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Systemic Pharmacology Reveals the Potential Targets and Signaling Mechanisms in the Adjuvant Treatment of Brucellosis with Traditional Chinese Medicine

Tianyi Zhao,[#] Yu Zhang,[#] Liangbo Liu, Xingmei Deng, Jia Guo, Shuzhu Cao, Dexin Zhu, Jian Xu, Usevich Vera Nikolaevna, Suleimenov Maratbek, Zhen Wang, Zhihua Sun,* Xinli Gu,* and Hui Zhang*

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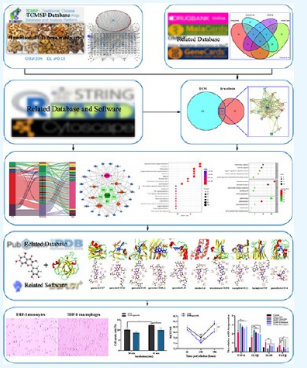
ABSTRACT: Human brucellosis is one of the world's most common zoonoses, caused by *Brucella* infection and characterized by induced inflammation, which in severe cases can lead to abortion and sterility in humans and animals. There is growing evidence that traditional Chinese medicine (TCM) is beneficial as an adjunct to the treatment of brucellosis. However, its specific targets of action and molecular mechanisms remain unclear. In this study, a systematic pharmacological approach was applied to demonstrate pharmacological targets, biological functions, and signaling pathways of TCM as an adjunct to the treatment of brucellosis (TCMTB). The results of network pharmacology were further verified by in vitro experiments. Network analysis revealed that 133 active ingredients and 247 targets were screened in TCMTB. Further data analysis identified 21 core targets and 5 core compounds in TCMTB, including beta-sitosterol, quercetin, kaempferol, luteolin, and paeoniflorin. Gene ontology and the Kyoto Encyclopedia of Gene and Genome analysis showed that TCMTB might actively treat brucellosis by regulating inflammatory response, enhancing immune function, and targeting signaling pathways such as tuberculosis and TNF. Molecular docking results showed that multiple compounds could bind to multiple targets. Further, in vitro experiments confirmed that quercetin, among the active compounds screened, induced the strongest immunomodulatory and pro-inflammatory cytokine production during *Brucella abortus* infection. Further, quercetin induced nitric oxide production, which attenuated the ability of *B. abortus* to internalize THP-1 cells as well as intracellular survival. This study reveals the mechanism by which TCMTB aids in the treatment of brucellosis through a synergistic multicomponent, multipathway, and multitarget action. The contribution of quercetin treatment to *B. abortus* infection was demonstrated for the first time, which may be related to the quercetin-induced production of nitric oxide and immunomodulatory and inflammatory cytokines. These predictions of the core compounds and targets may be used in the future for the clinical treatment of brucellosis.



1. INTRODUCTION

Brucellosis is a zoonotic infection caused by Gram-negative *Brucella abortus* (*B. abortus*). *Brucella*-contaminated animals and products are the main sources of infection.[1] It is estimated that approximately 3.5 billion people worldwide are at risk of infection, with approximately 500,000 new cases of human brucellosis diagnosed each year.[2] The clinical manifestations of brucellosis are joint and muscle pain, fatigue, sweating, fever, and changes in bone and joint inflammation. Lack of timely diagnosis and treatment can lead to high rates of disability.[3] The disease is also a serious constraint on the economic development of the affected areas, such as the medical costs to be paid by the patients and the economic losses from the culling of the affected animals.[4] The lack of an adequate human vaccine and the ineffectiveness of the available animal vaccines have prevented the complete elimination of animal brucellosis globally. Therefore, treatment is crucial in the management of patients with brucellosis.[5]

The World Health Organization recommended the use of doxycycline alone or in combination with rifampicin for the treatment of brucellosis in 1989, and this approach is still recommended today. Rifampicin, isoniazid, oxytetracycline, and streptomycin are currently commonly used in clinical treatment.[6] However, despite the effectiveness of treatment with antibiotics, relapse and treatment failure leading to drug resistance can still occur to some extent. Rifampicin-resistant *B. abortus* has been found in several countries, including Turkey, China, and Egypt.[7,8] At the same time, adverse drug reactions, especially severe toxicity in the liver, kidney, and spleen, to mention a few, may cause more serious and irreversible damage to patients, suggesting an even greater challenge to preventing and controlling brucellosis. Therefore, it is important to discover

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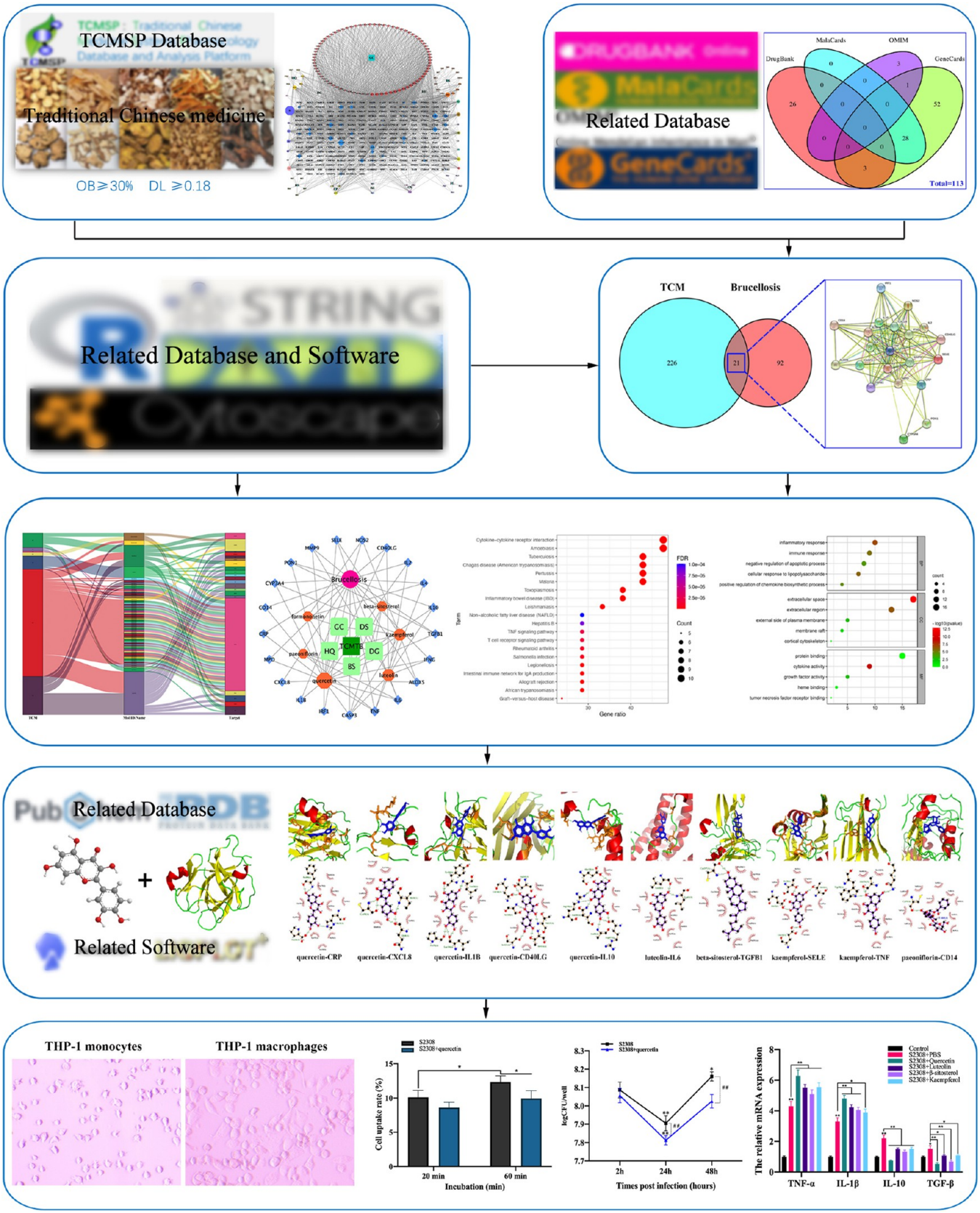

Figure 1. Workflow diagram. The figure indicates the role and mechanism of TCM in the treatment of Brucellosis using bioinformatics and network pharmacology analysis methods shown.

new natural compounds with the potential for treating brucellosis.

In China, the effectiveness of traditional Chinese medicine (TCM) has been demonstrated in the treatment of several

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infectious diseases, such as the therapeutic effect of artemisinin isolated from *Artemisia annua* on malaria and formulated herbal medicines for the prevention and treatment of Severe Acute Respiratory Syndrome and Coronavirus Disease 2019 that have been shown to shorten hospital stays and relieve respiratory distress and discomfort.[9−11] Due to the low adverse effects of TCM and the low susceptibility to drug resistance, several hospitals combine traditional TCM formulations and antibiotics to treat brucellosis with good results.[12,13] Zhang et al. counted the top 10 traditional Chinese medicines used for the treatment of brucellosis (TCMTB) using frequency of use as a reference indicator. In descending order, they are Gan Cao (GC), Dang Gui (DG), Fu Ling (FL), Chen Pi (CP), Bai Shao (BS), Chuan Xiong (CX), Bai Zhu (BZ), Huang Qi (HQ), Dang Shen (DS), and Di Huang (DH).[14] These herbs have anti-inflammatory, antioxidant, antibacterial, antiviral, immunomodulatory, and hepatoprotective properties; in addition, different TCMTB have some different biological activities. For example, GC is anti-arthritic;[15] DG is anti-fibrotic and prevents myocardial infarction;[16] FL is anti-diabetic;[17] CX is anti-atherosclerotic, anti-thrombotic, and anti-cancer;[18] BZ strengthens the spleen;[19] HQ and DS have anti-tissue damage and kidney-protective and lung-protective effects;[20,21] and DH is anti-tumor, anti-diabetic, and anti-osteoporosis.[22] There is a consensus that TCM exert their therapeutic effects through the pharmacological compounds that they contain.[23] Thus, these pharmacological benefits suggest that these TCM contain one or more active ingredients for the treatment of *B. abortus*.

Due to the emergence and development of techniques such as the extraction and identification of the active ingredients of herbal medicines,[24] network pharmacology, and molecular docking,[25] the prediction of the active chemical components of herbal medicines for various diseases with high efficiency and low cost has been made possible.[26] Network pharmacology is an effective approach for the study of pharmacology, a discipline based on the theory of systems biology for the discovery and design of drug molecules, which reveals the mystery of multimolecular drugs for the synergistic treatment of diseases through multicomponent, multitarget, and multipathways.[27] Molecular docking is one of the effective tools for drug discovery and development, which uses computer technology to predict the binding site and binding affinity from the interaction data, such as spatial and energy matching data, between the receptor and the drug molecule acting as a ligand.[28]

The aim of this study is to identify the core pharmacodynamic component targets and mechanism of action of TCMTB for the treatment of brucellosis through data mining and analysis techniques such as systematic network pharmacology, gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and in vitro experimental validation, so as to provide a theoretical basis for clinical TCM treatment of brucellosis and new drug development. The workflow is shown in Figure 1.

2. MATERIALS AND METHODS

2.1. TCMTB Active Ingredient Database Establishment. TCM Database and Analysis Platform (TCMSP, https://tcmsp-e.com) is an important platform for studying the systemic pharmacology of TCM, capturing information on the medicinal chemicals contained in TCM, and the targets corresponding to these chemicals. In addition, TCMSP shows pharmacokinetic properties of the medicinal chemicals such as water solubility, drug similarity, blood−brain barrier permeability, oral bioavailability, and intestinal epithelial permeability. In this study, 10 TCMTB were searched separately from TCMSP. Oral bioavailability ≥30% and drug similarity ≥0.18 were used as screening conditions to establish a herbal medicine−compound−target database.

2.2. Clustering of TCMTB and Brucellosis-Related Target Genes. Through the UniProt database (https://www.uniprot.org/),[29] we obtained the validated target names and corresponding standard symbols for 20,375 human proteins. The compounds corresponding to TCMs in the TCMSP database and the associated target names were de-duplicated and clustered to some extent. The protein names were then compared with those in the UniProt database to obtain the standard symbols of all compounds corresponding to the target proteins in the TCMTB.

Brucellosis-related target genes were collected from four databases, namely, DrugBank (https://go.drugbank.com/), GeneCard (https://www.genecards.org/), MalaCards (the human disease database, https://www.malacards.org/), and OMIM (Online Mendelian Inheritance in Man, https://omim.org/).[30] Potential target genes (i.e., overlapping target genes) for TCMTB treatment of brucellosis were obtained by crossover after installation of the VennDiagram package in the R software.

2.3. Protein−Protein Interaction (PPI) Network Map of the TCMTB−Brucellosis−Potential Target Genes. PPI network functional enrichment analysis of target genes common to TCMTB and *Brucella* was performed using the STRING database (https://cn.string-db.org/). PPI was used to predict co-expression, fusion, neighborhood, and co-localization of potential target genes for gene interactions.[31] The analysis results were obtained by entering the name of the target gene through the STRING database and selecting "*Homo sapiens*" for the organism. Each node represented a protein in the PPI network graph, and each edge represented a functional association between potential target genes.

2.4. Construction of TCMTB−Compound−Target Network and TCMTB−Brucellosis−Potential Target Gene Network. The 10 closely related TCMTB, alongside information on the compounds and targets that they contain, were analyzed by Cytoscape software (version 3.7.1) to obtain a clear visualization network reflecting the complex relationships between them.[32] Each node in the Cytoscape software represented the name of the herbal medicine, compound, and target gene, and the lines indicated molecular interactions. Key compounds and target genes were screened by analyzing the network topology parameters.

The compounds and TCM corresponding to the intersection of TCMTB and brucellosis targets were collated. The Sankey diagrams were displayed by installing the "ggplot2" and "ggalluvial" packages using R software to obtain a visual network reflecting their complex relationships. The network was analyzed to screen for key compounds and target genes. Further, the screened TCMTB−brucellosis core compounds and target genes were demonstrated by the Cytoscape software.

2.5. GO and KEGG Pathway Enrichment Analysis. Using the Database for Annotation, Visualization, and Integrated Discovery website (https://david.ncifcrf.gov/), we entered the TCMTB and brucellosis-common target, selected the "*Homo sapiens*" organism, and then submitted the analysis to obtain the KEGG pathway and GO function data. The KEGG pathway enrichment analysis was visualized using the R package "ggplot2". The GO Biology Functional Heat Map was plotted by http://www.bioinformatics.com.cn, a free online platform for data analysis and visualization.

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2.6. Determination of Binding Capacity between Active Ingredients and Key Target Genes by Molecular Docking. The three-dimensional structures of the target targets were downloaded separately from the Protein Data Bank (https://www.rcsb.org/), and the chemical structures of the compounds were downloaded separately from PubChem (https://Pubchem.ncbi.nlm.nih.gov/). Using ChemBio3D Ultra7.0[33] and Autodock Tool 1.5.6,[34] the structures of compounds and target proteins were optimized by performing energy minimization, removal of water molecules, and incorporation of non-polar hydrogen. Blind docking was subsequently performed using the Autodock Tool 1.5.6 software, and 10 predictions were recorded. The docking model with the lowest binding energy score was selected and visualized using PyMOL 1.7[35] to clarify the binding sites and interactions between the key targets and compounds.

2.7. In Vitro Experiments to Further Define the Regulatory Role between Core Active Ingredients and Key Target Genes. *2.7.1. Bacterial Strains and Cells Culture.* The *B. abortus* strain 2308 (S2308) was obtained from the Center for Chinese Disease Prevention and Control (Beijing, China). S2308 were cultured in tryptic soy agar or tryptic soy broth (Difco, MI, USA) at 37 °C in 5% CO_2. All analyses of the live S2308 were conducted in a biosafety level 3 laboratory.

THP-1 cell lines were obtained from the Cell Resource Center, IBMS, CAMS/PUMC, Beijing, China, and cultured in RPMI-1640 medium (Hyclone) containing 10% fetal bovine serum (Gibco Life Technologies, Rockville, MD, USA) at 37 °C with 5% CO_2 (v/v).

2.7.2. Cell Viability Assay. THP-1 monocytes (1×10^5/well) were differentiated into macrophages with 200 nM PMA (Beyotime, Shanghai, China) for 48 h. Thereafter, THP-1 cells were exposed to various concentrations (25, 50, 75, 100, 125, and 150 μM) of quercetin (Solarbio, #Q8010) in RPMI-1640 medium (Hyclone) containing 10% fetal bovine serum (Gibco). The medium was replaced with a fresh one after culturing for 48 h. CCK-8 (10 μL/well) was then added to the culture medium followed by incubation for 2 h. An automated microplate reader (Tecan Sunrise, CH) was subsequently used to read the optical density at 450 nm. In addition, the cell viability of luteolin (100 μM, Solarbio, #SL8300), β-sitosterol (100 μM, Solarbio, #SS8580), and kaempferol (100 μM, Solarbio, #SK8030) was assayed according to the same method.

2.7.3. Bactericidal Assay. *B. abortus* (5×10^4 CFU/well) were incubated with different concentrations of quercetin (0, 50, 100, and 200 μM) in 24-well plates with PBS for 0, 12, 24, and 48 h. Samples were serially diluted with PBS and coated with *Brucella* agar, and the direct effect of quercetin on *Brucella* growth was determined by calculating bacterial survival (percentage of treated group relative to control samples), and the survival of control samples was set at 100%.

2.7.4. Infection Assay. THP-1 monocytes were treated with 200 nM PMA for 48 h to differentiate into macrophages for subsequent experiments. To determine the uptake efficiency of S2308, differentiated macrophages were pretreated with quercetin for 4 h before infection with strain S2308 (MOI = 100) and then incubated at 37 °C with 5% CO_2 for 20 and 60 min. After washing twice with PBS, fresh medium containing 50 mg/mL gentamicin was added. Incubation was continued for 30 min, and then the sample was lysed with triton. To determine the growth efficiency of S2308 within the differentiated macrophages and the expression of associated inflammatory factors, the S2308 strain was infected with macrophages as described above, but quercetin was added at a stage defined as time zero and incubation was continued for 0, 24, and 48 h. Cells were cultured as in the invasion assay, but cells needed to be infected for 1 h and then treated with gentamicin for 30 min to kill extracellular bacteria before adding fresh medium containing quercetin (100 μM) for 2, 24, and 48 h incubation. Cells collected from each group were divided into two portions. One portion was used to lyse the cells with triton, serially diluted and plated onto *Brucella* agar to determine the number of surviving bacteria by counting CFUs. The other copy was used to extract RNA and protein and to determine the expression of relevant inflammatory factors.

2.7.5. NO and Nitrite Detection. The method of collecting culture supernatants at 2, 24, and 48 h after *B. abortus* infection is similar to the method used in the infection assay section to detect the growth efficiency of *Brucella* within differentiated macrophages. Nitric oxide (NO) production was assessed by measuring nitrite accumulation through the Griess reaction using an NO Content Assay Kit (Applygen Corp, E1030, China) according to the manufacturer's instructions.

2.7.6. RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction. Total cellular RNA was isolated from THP1 macrophages using a TRIzol reagent (Takara, Japan), and the first strand of cDNA was synthesized using a PrimeScript RT kit (Takara, Japan). SYBR Green Master Mix (Promega, Beijing, China) was used to conduct a real-time quantitative polymerase chain reaction (qRT-PCR) in a 10 μL reaction system. All experiments were performed in triplicate. The relative expression levels of mRNAs were normalized to those of the internal control GAPDH using the $2^{-\Delta\Delta Ct}$ cycle threshold method. All the related gene sequences are listed in Table 1.

Table 1. Nucleotide Sequences of the Primer Pairs Used for Quantitative Gene Expression

gene name	forward and reverse primer (5′ to 3′)	amplicon length
TNF-α-F	CCTCTCTCTAATCAGCCCTCTG	220
TNF-α-R	GAGGACCTGGGAGTAGATGAG	
IL-1β-F	ATGATGGCTTATTACAGTGGCAA	132
IL-1β-R	GTCGGAGATTCGTAGCTGGA	
TGF-β-F	CAATTCCTGGCGATACCTCAG	86
TGF-β-R	GCACAACTCCGGTGACATCAA	
IL-10-F	TCAAGGCGCATGTGAACTCC	176
IL-10-R	GATGTCAAACTCACTCATGGCT	
GAPDH-F	ACAACTTTGGTATCGTGGAAGG	101
GAPDH-R	GCCATCACGCCACAGTTTC	

2.7.7. Western Blotting. Total protein was extracted from the cells by using RIPA lysis buffer (Solarbio Science & Technology, #R0010, China) containing 1% PMSF, and the protein concentration was determined using the BCA kit (Thermo Fisher Scientific). Thereafter, 20 μg of protein separated by 12% SDS-PAGE was transferred to the PVDF membrane (Millipore, MA, USA) by a wet transfer system. The membranes were closed overnight at room temperature in 5% (w/v) skim milk in TBS-Tween 20 buffer. The following primary antibody rooms were used to incubate for 2 h:anti-TNF-α (60291-1-Ig, 1:1000, Proteintech, USA), anti-IL-1β (16806-1-AP, 1:1000, Proteintech, USA), anti-TGF-β (21898-1-AP, 1:1000, Proteintech, USA), anti-IL-10 (60269-1-Ig, 1:1000, Proteintech, USA), and anti-GAPDH (60004-1-Ig, 1:1000, Proteintech, USA). The membrane was washed with TBS-Tween 20 and incubated with HRP-labeled secondary antibody for 1.5 h at room temperature.

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Image Lab 3.0 software (Bio-Rad, CA, USA) was used to image the protein bands. Image J software was used to analyze the relative expression of each protein, and GAPDH was used as the control and normalized treatment.

2.7.8. Statistical Analysis. The statistical analysis to determine the significance of differences between parameters was performed with GraphPad Prism 8 software using a one-tailed *t*-test. Data are presented as the mean values ± standard deviation; $p < 0.05$ was considered to indicate statistical significance. Each treatment was repeated at least three times.

3. RESULTS

3.1. Main Chemical Compounds and Protein Targets of TCMTB. Ten TCMTB were searched through the TCMSP database. The active compounds and their corresponding protein targets were screened using oral bioavailability ≥30% and drug similarity ≥0.18 as the screening conditions (Table 2).

Table 2. Constitution and Protein Targets of TCMTB

Chinese names	Latin names	no. of total ingredients	no. of ADME filtration	no. of protein targets
Gan Cao (GC)	Radix Glycyrrhizae	280	92	215
Dang Gui (DG)	Radix Angelicae Sinensis	125	2	52
Fu Ling (FL)	Indigo Naturalis	34	15	22
Chen Pi (CP)	Citri Reticulatae Pericarpium	63	5	64
Bai Shao (BS)	Radix Paeoniae Alba	85	13	76
Chuan Xiong(CX)	Rhizoma Chuanxiong	189	7	30
Bai Zhu (BZ)	Rhizoma Atractylodis Macrocephatae	55	7	20
Huang Qi (HQ)	Radix Astragali	87	20	193
Dang Shen (DS)	Radix Codonopsis Pilosulae	134	21	105
Di Huang (DH)	Radix Rehmanniae	76	2	29

The results showed that after the screening, GC contained 92 active ingredients and 215 protein targets followed by DS, which possessed 21 active compounds and 105 protein targets. Although DG and DH had the lowest number of active ingredients, two each, they possessed a very large number of targets, 52 and 29, respectively.

3.2. TCMTB−Compound−Target Network Analysis. Further, by removing duplicate compounds and active compounds that failed to match target proteins, we obtained a total of 133 active compounds and 247 protein targets. The TCMTB−chemical compound−protein target network was constructed using Cytoscape software (Figure 2). The results showed that the compound with the most targets in GC and HQ was quercetin (B1), targeting 144 targets. The most targeted compounds in BS and DS were kaempferol (C2) and 7-methoxy-2-methyl isoflavone (J1), targeting 51 and 44 targets, respectively. The most targeted compounds in DG and CP were beta-sitosterol (E1) and naringenin (K1), targeting 40 and 36 targets, respectively. The most targeted compounds in DH and CX were stigmasterol (H1) and myricanone (CX2), targeting 32 and 26 targets, respectively. The most targeted compounds in FL and BZ were hederagenin (D1) and 3β-acetoxyatractylone (BZ1), targeting 23 and 17 proteins, respectively. In the study of compound-targeted, we identified 103 active compounds targeting *PTGS2*. In addition, over 70 compounds derived from different herbal medicines targeted *ESR1*, *HSP90AA1*, *AR*, *PPARG*, and NOS_2 proteins. Our data suggest the pharmacological effects of multiple compounds and multiple targets of TCM and the mechanism of action by which the combination of different TCM can act on the same target and thus exert synergistic and increased effects. In addition, detailed information about the compounds and target proteins obtained by filter screening in TCMTB is provided in Supplementary Table 1.

3.3. Potential Target Genes and PPI Network Map of TCMTB Therapy for Brucellosis. A total of 169 brucellosis target genes were obtained by searching GeneCards, OMIM, MalaCards, and DrugBank with brucellosis as the search term (Supplementary Table 2). After validation and removal of duplicate sequences, 113 brucellosis target genes were obtained (Figure 3A). The 113 target genes screened and the 247 herbal targets screened in TCMTB were crossed using R software, and 21 intersecting genes were obtained (Figure 3B) (Table 3). The 21 intersecting genes were imported into the STRING database to obtain the PPI network map (Figure 3B). The results showed that these intersecting genes included *IFNG*, *TNF*, *IL6*, *IL10*, *and IL4*, to mention a few, and there were multiple interactions.

3.4. Construction and Analysis of the TCMTB−Brucellosis−Potential Target Gene Network. We showed the compounds and herbs corresponding to the 21 intersecting genes by Sankey diagrams (Figure 4A). The results showed that six herbs and 75 core compounds showed targeting effects with 21 genes. The 65 active compounds in GC targeted all intersectional targets except CD14, suggesting a central role for licorice in the treatment of brucellosis. The common component of GC and HQ, quercetin, targeted 18 genes and was the compound with the most targets. The target gene, NOS_2, was the common target of the remaining six herbs except DG; however, 70 other active compounds targeted this gene. We further constructed the TCMTB−brucellosis core network (Figure 4B) using Cytoscape software (version 3.7.1) for the TCMs and targets corresponding to compounds with more than three targets. The results showed that a total of 34 nodes and 78 lines were obtained. Further analysis revealed that the targets *CASP3*, *TNF*, and IL6 were in this process to a greater extent, which explains their significance in the network (degree >3) (Table 4).

3.5. GO and KEGG Pathway Enrichment Analysis. KEGG and GO analysis of 21 intersecting genes was performed through the DAVID Bioinformatics Resources website. GO enrichment analysis yielded biological processes for 146 genes, cellular components for six genes, and molecular function for five genes. The top five significantly enriched catalogs in each broad category were selected separately to generate a scatter plot (Figure 5A). The functions of these genes mainly include inflammatory responses (10 genes enriched), immune responses (9 genes enriched), cytokine activity (9 genes enriched), etc. These results suggest that TCMTB is involved in the treatment of brucellosis mainly through the regulation of multiple biological functions such as inflammatory and immune responses, thereby contributing to the understanding of the anti-inflammatory and immunomodulatory mechanisms of *B. abortus*.

To gain insights into the mechanism of action of TCMTB in the treatment of brucellosis, a total of 46 signaling pathways

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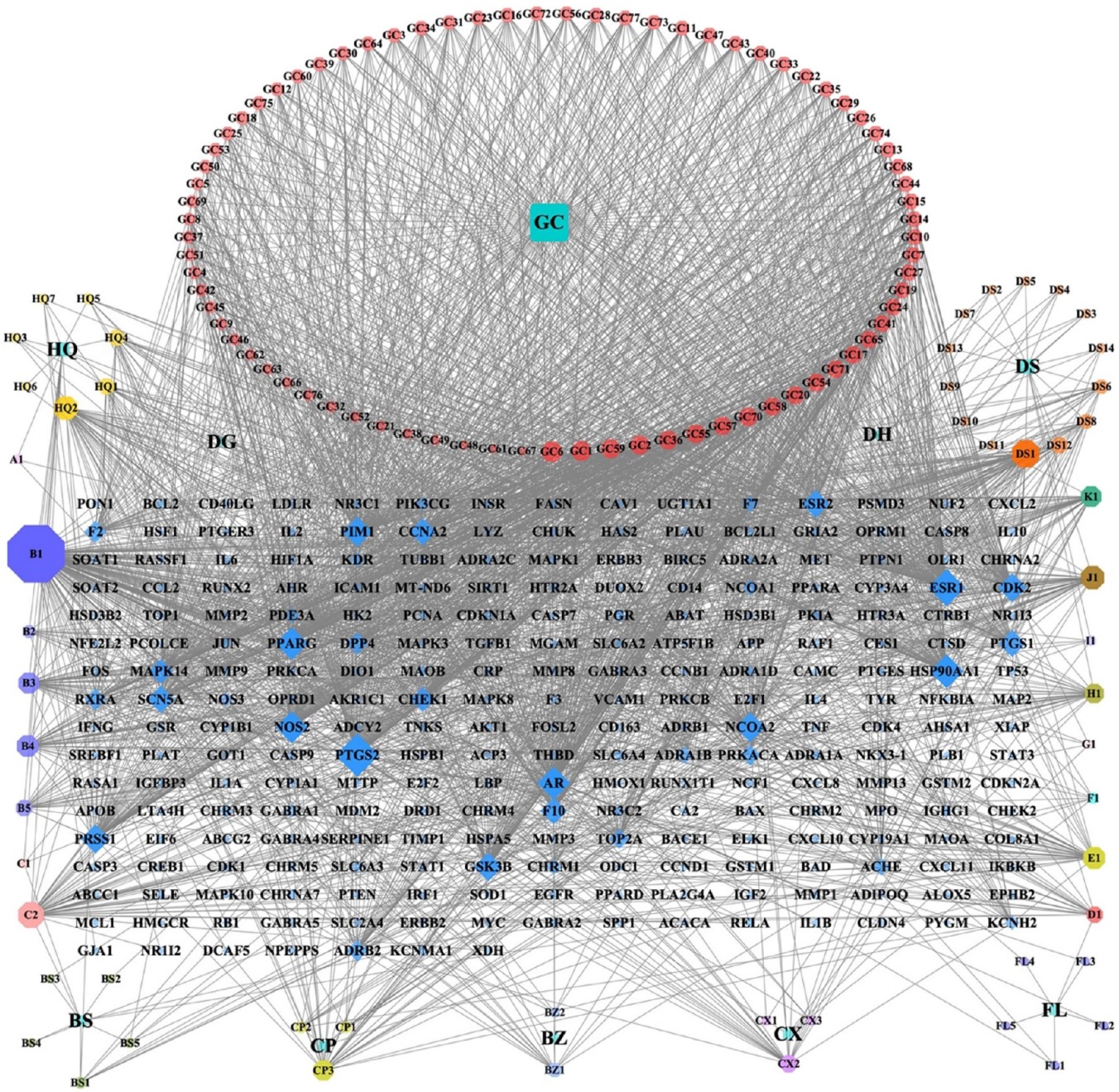

Figure 2. Compound-target network diagram of TCMTB. Nodes with regulatory relationships in the network are connected to each other with gray connecting lines, and the magnitude of the degree value is indicated by the size of the node. TCMTB: Gan Cao (GC), Dang Gui (DG), Fu Ling (FL), Chen Pi (CP), Bai Shao (BS), Chuan Xiong (CX), Bai Zhu (BZ), Huang Qi (HQ), Dang Shen (DS), Di Huang (DH).

were obtained by KEGG pathway enrichment analysis. Scatter plots are shown for the top 20 important signaling pathways (Figure 5B). The results showed that many signaling pathways are closely related to brucellosis treatment, such as tumor necrosis factor (TNF), tuberculosis, and T cell receptor signaling pathways, to mention a few. In addition, the important tuberculosis and TNF signaling pathways are shown in Figure 5C,D. Detailed data about KEGG and GO analysis are shown in Supplementary Table 3.

3.6. Molecular Docking Studies on the Ability of Active Ingredients to Bind to Target Genes Related to Inflammatory Responses. The five active components screened from the TCMTB−brucellosis potential target gene network all had strong binding energy to target genes involved in inflammatory responses (Table 5). The lower the binding energy, the stronger the interaction between the compound and the target protein. We presented 3D and 2D binding profiles of the 10 target proteins most involved in inflammation and the compounds that bound most strongly to them (Figure 6). The results showed that all active compounds bind to target proteins via hydrogen bonds. The target proteins IL6, TGFB1, and CD14 were most strongly bound to the active compounds luteolin, beta-sitosterol, and paeoniflorin, respectively. SELE and TNF proteins had the strongest binding capacity to the active compound kaempferol. Most importantly, the five target proteins CRP, CXCL8, IL1B, CD40LG, and IL10 bound most

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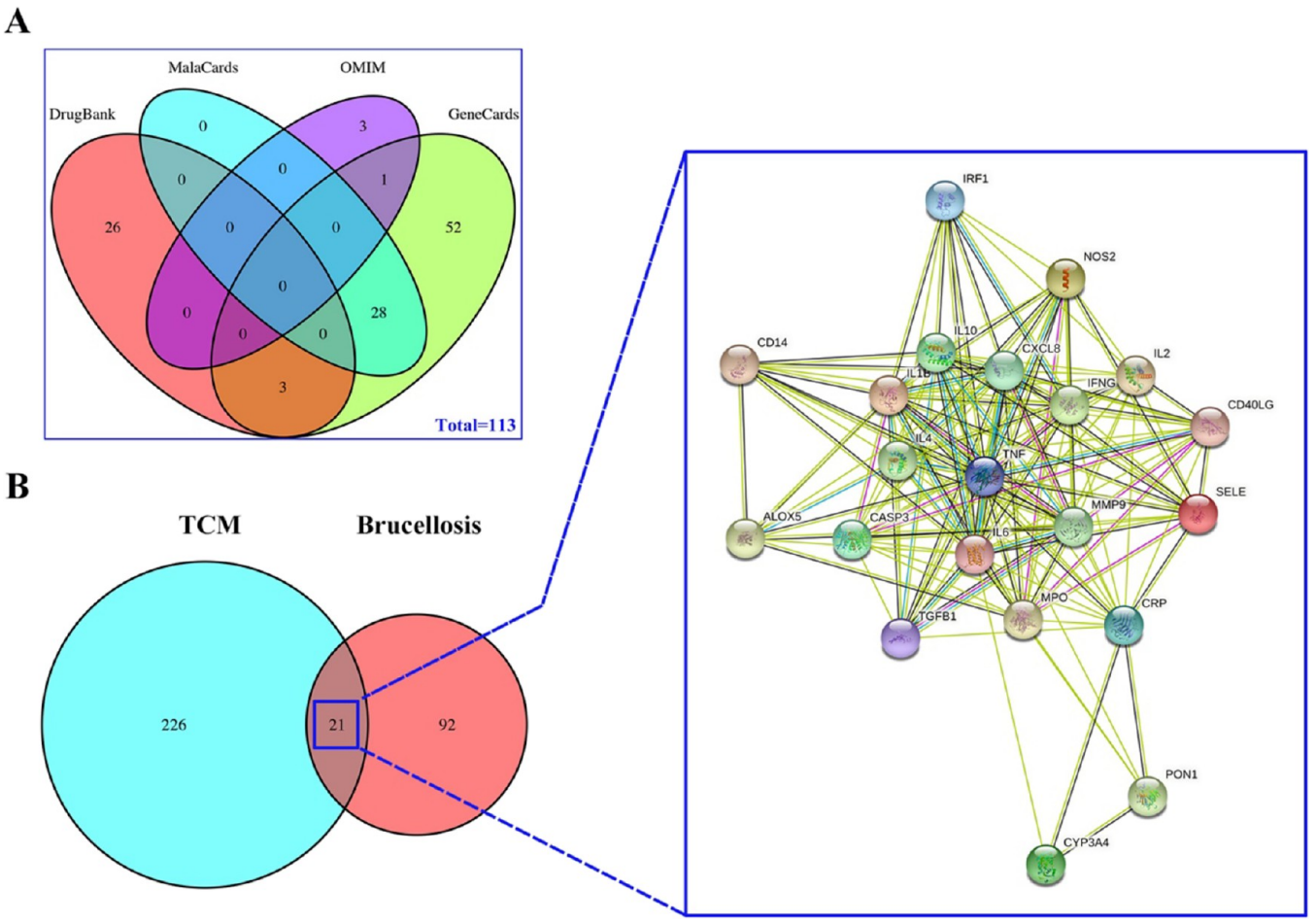

Figure 3. Potential target genes and PPI network map of TCMTB therapy for brucellosis. (A) Venny results of potential target genes of TCMTB therapy for brucellosis. (B) PPI network map of 21 target genes.

Table 3. 21 Potential Target Genes of TCMTB Therapy for Brucellosis

no.	target	symbol	gene ID	no.	target	symbol	gene ID
1	myeloperoxidase	MPO	4353	12	transforming growth factor beta-1	TGFB1	7040
2	interferon gamma	IFNG	3458	13	CD40 ligand	CD40LG	959
3	interleukin-6	IL6	3569	14	tumor necrosis factor	TNF	7124
4	interleukin-10	IL10	3586	15	monocyte differentiation antigen CD14	CD14	929
5	interleukin-4	IL4	3565	16	nitric oxide synthase, inducible	NOS2	4843
6	interleukin-1 beta	IL1B	3553	17	matrix metalloproteinase-9	MMP9	4318
7	interleukin-2	IL2	3558	18	serum paraoxonase/arylesterase 1	PON1	5444
8	E-selectin	SELE	6401	19	interferon regulatory factor 1	IRF1	3659
9	interleukin-8	CXCL8	3576	20	cytochrome P450 3A4	CYP3A4	1576
10	caspase-3	CASP3	836	21	arachidonate 5-lipoxygenase	ALOX5	240
11	C-reactive protein	CRP	1401				

strongly to the active compound quercetin. These results suggested that quercetin, the active compound derived from GC and HQ, may play a crucial role in the resistance to brucellosis.

3.7. Effect of Quercetin, the Core Active Compound of TCMTB, on *B. abortus* In Vitro Validation. *3.7.1. Effect of Quercetin on THP-1 Cell Viability.* The mechanism of quercetin inhibition of inflammation caused by *B. abortus* was further validated by in vitro experiments, selecting the TH1 cell line and TCMTB core active compound, quercetin. As shown in Figure 7A, TH1 monocytes were significantly differentiated into macrophages after 48 h of PMA induction. To determine the toxic effect of quercetin on THP1 macrophages, a CCK8 assay was performed. As shown in Figure 7B, there was no significant change in cell viability in all groups of THP-1 macrophages exposed to different tested concentrations of quercetin compared to untreated controls. Solvent control DMSO also had no effect on cell viability. These results indicated that all concentrations of quercetin tested were not toxic to THP-1 macrophages. Therefore, we chose 100 μM as the quercetin intervention concentration for the subsequent experiments.

3.7.2. Effect of Active Compounds on Cytokine Production in THP-1 Cells. The mRNA levels of key targets (*TNF-α*, *IL-1β*, *IL-10*, and *TGF-β*) regulated by active compounds were assessed by qRT-PCR. These targets are involved in immune regulation

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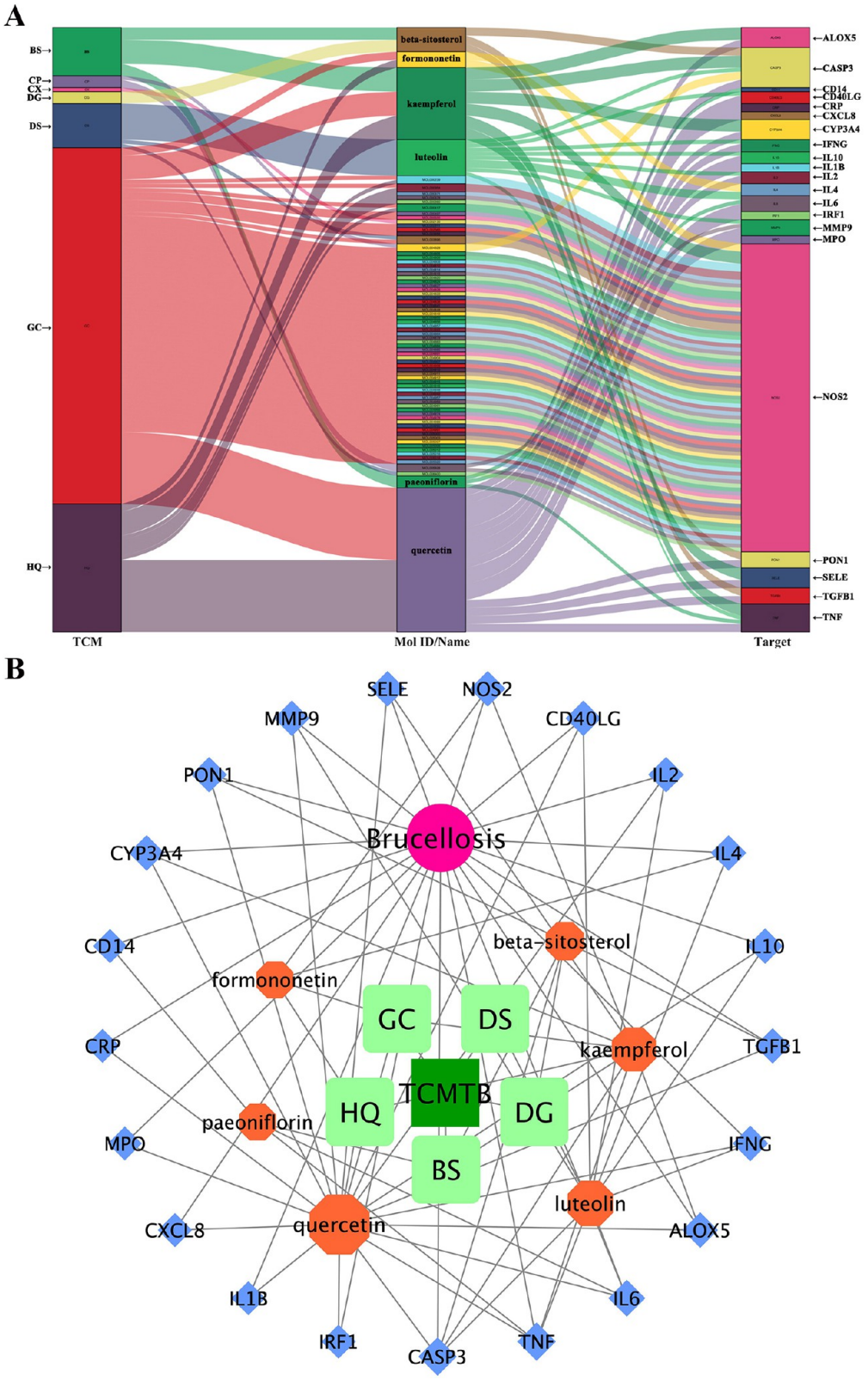

Figure 4. Construction and analysis of the TCMTB−brucellosis−potential target gene. (A) Sankey diagram showing the relationship between TCMTB, compounds, and target genes. (B) Network analysis of core TCMTB−compound−brucellosis potential target gene: pink represents brucellosis, blue represents the target gene, orange represents the compound, and light green represents TCM.

and inflammatory responses. After 24 h of cell culture in each group, quercetin significantly promoted *TNF-α* and *IL-1β* mRNA expression and inhibited *TGF-β* and *IL-10* mRNA expression compared with the *Brucella* infection group (Figure 7C). In addition, other active compounds, such as luteolin, *β*-sitosterol, and kaempferol, have been shown to play an important role in the anti-*Brucella* and anti-*Mycobacterium tuberculosis* activity.[36,37] The results of our bioinformatics

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Table 4. Top 15 High Degree Genes in the Network

no.	target	symbol	degree
1	caspase-3	CASP3	5
2	tumor necrosis factor	TNF	5
3	interleukin-6	IL6	4
4	arachidonate 5-lipoxygenase	ALOX5	3
5	cytochrome P450 3A4	CYP3A4	3
6	serum paraoxonase/arylesterase 1	PON1	3
7	matrix metalloproteinase-9	MMP9	3
8	E-selectin	SELE	3
9	nitric oxide synthase, inducible	NOS2	3
10	CD40 ligand	CD40LG	3
11	interleukin-2	IL2	3
12	interleukin-4	IL4	3
13	interleukin-10	IL10	3
14	transforming growth factor beta-1	TGFB1	3
15	interferon gamma	IFNG	3

analysis also suggest that all of these active compounds are involved in immune regulation and tuberculosis signaling pathways. On the other hand, qRT-PCR results showed that these compounds had no effect on cell viability at concentrations of 100 μM (data not shown), but also exhibited the same target-regulatory effects as quercetin, both of which were weaker than those of quercetin. However, nothing has been reported about the regulation of *Brucella* infection by quercetin. Western blot results showed that quercetin significantly promoted the expression of TNF-α and IL-1β proteins and inhibited the expression of TGF-β and IL-10 proteins (Figure 7D). These findings suggest that these screened active compounds induce immunomodulatory and pro-inflammatory cytokine production during *Brucella* infection, and that the compound quercetin has the strongest induction effect.

3.7.3. Effect of Quercetin on B. abortus Growth and NO Production. Treatment of *B. abortus* with different concentrations of quercetin (50, 100, and 200 μM) did not cause significant changes in *B. abortus* survival (Figure 7E). Furthermore, in *B. abortus*-infected THP-1 cells, the difference in nitric oxide accumulation at 2 h was not significant in the group whose cells were treated with quercetin compared to the untreated group, while it was significantly increased at 24 and 48 h (Figure 7F). It has been shown that NO plays an important role in the clearance of intracellular bacteria, including *B. abortus*.[38] In summary, quercetin treatment did not directly affect the growth of *B. abortus* but significantly induced NO production in *B. abortus*-infected THP-1 cells.

3.7.4. Effect of Quercetin in B. abortus Uptake and Intracellular Survival within THP-1 Cells. Compared to the S2308 strain-infected control group, the difference in cell uptake rate between the macrophage-treated group pretreated with quercetin and then infected with S2308 strain was not significant at 20 min, while the cell uptake rate was significantly lower at 60 min ($p < 0.01$). Compared with 20 min, there was no significant difference in the cell uptake rate at 60 min in the control group, but there was a significant difference in the treatment group ($p < 0.01$) (Figure 7G). The intracellular survival assay of *B. abortus* S2308 showed that quercetin significantly reduced the intracellular survival of the bacteria compared to the S2308-infected control without quercetin at 24 and 48 h, while the difference was not significant at 2 h. Intracellular survival of *B. abortus* was significantly reduced at 24 h and significantly increased at 48 h in all experimental groups ($p < 0.01$) (Figure 7H).

4. DISCUSSION

Recently, the high incidence of brucellosis in developing countries seriously affects the development of animal husbandry and seriously threatens the physical and mental health of humans.[4] The mechanism of *Brucella* infecting human beings and animals involves immune responses, inflammatory responses, and cytokines/receptor signaling pathways.[39] TCMTB has become popular among clinicians because of its advantages, such as fewer adverse effects, less susceptibility to drug resistance, and better effect when combined with antibiotic drugs for brucellosis treatment. In this study, we used bioinformatics analysis of network pharmacology to identify five core active ingredients in TCMTB that play important roles in the treatment of *B. abortus*. These active ingredients are related to a variety of signaling pathways and proteins and have potential research value.

The results of the TCMTB−compound−potential target network (Figure 2) showed that the 10 TCMTB contained a very large number of compounds, up to 133. Most of these compounds were common components of different TCM, such as sitosterol (F1) in GC, CP, BS, CX, and DH; quercetin (B1) in GC and HQ; stigmasterol (H1) in DG, DS, and DH, etc. In addition, compounds often have multiple targets; for instance, F1 contained three target genes, *NCOA2*, *NR3C2*, and *PGR*; H1 contained 29 target genes, and B1 contained 142 target genes, to mention a few. Many target genes could be co-regulated by multiple compounds; 103 compounds co-regulate *PTGS2*, 70 compounds co-regulate NOS_2, 84 compounds co-regulate *ESR1*, etc. These results suggest that TCM has multicomponent and multitarget properties for the treatment of brucellosis. The PPI results (Figure 3) showed that the 21 target genes cross-linked in the brucellosis-related target gene database and TCMTB were interrelated and interacted with each other.[40] For the 21 important target genes, the TCMTB−compound−target network was constructed (Figure 4), which showed GC, HQ, BS, and DS as core herbal medicines, beta-sitosterol, quercetin, kaempferol, luteolin, and paeoniflorin as core compounds, and 21 genes including *NOS2*, *CASP3*, and *TNF* as core targets in the treatment of brucellosis.

GO and KEGG enrichment analysis of the 21 key targets showed that 157 biological functions and 46 signaling pathways are involved in the development and progression of brucellosis, including inflammatory and immune responses, biological functions such as cytokine activity, and signaling pathways involving cytokine interactions, TNF, tuberculosis, and T cell receptors (Figure 5). Despite the lack of typical pathogenic factors in brucellosis, inflammation is a hallmark of brucellosis, particularly in osteoarticular brucellosis.[41] TNF-α and IL-1β, which are highly expressed in bacterial osteoarthritic synovial fluid, stimulate inflammatory cells such as macrophages and neutrophils to release proteases, and together with IL-6, promote osteoclast differentiation and bone resorption.[42,43] Cytokine interaction signaling pathways are primarily involved in regulating immunity, inflammatory responses, and wound healing, in addition to amplifying responses to pathogenic infections through cytokine interactions.[44] Innate immunity is the first line of defense against pathogens, and when host macrophages recognize *Brucella* infection, they first initiate an innate immune response by releasing large amounts of pro-inflammatory cytokines to control the infection.[45] Then, adaptive immunity was activated, and early in *Brucella* infection, T cell receptors recognize relevant bacterial peptides and MHC

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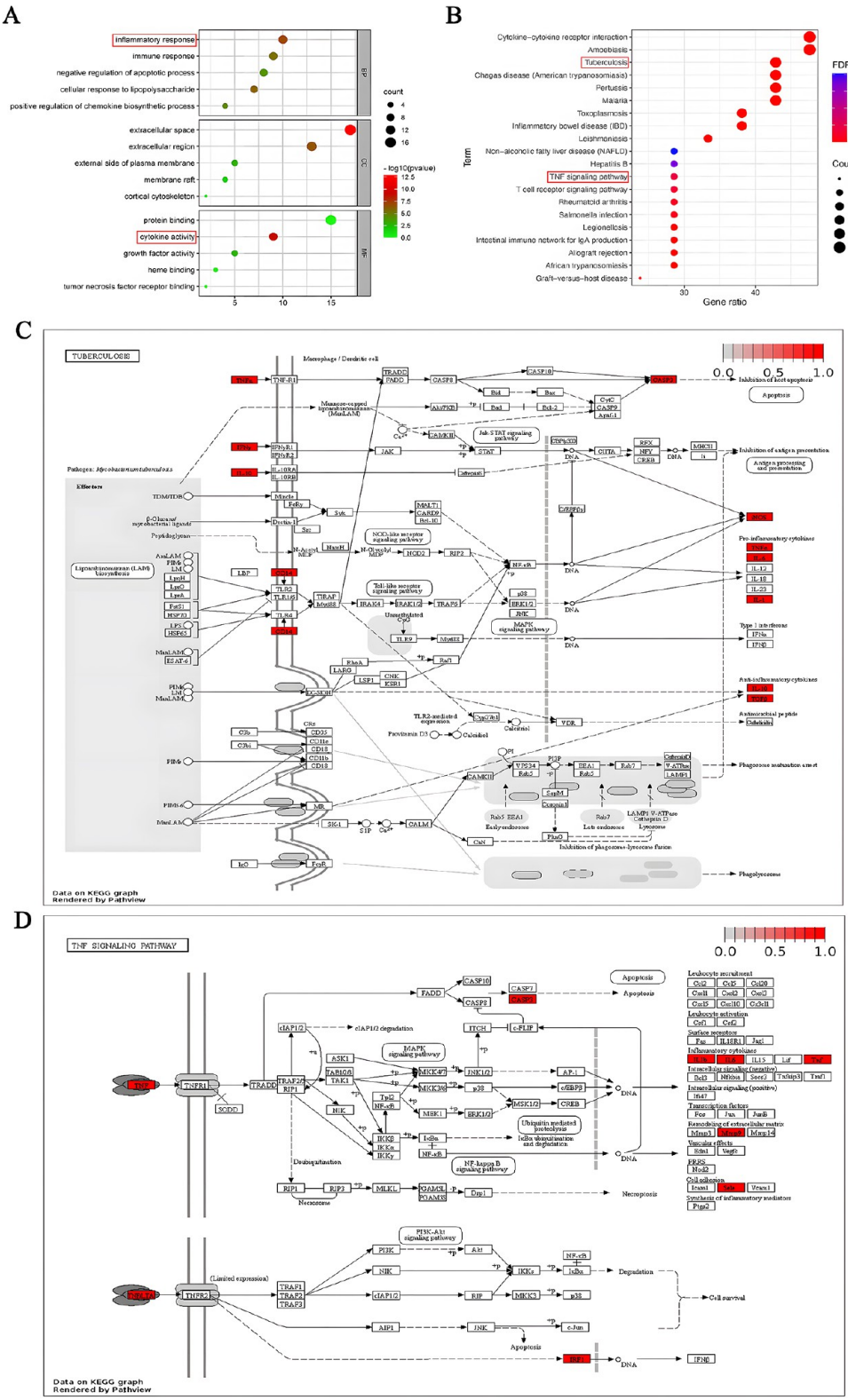

Figure 5. Functional characterization of TCMTB against brucellosis intersecting genes. (A) GO analysis of the top 18 significantly enriched potential target genes of TCMTB in brucellosis. (B) KEGG analysis of potential TCMTB target gene signaling pathways in the top 20 significantly enriched brucellosis. (C, D) Suggested KEGG signaling pathways for tuberculosis and TNF. The red rectangular node represents the core gene.

complexes, causing naive T cells to differentiate and overexpress into T helper type 1 (Th1) cells that produce IL-2 and IFN-γ, thereby clearing *Brucella*.[46] In the late stages of the infection, overexpressed Th1 transforms into Th2 cells that produce IL-4, IL-5, and TGF-β, thus reducing inflammation caused by *Brucella*.[47] B lymphocytes, which have an immunomodulatory role, are also key cells in adaptive immunity. Studies have shown that reducing B cell production of IL-10 and TGF-β increases the efficiency of *Brucella* clearance.[48] These functions and

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Table 5. Molecular Docking Results of Five Core Active Ingredients in TCMTB with 10 Target Genes of Inflammatory Response

no.	TCMTB	Mol ID	Mol	target	PDB ID of target	binding energy
1	DG, BS	MOL000358	beta-sitosterol	TGFB1	5VQP	−5.81
2	GC, HQ	MOL000098	quercetin	TGFB1		−4.49
3	GC, HQ, BS	MOL000422	kaempferol	TNF	5UUI	−5.08
4	DS	MOL000006	luteolin	TNF		−4.99
5	BS	MOL001924	paeoniflorin	TNF		−3.12
6	GC, HQ	MOL000098	quercetin	TNF		−4.27
7	DS	MOL000006	luteolin	IL6	1ALU	−6.56
8	BS	MOL001924	paeoniflorin	IL6		−4.7
9	GC, HQ	MOL000098	quercetin	IL6		−6.36
10	DS	MOL000006	luteolin	IL10	2ILK	−5.73
11	GC, HQ	MOL000098	quercetin	IL10		−6.00
12	BS	MOL001924	paeoniflorin	CD14	4GLP	−4.68
13	GC, HQ	MOL000098	quercetin	CRP	1B09	−8.24
14	GC, HQ	MOL000098	quercetin	CXCL8	4XDX	−8.21
15	GC, HQ, BS	MOL000422	kaempferol	SELE	1G1T	−5.38
16	GC, HQ	MOL000098	quercetin	SELE		−5.37
17	GC, HQ	MOL000098	quercetin	IL1B	5R8Q	−8.06
18	GC, HQ	MOL000098	quercetin	CD40LG	1ALY	−6.84

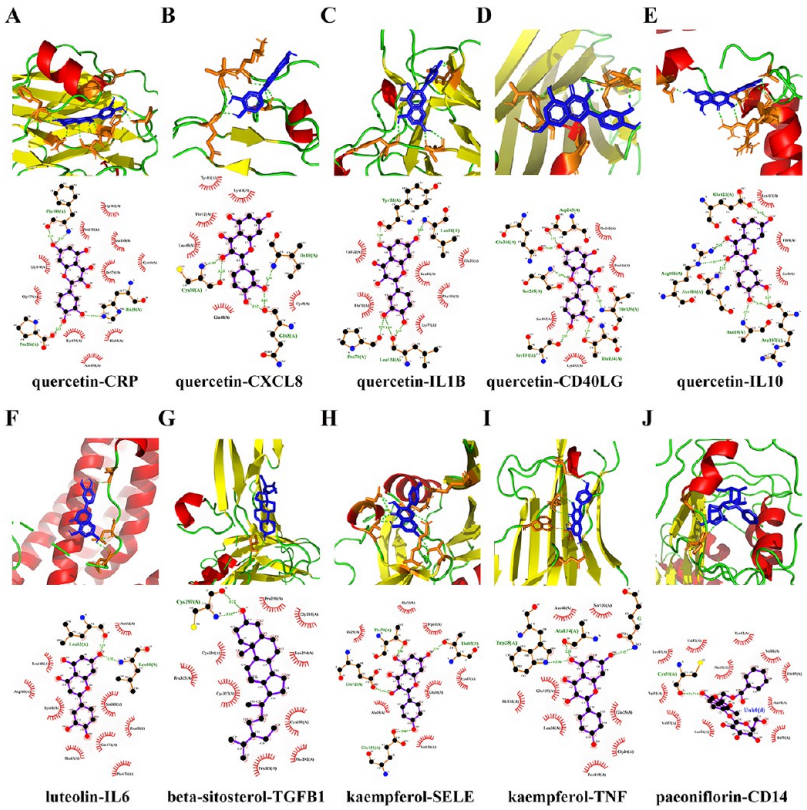

Figure 6. (A−J) 3D and 2D maps of active compound binding to target proteins. The blue color indicates the active compound, the green dashed line indicates the hydrogen bond, and the brown color indicates the region where the active compound binds to the target protein.

pathways provide potential mechanisms for the suppression and immune regulation of inflammation caused by *Brucella*.

The core compounds in TCMTB have been widely demonstrated to have important roles in the inhibition of pathogenic bacteria and the regulation of inflammation and immunity. Studies have shown that luteolin, an immunomodulator, in combination with the anti-tuberculosis drug isoniazid, can promote anti-tuberculosis immunity, shorten the duration of treatment, and prevent disease relapse.[37] In addition, luteolin is sensitive to septic bacteria and Salmonella and has a strong inhibitory effect.[49] Luteolin exert anti-inflammatory effects by inhibiting nuclear factor kappa-B signaling pathways.[50] Kaempferol has good activity anti-*mycobacteria*, anti-*Escherichia coli*, anti-*Bacillus subtilis*, and anti-*Staphylococcus aureus*. In addition, kaempferol exerts anti-inflammatory effects by inhibiting the mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways and suppressing inflammatory factors such as *TNF-α*, *IL6*, and *IL10*.[51] Beta-sitosterol increases resistance to *M. tuberculosis* and *Brucella* by inducing the production of pro-inflammatory cytokines.[36,52] Beta-sitosterol

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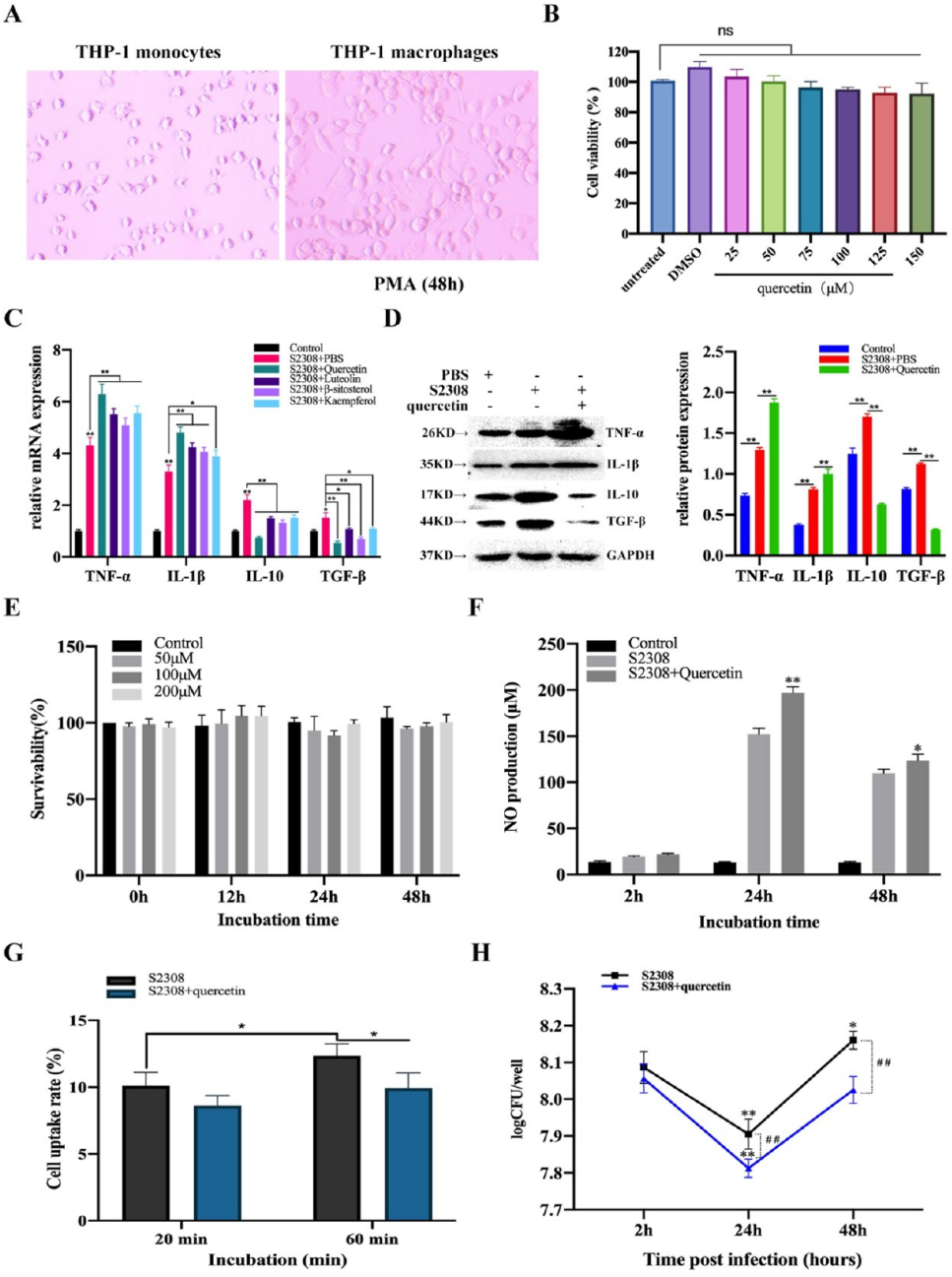

Figure 7. TCMTB core active compound inhibits *B. abortus*-induced inflammation in THP1 cells. (A) PMA induces differentiation of THP1 monocytes into macrophages. (B) Effect of quercetin on cell viability of THP-1 macrophages. Values are expressed as mean ± SEM of three independent experiments. (C, D) Expression of mRNA and protein levels of immunity and inflammation-related targets after active compound treatment of *B. abortus*-infected THP1 macrophages. (E) *B. abortus* was incubated with different concentrations of quercetin, and bacterial survival rates were determined. (F) Cells were infected with *B. abortus* for 1 h. Quercetin was optionally added or not, and NO accumulation was detected after 2, 24, and 48 h of incubation. (G) Bacterial internalization efficiency. After 1 h of infection with *B. abortus*, cells were incubated with or without quercetin for 20 and 60 min. (H) Bacterial intracellular survival efficiency. $*p < 0.05$, $**p < 0.01$.

significantly reduced intracellular survival of *Salmonella typhimurium* and inflammation by inhibiting inflammatory cytokines by regulating macrophage polarization.[53,54] Paeoniflorin has a strong inhibitory effect on pathogenic organisms such as *Candida albicans*, *S. aureus*, and *Escherichia coli*.[55] Paeoniflorin exerts anti-inflammatory and immunomodulatory effects by reducing inflammatory factor production and suppressing immune cells.[56] These findings suggest that multiple active compounds in TCMTB can synergize to modulate cytokines and signaling pathways to exert therapeutic effects against brucellosis.

In our study, quercetin, the core compound of the herbs GC and HQ, targeted 18 of the 21 core targets and thus received special attention from us. Quercetin has been reported to have anti-*M. tuberculosis*, anti-*Pseudomonas aeruginosa*, anti-*Enterococcus faecalis*, anti-*Streptococcus pyogenes*, anti-*S. aureus*, and anti-*E. coli* by a mechanism related to the inhibition of biofilm formation.[57,58] In recent years, the anti-inflammatory, anti-liver and kidney damage, and immune promoting effects of quercetin have become a hot research topic. Studies have shown that quercetin antagonizes hepatotoxicity induced by combinations of antituberculosis drugs, including rifampin, pyrazinamide,

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isoniazid, and ethambutol, in mice.[59] Its mechanism is related to the inhibition of oxidative stress and nitrosative stress by inhibiting mitogen-activated protein kinase and NF-κB signaling pathways to reduce inflammatory cytokines to regulate oxidative stress and liver damage induced by acetaminophen overdose.[60,61] These findings suggest that multiple active compounds in TCMTB can synergize to modulate cytokines and signaling pathways to exert therapeutic effects against brucellosis. Currently, there are fewer studies on the mechanism of quercetin anti-*Brucella*, and it is well known that macrophages are the main hosts of *Brucella*. Therefore, the study of the modulatory effect of quercetin on macrophage inflammation induced by *Brucella* infection is important for the validation of the compound quercetin in regulating *Brucella*-related targets and the subsequent investigation of the mechanism of action of quercetin in anti-brucellosis.

To further rationalize the pharmacodynamic basis of TCMTB against brucellosis, the core compounds of TCMTB obtained from the network pharmacological approach screening were docked with the corresponding targets for validation analysis using molecular simulation docking techniques (Figure 6). The results indicated that multiple compound components bind to the corresponding targets in an individual or synergistic manner. Also, based on the analytical study of the binding energy values, we identified the structures of the core compounds and the targets for which the best binding activity existed for the core compounds involved in a variety of TCM. The results of the network pharmacology, bioinformatics, and molecular docking analysis identified the core active compounds and potential key targets and pathways of TCMTB for the treatment of brucellosis. The compounds beta-sitosterol, kaempferol, luteolin, paeoniflorin, and especially quercetin may play a key role in the resistance to brucellosis. The anti-*Brucella* effect of some of the compounds has been experimentally validated; however, the specific mechanisms by which these compounds exert their effects are not well understood.[36,52] To verify the reliability of the predicted results, we further validated the contribution of quercetin treatment in *B. abortus* by in vitro experiments, selecting the TH1 cell line and TCMTB active compounds (Figure 7). In addition, in vitro experiments confirmed that the core compound quercetin attenuated the ability of *B. abortus* to internalize THP-1 cells as well as intracellular viability. It significantly increased nitric oxide and pro-inflammatory targets TNF-α and IL-1β and significantly inhibited mRNA and protein expression of IL-10 and TGF-β. TGF-β inhibition has been reported to reduce bone and joint inflammation.[62] Inhibition of IL-10 facilitates the clearance of *B. abortus*.[63] Our results showed that multiple herbal components bind to their corresponding targets in an individual or synergistic manner to treat or alleviate brucellosis by participating in the regulation of the body's cytokines, immune responses, and inflammation-related signaling pathways.

In conclusion, based on systematic pharmacology findings, this study first revealed that TCMTB, through beta-sitosterol, quercetin, kaempferol, luteolin, paeoniflorin, and other multicomponents, could exert the molecular mechanism of synergistic treatment of brucellosis by regulating inflammatory response and immune function and regulating TNF, tuberculosis, and other multipathways including *TGFB*, *TNF*, *IL6*, *IL10*, *CRP*, *IL1B*, and other multitargets. Molecular docking and in vitro experiments initially validated the reliability of the results obtained from network pharmacology and bioinformatics analysis. The potential of quercetin therapy in the control of *Brucella* infection was demonstrated for the first time. Hence, quercetin could be investigated as the most suitable natural anti-brucellosis agent and a lead compound for further structural modifications and drug development. These predictions may be useful in the treatment of brucellosis in future clinical practice.

■ ASSOCIATED CONTENT

Data Availability Statement

All relevant data are within the manuscript and its additional files.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c03716.

Detailed information about the compounds and target proteins obtained by filter screening in TCMTB (Supplementary Table 1); brucellosis target gene information obtained from GeneCards, OMIM, MalaCards, and DrugBank database searches (Supplementary Table 2); detailed information about KEGG and GO analysis (Supplementary Table 3) (ZIP)

■ AUTHOR INFORMATION

Corresponding Authors

Zhihua Sun − *State International Joint Research Center for Animal Health Breeding, College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang 832003, China*; Email: zhihuasun918@shzu.edu.cn

Xinli Gu − *State International Joint Research Center for Animal Health Breeding, College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang 832003, China*; Email: xinligu123@sina.com

Hui Zhang − *State International Joint Research Center for Animal Health Breeding, College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang 832003, China*; orcid.org/0000-0002-4580-0824; Email: prof.zhang@foxmail.com

Authors

Tianyi Zhao − *State International Joint Research Center for Animal Health Breeding, College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang 832003, China*

Yu Zhang − *State International Joint Research Center for Animal Health Breeding, College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang 832003, China*

Liangbo Liu − *State International Joint Research Center for Animal Health Breeding, College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang 832003, China*

Xingmei Deng − *State International Joint Research Center for Animal Health Breeding, College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang 832003, China*

Jia Guo − *State International Joint Research Center for Animal Health Breeding, College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang 832003, China*

Shuzhu Cao − *State International Joint Research Center for Animal Health Breeding, College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang 832003, China*

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Dexin Zhu − *State International Joint Research Center for Animal Health Breeding, College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang 832003, China*

Jian Xu − *Herbivorous Animal Bacterial Disease Innovation Team, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu 730046, China*

Usevich Vera Nikolaevna − *State International Joint Research Center for Animal Health Breeding, College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang 832003, China; College of Veterinary, Ural State Agricultural University, Yekaterinburg 620000, Russia*

Suleimenov Maratbek − *State International Joint Research Center for Animal Health Breeding, College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang 832003, China; College of Veterinary, Kazakh National Agricultural University, Nur Sultan 050001, Kazakhstan*

Zhen Wang − *State International Joint Research Center for Animal Health Breeding, College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang 832003, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c03716

Author Contributions

[#]T.Z. and Y.Z. equally contributed to this work as a first author. T.Z. and Y.Z.: conceptualization, data curation, formal analysis, methodology, visualization, writing—original draft. L.L. and X.D.: vitro experiment, data analysis. J.G., S.C., D.Z., and J.X.: data curation, formal analysis, methodology, writing—review & editing. U.N., S.M., and Z.W.: data curation, software, visualization. Z.S., X.G., and H.Z.: supervision, writing—review & editing.

Notes

The authors declare no competing financial interest.

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