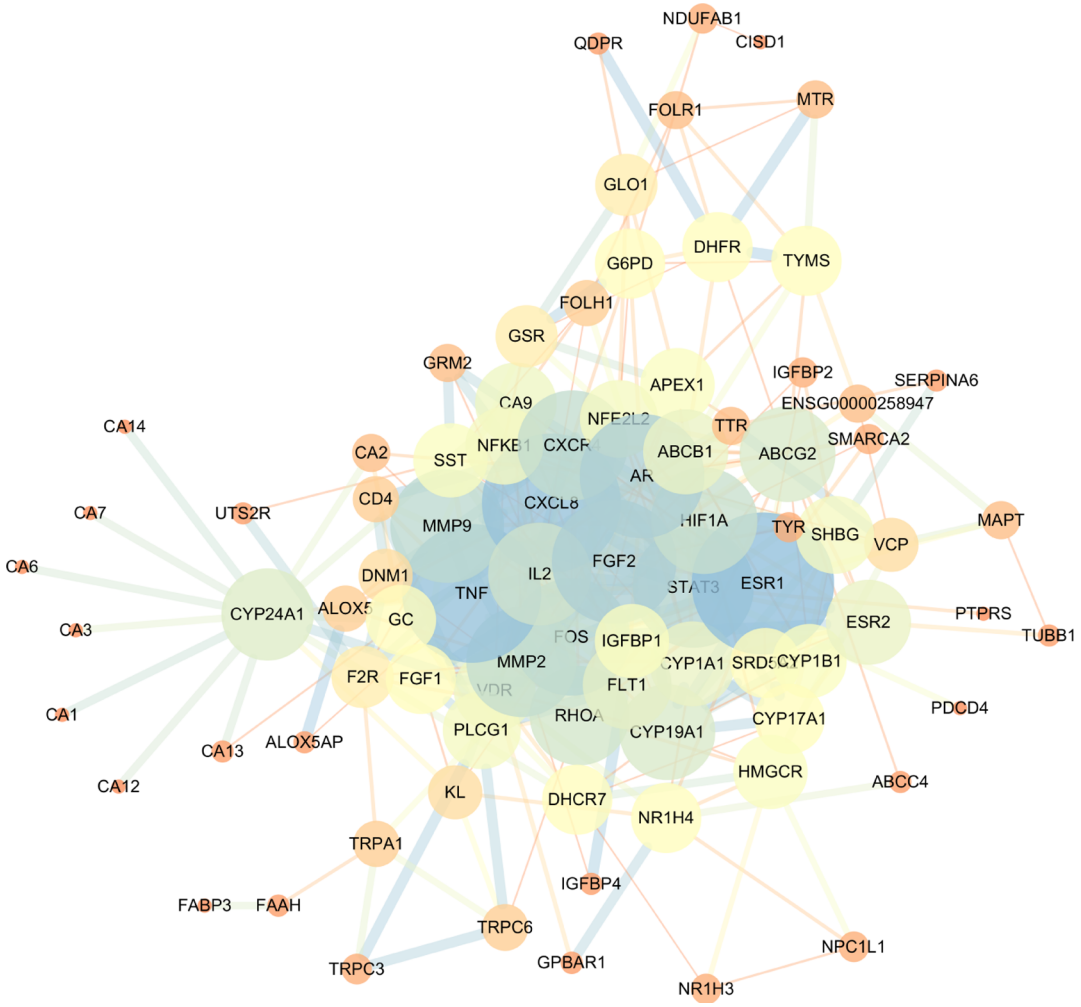

Figure 4. PPI network of intersection targets.

necessary to explore the basis and mechanism of DC responsible for the pharmacological activities, which will be conducted in our further study.

3. CONCLUSIONS

Danggui and Chuanxiong are blood-activating drugs and usually used for treating cardiovascular diseases. Danggui still has a certain effect of nourishing blood. The effects of promoting blood circulation, removing blood stasis, and nourishing blood can be enhanced with paired use of these two drugs. In this study, we used network pharmacology and zebrafish models to analyze the mechanism of DC in the treatment of thrombus. In the results of network pharmacology, 24 active ingredients and 89 related targets were obtained for DC treatment of thrombus. By combining the PPI and KEGG pathway analysis, the main targets for the herb were mainly related to inflammation, vasculogenesis, immunity, hormones, and so forth. In addition, the zebrafish experiment results showed that DC had antithrombotic, anti-inflammatory, antioxidant, and vasculogenesis activities. The main compounds of DC, DC2, DC7, and DC17 had different effects on zebrafish activities. The antithrombotic mechanisms of DC may be through its phytochemical components acting on different targets of anti-inflammatory, antioxidation, vasculogenesis, and so forth. These results offer a support for the medicinal uses of DC.

4. MATERIALS AND METHODS

4.1. Network Pharmacology Analysis. *4.1.1. Chemical Ingredient Database Building and Active Ingredient Screening.* The construction of Danggui (the radix of *A. sinensis* (Oliv.) Diels) and Chuanxiong (the radix of *L. chuanxiong* Hort) chemical composition database is mainly based on the TcmSP (http://tcmspw.com/tcmsp.php),[57] Chinese Academy of Sciences database (www.organchem.csdb.cn), Scifinder (https://www.cas.org/products/scifinder), and China Knowledge Network databases. The compounds were saved using the Drug Bank server (https://www.drugbank.ca/) or Chemdraw software in the mol2 format and SMILES format for later analysis. Meanwhile, important pharmacological parameters of the compound were obtained from TcmSP, including the molecular weight, OB, DL, number of donor atoms for H-bonds (nHDon), and number of acceptor atoms for H-bonds (nHAcc).

The chemical components with physical and chemical parameters OB > 30% and DL ≥ 0.2 were selected as active ingredients. Additionally, chemical constituents with cardiovascular pharmacological activity found in previous literature investigations were also selected as active ingredients for subsequent analysis.

4.1.2. Target Fishing. To predict potential targets of active compounds in DC, compounds in the SMILES format were imported into the databases of SEA (http://sea.bkslab.org/)

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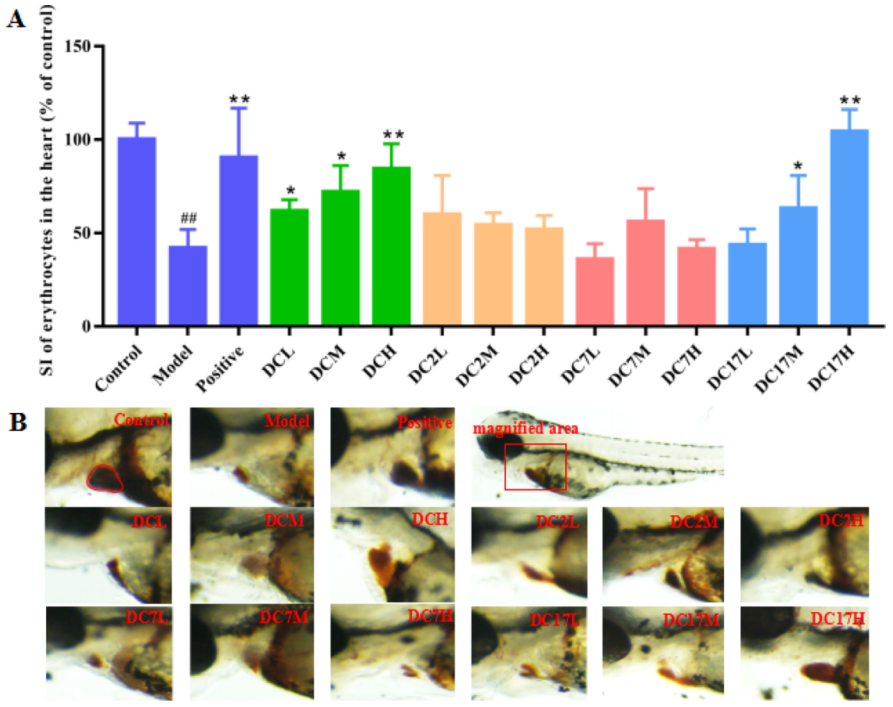

Figure 5. (A) Antithrombotic activity of DC, DC2, DC7, and DC17. (B) Representative pictures of DC, DC2, DC7, and DC17 groups. ##P < 0.01, compared with the normal control group. **P < 0.01; *P < 0.05, compared with the model control group. DCL, DCM, and DCH, administrated with DC of 20, 40, and 80 μg/mL; DC2L, DC2M, and DC2H, treated with DC2 of 1, 5, and 10 μg/mL; DC7L, DC7M, and DC7H, given DC7 of 1, 5, and 10 μg/mL; and DC17L, DC17M, and DC17H, administrated with DC17 of 1, 5, and 10 μg/mL.

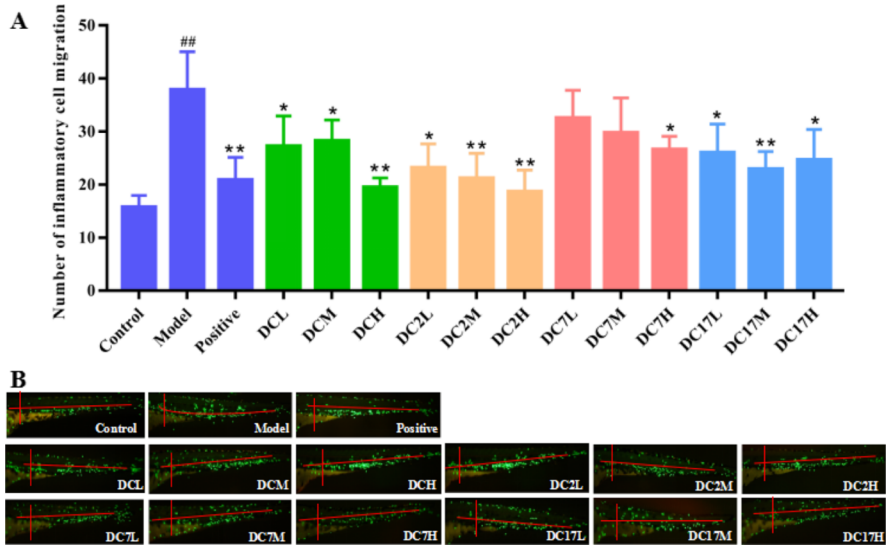

Figure 6. (A) Anti-inflammatory activity of DC, DC2, DC7, and DC17. (B) Representative pictures of DC, DC2, DC7, and DC17 groups. ##P < 0.01, compared with the normal control group. **P < 0.01; *P < 0.05, compared with the model control group. DCL, DCM, and DCH, administrated with DC of 20, 40, and 80 μg/mL; DC2L, DC2M, and DC2H, treated with DC2 of 1, 5, and 10 μg/mL; DC7L, DC7M, and DC7H, given DC7 of 1, 5, and 10 μg/mL; and DC17L, DC17M, and DC17H, administrated with DC17 of 1, 5, and 10 μg/mL.

and STITCH (http://stitch.embl.de/).[58,59] The potential targets were filtrated using the TTD (http://db.idrblab.net/ttd/) and CTD (http://ctdbase.org/) (screening species was "*Homo sapiens*"), combining with the existing literature reports to obtain targets related to the CVD process. The target proteins selected above were used to build a PPI network model on the STRING (https://string-db.org/) platform.[44]

4.1.3. KEGG Pathway Analysis. The selected target protein information was imported into the DAVID (http://david.abcc.ncifcrf.gov) to get the pathways of the target enrichment.[60] OFFICIAL GENE SYMBOL and *Homo sapiens* were selected as the background. A wide range of pathways were excluded using P < 0.05 as the screening condition.

4.1.4. Network Construction. The data of the correlation between the active ingredient and selected target and the selected target and pathway obtained by screening were integrated in the excel tables. Then, C−T and T−P networks of the data were constructed using Cytoscape 3.6.1 software. The PPI of target proteins selected was built from the

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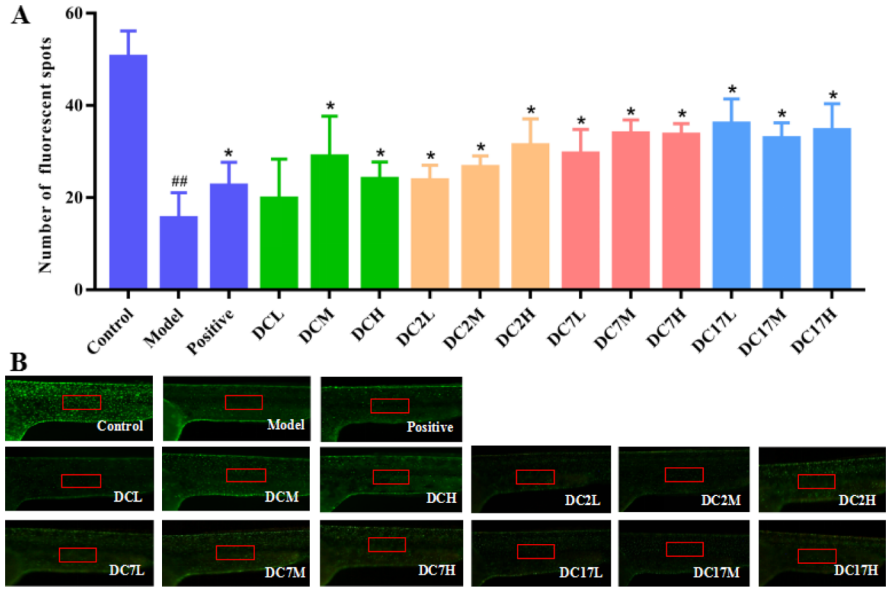

Figure 7. (A) Antioxidant activity of DC, DC2, DC7, and DC17. (B) Representative pictures of DC, DC2, DC7, and DC17 groups. ##$P < 0.01$, compared with the normal control group. *$P < 0.05$, compared with the model control group. DCL, DCM, and DCH, administrated with DC of 20, 40, and 80 μg/mL; DC2L, DC2M, and DC2H, treated with DC2 of 1, 5, and 10 μg/mL; DC7L, DC7M, and DC7H, given DC7 of 1, 5, and 10 μg/mL; and DC17L, DC17M, and DC17H, administrated with DC17 of 1, 5, and 10 μg/mL.

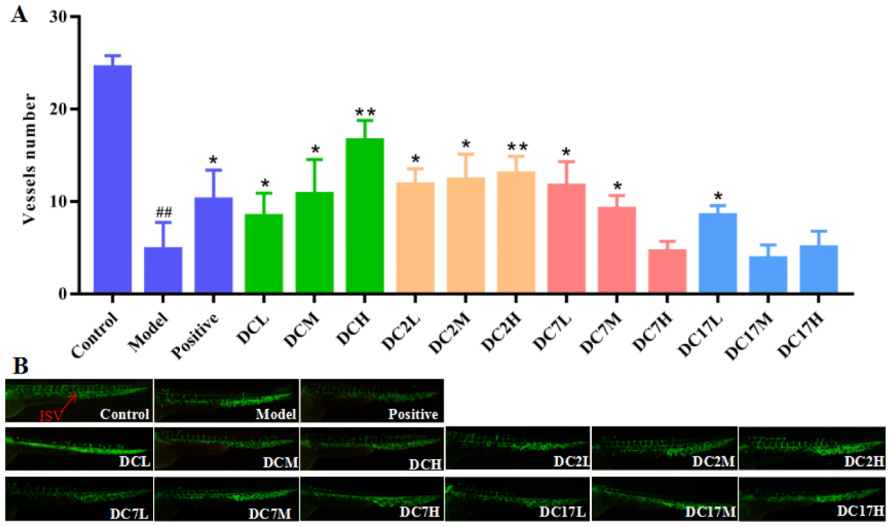

Figure 8. (A) Vasculogenesis activity of DC, DC2, DC7, and DC17. (B) Representative pictures of DC, DC2, DC7, and DC17 groups. ##$P < 0.01$, compared with the normal control group. **$P < 0.01$; *$P < 0.05$, compared with the model control group. DCL, DCM, and DCH, administrated with DC of 20, 40, and 80 μg/mL; DC2L, DC2M, and DC2H, treated with DC2 of 1, 5, and 10 μg/mL; DC7L, DC7M, and DC7H, given DC7 of 1, 5, and 10 μg/mL; and DC17L, DC17M, and DC17H, administrated with DC17 of 1, 5, and 10 μg/mL.

STRING (https://string-db.org/) platform, and the PPI network was obtained through Cytoscape 3.6.1 software.

4.2. Chemicals and Regents. MTZ, *o*-dianisidine, tricaine, Vc, and AA were obtained from Solarbio (Beijing, China); aspirin, copper sulfate, and ibuprofen were from Bioleaf Biotech Co., Ltd. (Shanghai, China); PTK787 was from Abcam (Cambridge, UK); Danhong injection was from Heze Buchang Pharmaceutical Co., Ltd. (Shandong, China); ferulic acid, *cis*-ligustilide, and levistolid A were from Shanghai Jianglai Biochemical Co., Ltd. (Shanghai, China); and dimethyl sulfoxide (DMSO) was supplied by Shanghai Shenggong Co., Ltd. (Shanghai, China). The other chemicals and reagents used in this study were of analytical grade.

4.3. Medicinal Materials and Extraction. Danggui (voucher specimen no. 2020042001) and Chuanxiong (voucher specimen no. 2020042002) medicinal materials were bought from Jinan Jianlian Chinese Medicine Shop and stored at Biology Institute, Qilu University of Technology (Shandong Academy of Sciences), Jinan, China. These two materials were ground into crude powder, mixed in proportion (1:1), and then extracted with distilled water (1:8, w/v) twice under reflux for 2 h. The filtrate was merged and concentrated at 40 °C and then freeze-dried to obtain the extract of DC.

4.4. Animals and Treatment. The zebrafish of AB strains, Tg: Lyz-EGFP, Tg: (krt4:NTR-hKikGR)^{cy17} (supplied by Chung-Der Hsiao from Chung Yuan Christian University), and Tg: fli1-EGFP were used for antithrombotic, anti-

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inflammatory, antioxidant, and vasculogenesis activity experiments, respectively. The zebrafish were kept under a 14 h light/10 h dark cycle at 28.0 ± 0.5 °C. Healthy larvae were selected and then maintained in a light incubator at 28.0 ± 0.5 °C for the activity experiments. The experiments were conducted according to the standard ethical guidelines. The procedures were approved by the Ethics Committee of the Biology Institute of Shandong Academy of Science (no. SWS20200601).

4.4.1. Antithrombotic Activity Experiment. The intensity of RBCs of the heart in the antithrombotic activity experiment was used to assess the activating blood activity capability of DC. A total of 15 groups were contained in the antithrombotic experiment, including the normal control group; positive control group; model control group; and low-, medium-, and high-dosage of DC, DC2, DC7, and DC17 groups (DCL, DCM, and DCH; DC2L, DC2M, and DC2H; DC7L, DC7M, and DC7H; and DC17L, DC17M, and DC17H, respectively). AB strain zebrafish larvae of 3 dpf were randomly assigned to 24-well microplates (10 per well). The normal control group was given DMSO (0.1% v/v), and the positive control group was given aspirin (30 μg/mL). The larvae in the DC groups were administrated with three concentrations of DC of 20, 40, and 80 μg/mL. The larvae in the DC2, DC7, and DC17 groups were treated with three concentrations of DC2, DC7, and DC17 of 1, 5, and 10 μg/mL, respectively. After incubation for 6 h in the light incubator, all groups except the normal control group were treated with AA (80 μM). Incubating for another hour, the zebrafish larvae were stained with 1.0 mg/mL *o*-dianisidine for 10 min. The venous thrombosis of the zebrafish larvae in each group was observed and photographed using an inverted fluorescence microscope (Olympus IX53, Japan). Image Pro Plus 5.1 processing software was used to quantitatively analyze the erythrocytes in the heart.

4.4.2. Anti-inflammatory Activity Experiment. The divided groups and the dosages of DC were the same as those in the antithrombotic activity experiment. Tg (Lyz:EGFP) strain zebrafish larvae of 3 dpf were randomly divided into 24-well microplates (10 per well). The normal control group was given DMSO (0.1% v/v), and the positive control group was given ibuprofen (20 μM). The larvae in the DC groups were treated with three concentrations of DC of 20, 40, and 80 μg/mL. The larvae in the DC2, DC7, and DC17 groups were given three concentrations of DC2, DC7, and DC17 of 1, 5, and 10 μg/mL, respectively. After incubation for 6 h in the light incubator, all groups except the normal control group were administrated with $CuSO_4$ (20 μM). After incubating for another hour, the zebrafish larvae were anesthetized with tricaine (0.16%, w/v) and photographed using the inverted fluorescence microscope. The number of inflammatory cells that migrated above the caudal notochord was counted.

4.4.3. Antioxidant Activity Assay. The divided groups and dosages of DC were both the same as those in the antithrombotic activity experiment. The zebrafish larvae of 1 dpf of Tg (krt4:NTR-hKikGR)^{cy17} were used and randomly divided into 24-well microplates (10 per well). The normal control group was treated with DMSO (0.1% v/v), the model control group was treated with MTZ (5 mM), and the positive control group was treated with MTZ (5 mM) and Vc (0.2 mM). The larvae in the DC groups were administrated with MTZ (5 mM) and three concentrations of DC of 20, 40, and 80 μg/mL. The larvae in the DC2, DC7, and DC17 groups were treated with MTZ (5 mM) and three concentrations of DC2, DC7, and DC17 of 1, 5, and 10 μg/mL, respectively. After incubation for 24 h, we observed the skin FSs using an inverted fluorescence microscope and counted their number using Image Pro Plus 5.1.

4.4.4. Vasculogenesis Activity Experiment. In the vasculogenesis activity experiment, the divided groups were the same as those in the antithrombotic activity experiment. The zebrafish larvae of 24 hpf of Tg (fli1:EGFP) strain were used and randomly divided into 24-well microplates (10 per well). The normal control group was treated with DMSO (0.1% v/v), the model control group was treated with PTK787 (0.225 μg/mL), and the positive control group was treated with PTK787 (0.225 μg/mL) and Danhong injection (9 μL/mL). The larvae in the DC groups were given PTK787 (0.225 μg/mL) and three concentrations of DC of 20, 40, and 80 μg/mL. The larvae in the DC2, DC7, and DC17 groups were administrated with PTK787 (0.225 μg/mL) and three concentrations of DC2, DC7, and DC17 of 1, 5, and 10 μg/mL, respectively. After incubation for 24 h in the light incubator, the zebrafish larvae were imaged using the fluorescence microscope. The number of ISV was counted to assess the angiogenic activity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c01847.

Detailed information of all compounds in DC; total ion chromatograms of the active compounds of DC by ultrahigh-performance liquid chromatography Q-Exactive HF mass spectrometry (UHPLC-Q-Exactive HF/MS) in negative and positive ion modes; and UHPLC-Q-Exactive HF/MS analysis of DC (PDF)

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Author Contributions

M.Z. and P.L. contributed equally to this work. K.L., X.L., P.L., and M.Z. established research ideas and designed methods. M.Z., S.Z., and X.Z. conducted experimental operations; P.L. and L.W. collected data; P.L. and Y.Z. analyzed the data; and M.Z, K.L., and X.L. led the manuscript writing. All authors reviewed the manuscript.

Notes

The authors declare no competing financial interest.

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