Combined with network pharmacology to study the effect and mechanism of Sanhuang ointment on MRSA infection in the skin and soft tissue

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Article

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Abstract

Overview. Skin and soft tissue infection SSTI is a frequent clinical disease. Sanhuang ointment is a traditional Chinese medicine used to treat SSTI. However, the pharmacological effect and mechanism of Sanhuang ointment on SSTI remain unclear. In this study, we investigated the protective effect and mechanism of Sanhuang ointment on MRSA in the skin and soft tissue infections by network pharmacological analysis, followed by in vivo experimental validation.

Methods. Using network pharmacology, the active ingredients and disease targets of Sanhuang ointment were screened and intersected for GO and KEGG enrichment analysis. A rat model of skin and soft tissue infection was established and the pathological features were observed. Sanhuang ointment large, medium and small dose groups (1g, 0.5g, and 0.25g/animal, with the total amount of Vaseline, dispensed 1g/animal) and Mupirocin Ointment positive control group (0.5g/animal, with the total amount of Vaseline, dispensed 1g/animal) were used, respectively. The expression of key proteins of the IL-17/NF-κB signaling pathway and downstream inflammatory factors were analyzed by histomorphological observation, enzyme-linked immunosorbent assay, polymerase chain reaction, and Western blotting.

Results. Network pharmacology analysis confirmed that 119 active components and 275 target genes of Sanhuang ointment were identified and intersected with MRSA infection-related genes, and 34 target genes of Sanhuang ointment were found to be used for skin and soft tissue infection with MRSA. Sanhuang ointment (1g/mouse) could effectively ameliorate histopathological changes and significantly inhibit the expression of key proteins of the IL-17/NF-κB signaling pathway and downstream inflammatory factors (p < 0.05). Discussion and Conclusion This study suggests that Sanhuang ointment protects against MRSA infection and inhibits inflammation by inhibiting IL-17/NF-κB signaling pathway. This is important for the secondary development and new drug development of Sanhuang ointment.

Conclusion. Sanhuang ointment has a protective effect on MRSA infection and inhibits inflammation by inhibiting IL-17/NF-κB signaling pathway. This is important for the secondary development and new drug development of Sanhuang ointment.

1. Introduction

Skin and soft tissue infections (SSTIs) are common clinical diseases. Most soft tissue infections are inflammatory reactions mainly caused by pathogen invasion and reproduction, of which bacterial infections (most commonly Staphylococcus aureus and hemolytic streptococci) are common problems faced in the treatment of soft tissue infections on the body surface. Methicillin-resistant Staphylococcus aureus (MRSA) is the most common drug-resistant Staphylococcus aureus, with increasing detection rates in body surface infected tissues, secretions, and pus, and MRSA has become one of the bacteria with the highest incidence of nosocomial infections in the world. According to the Global Surveillance System for Antibiotic Resistance and Use (GLASS) report survey statistics in 2020, about 700,000 people die of antibiotic-resistant bacterial infections each year worldwide, and MRSA is
one of its major risk factors \(^4\). At present, vancomycin is the first-line antibiotic for the clinical treatment of MRSA infection, but the increasing number of vancomycin-resistant strains has increased the complexity of clinical anti-MRSA infection treatment \(^5,6\). Although some progress has been made in the research and development of new antibiotics for MRSA in recent years, it is important to find new treatment strategies to prevent the emergence of new antibiotic-resistant bacteria.

"Sanhuang ointment" (in-hospital preparation of the Affiliated Hospital of Gansu University of Traditional Chinese Medicine, Ganyao Zhunzi Z04010878): It is based on the theory of "clearing heat and detoxifying to relieve swelling and ulcers" in traditional Chinese medicine. Coptidis Rhizoma, Phellodendri Chinensis Cortex, Scutellariae Radix, Borneol, Sesame Oil, Vaseline, etc. are prepared in specific proportions. Sanhuang ointment has the functions of clearing away heat and detoxifying, promoting blood circulation and relieving pain, reducing swelling and discharging pus, etc. It has been used clinically for more than 35 years, with significant curative effects and few side effects, especially for boils, carbuncles, erysipelas, abscesses, and other acute and chronic purulent infections, burns, soft tissue injuries, fluid extravasation, body surface ulcers, banding caused by infusion and chemotherapy. Herpes and other infectious diseases have a significant curative effect \(^7\). The chemical analysis of Coptidis Rhizoma by ultra-performance liquid chromatography-mass spectrometry (UPLC-Q-TOF/MS) found that the main components of Coptidis Rhizoma were alkaloids, among which magnolia, berberine, coptisine, jatrorrhizine, palmatine, and Berberine inhibits Staphylococcus aureus \(^8\). The main components of Phellodendri Chinensis Cortex are alkaloids (such as berberine, palmatine, and Phellodendron), which are known as anti-inflammatory agents \(^9,10\), by inhibiting NF- Activation of \(\kappa B\) and MAPK (mitogen-activated protein kinase) downregulates NO (nitrogen monoxide) and iNOS (Inducible Nitric Oxide Synthase) for anti-inflammatory purposes \(^11\). The main active components of Scutellariae Radix are flavonoids such as baicalin, baicalein, wogonin, and oroxylin A \(^12-14\). Antioxidant and anti-inflammatory effects have been demonstrated in various disease models, including diabetes, cardiovascular disease, inflammatory bowel disease, gout and rheumatoid arthritis, asthma, neurodegenerative diseases, liver and kidney disease, cerebrospinal inflammation and carcinogenesis \(^15\).

Network pharmacology is an emerging pharmacological research method integrating traditional pharmacology, bioinformatics, chemoinformatics, and network biology. Network pharmacology can systematically reveal the active ingredients and potential mechanisms of action of traditional Chinese medicines \(^16,17\). Through network pharmacology, we predicted that Sanhuang ointment might treat skin and soft tissue infections with MRSA via IL-17/NF-\(\kappa B\) signaling pathway. A rat model of skin and soft tissue infection with MRSA was created by subcutaneous injection of MRSA bacterial suspension. After Sanhuang ointment intervention, histopathological chances of infection, as well as the expression of IL-17, TRAF6, TAK1, TAB1, IKK\(\beta\), NF-\(\kappa B\) p65, and inflammatory cytokines IL-1\(\beta\), IL-4, IL-5, IL-6, TNF-\(\alpha\), and IFN-\(\gamma\), key proteins in the IL-17/NF-\(\kappa B\) signaling pathway, were detected, thus confirming the role of Sanhuang ointment in the treatment of MRSA infected by the skin and soft tissue infection through IL-17/NF-\(\kappa B\) signaling pathway. The study flow is shown in Fig. 1.
2. Results

2.1 Construction and Analysis of the "Active Ingredient-Target" Network of Sanhuang Ointment

Seventy chemical components were obtained from the TCMSP database according to DL ≥ 0.18 Phellodendri Chinnsis Cortex, 31 from Coptidis Rhizoma, and 72 from Scutellariae Radix, and a total of 151 chemical components were obtained after removing repeated values (results are shown in Table 1). The active ingredients were searched in the database to obtain the corresponding targets, and imported into the UniProt database for gene name normalization to obtain 212 active ingredient targets Phellodendri Chinnsis Cortex, 192 Coptidis Rhizoma, and 168 Scutellariae Radix, and a total of 275 active targets were obtained by removing duplicate values after merging. The active ingredients and corresponding targets of Sanhuang ointment were imported into Cytoscape 3.7.2 software to construct the "active component-target" network diagram (Figure 2). The network consists of 382 nodes and 2116 edges, and the positive 8 edges represent the active ingredients selected by the three herbs, with larger graphs indicating more important active ingredients. Among them, the green plus 8 edge shape is the component derived from Phellodendri Chinnsis Cortex; the purple plus 8 edge shape is the component derived from Coptidis Rhizoma; the blue plus 8 edge shape is the component derived from Scutellariae Radix; the purple circle represents the common components jatrorrizine and coptisine of Phellodendri Chinnsis Cortex, Coptidis Rhizoma and Scutellariae Radix; the green circle represents the common components aborigine, columbine, Magnoorine, columberrubine, Phellodendri Chinnsis Cortex, Magnoorine grandiose, columbine, quercetin, and Worenine of Phellodendri Chinnsis Cortex and Scutellariae Radix; the yellow circle represents the common components of Phellodendri Chinnsis Cortex and Scutellariae Radix; the blue circle represents the common components ogluside, sitosterol and Snosterol of Phellodendri Chinnsis Cortex. The edges between nodes and nodes indicate the interaction relationship between active components and targets, and larger nodes are significant. From degree analysis, the top ten compounds were quercetin, apigenin, beta-sitosterol, Stigmasterol, magnoorine, wogonin, columbamine, palmatine, baicalein, and Isocorypalmine. Degree was 303, 79, 76, 64, 50, 46, 44, 40, 38, 37, respectively.

2.2 Potential Targets for Sanhuang Ointment in the Treatment of MRSA in Skin and Soft Tissue Infections

A total of 328 MRSA infection-related gene targets were identified through Gene cards, OMIM, TTD, Drug bank, and Disgent databases. Venny was used to intersect the selected active ingredient targets of Sanhuang ointment with MRSA infection targets to obtain 34 common targets of Sanhuang ointment-MRSA infection (Figure 3).

2.3 Construction and Analysis of Interaction Network

To comprehensively investigate the core pharmacological mechanism of Sanhuang ointment in the treatment of MRSA from the skin and soft tissue infections, we constructed a PPI network using overlapping genes. The intersection genes were introduced into the STRING database to construct a
protein interaction network, and the lowest interaction score was greater than "0.900". After hiding the free points, the first three bases of the PPI network map were IL-6, IL-1β, and TNF, respectively. The size of a gene node is related to the degree value. The larger the node, the more important the node is in the network (Figure 4).

2.4 Enrichment Analysis of Sanhuang Ointment on Therapeutic Targets of MRSA in Skin and Soft Tissue Infections

To further understand the pharmacological mechanism of Sanhuang ointment against MRSA in the skin and soft tissue infections, GO functional and KEGG pathway enrichment analysis of 34 common targets was performed using the DAVID database. The threshold was set at p < 0.05. We found that biological process functions were mainly related to inflammatory responses, responses to xenogenic stimuli, and defense responses to bacteria. Cellular components are mainly associated with membrane rafts (membrane rafts), membrane microdomains, and plasma membrane rafts. The molecular function is mainly related to cytokine activity, heme binding, and protein homodimer activity (Figure 5). The results of KEGG analysis showed that Sanhuang ointment treatment for MRSA infection was mainly associated with the inflammatory response. The top 10 pathways were the AGE-RAGE signaling pathway in diabetic complications, Fluid shear stress and atherosclerosis, Lipid and atherosclerosis, Pathways in cancer, IL-17 signaling pathway, Chagas disease, TNF signaling pathway, Malaria, and Rheumatoid arthritis (Figure 6). The intersection targets were shown in the IL-17/NF-κB signaling pathway in KEGG, and the results are shown in Figure 7.

2.5 Screening of Bioactive Compounds by UPLC-MS/MS Analysis

The present approach identified 743 bioactive compounds in Sanhuang ointment (Table 2), and some important bioactive compounds in the Sanhuang ointment compound-target network were identified, such as quercetin, apigenin, beta-sitosterol, Stigmasterol, magnoflorine, wogonin, columbamine, palmatine, baicalein, and Isocorypalmine.

2.6 Pathological Changes in Infected Skin Tissues

The skin tissue structure of rats in the blank group was clear. In the model group, the squamous epithelium of the epidermal layer of the skin tissue was generally thickened, the epidermal structure in the abscess area was destroyed and the level was unclear; the dermis layer became thinner and the staining was deepened, the collagen fibers accumulated into sheets and clumps, and a large number of inflammatory cells infiltrated in each layer; the subcutaneous tissue structure was reduced. In the Sanhuang ointment high dose group, collagen fiber accumulation in the infected tissue was significantly reduced, the surrounding granulation tissue was filled, and there were new thin-walled capillaries. In Sanhuang ointment low dose group, the degree of injury of infected tissue was more serious, collagen fibers accumulated into cords, the necrotic layer increased compared with Sanhuang ointment high dose group, and granulation tissue filling decreased compared with Sanhuang ointment high dose group. See Figure 8.
2.7 Eyme-linked immunosorbent assay (ELISA) was used to determine the contents of interleukin-1β, interleukin-4, interleukin-5, interleukin-6, interleukin-17, tumor necrosis factor-α and interferon-α in serum and skin tissues of rats

Compared with the blank group, the contents of IL-1β, IL-4, IL-5, IL-6, IL-17, TNF-α, and IFN-γ in the serum and skin tissues of rats in the model group were significantly increased, and the differences were statistically significant (P < 0.01); compared with the model group, the contents of IL-1β, IL-4, IL-5, IL-6, IL-6, IL-17, TNF-α, and IFN-γ in the serum and skin tissues of rats in the Sanhuang ointment high and medium dose groups were significantly reduced, and the differences were statistically significant (P < 0.05, P < 0.01). Results are shown in Figure 9.

2.8 Expression of Interleukin-1β, Interleukin-4, Interleukin-5, Interleukin-6, Interleukin-17, Tumor Necrosis Factor-α, Interferon Nucleus-γ, TNF receptor Associated Factor 6, Transforming Growth Factor Kinase 1, Transforming Growth Factor Kinase 1 Binding Protein 1, Kappa B Inhibitor Kinase β, Nuclear Factor-κB p65 mRNA in Rat Skin Tissue

Compared with the blank group, IL-1β, IL-4, IL-5, IL-6, IL-17, TNF-α, IFN-γ, TRAF6, TAK1, TAB1, IKKβ, and NF-κB p65 mRNA expression in the skin tissue of rats in the model group was significantly increased, and the difference was statistically significant (P < 0.01); compared with the model group, IL-1β, IL-4, IL-5, IL-6, IL-17, TNF-α, IFN-γ, TRAF6, TAK1, TAB1, IKKβ, and NF-κB p65 mRNA expression in the skin tissue of rats in the Sanhuang ointment high and medium dose groups was significantly decreased, and the difference was statistically significant (P < 0.05, P < 0.01). Results are shown in Figure 10.

2.9 Expression of TNF receptor-associated factor 6, transforming growth factor kinase 1, transforming growth factor kinase 1 binding protein 1, kappa B inhibitor kinase β, nuclear factor-κB p65 nuclear factor-κB p65 protein in rat skin infected tissues

Compared with the blank group, the expression of TNF receptor-associated factor 6, transforming growth factor kinase 1, transforming growth factor kinase 1 binding protein 1, kappa B inhibitor kinase β, and nuclear factor-κB p65 NF-κB p65 protein in the skin tissue of rats in the model group was significantly increased, and the difference was statistically significant (P < 0.01); compared with the model group, the expression of NF-κB p65 protein in the skin tissue of rats in the Sanhuang ointment high and medium dose groups was significantly decreased, and the difference was statistically significant (P < 0.05, P < 0.01). Results are shown in Figure 11.

3. Discuss

According to the characteristics of clinical symptoms of local soft tissue infection on the body surface, which are often accompanied by "redness, swelling, heat, and pain", it belongs to the category of yang syndrome of "swelling ulcer" in TCM and is mostly treated from the pathogenesis of "heat toxin", with significant effect.
In this study, 275 potential targets of Sanhuang ointment were screened using a network pharmacology approach. Thirty-four common targets were achieved when intersecting 328 methicillin-resistant S. aureus infection targets. Enrichment analysis identified 488 biological processes, 32 molecular functions, 20 cellular components, and 76 signaling pathways. We found that 254 active Sanhuang ointment components mainly regulate NOS2, IL6, TNF, NOS3, CXCL8, IL1B, CCL2, IFNG, IKBKG, ICAM1, and other related target proteins. They are also involved in regulating key biological pathways, such as inflammation, and bacterial infection, and may treat MRSA infection via the IL-17/NF-κB signaling pathway.

IL-17 is an effector cytokine of the innate and adaptive immune systems involved in antimicrobial host defense and maintenance of tissue integrity\(^{18}\). Signaling through the IL-17RA-IL-17RC heterodimeric receptor complex triggers homotypic interactions of IL-17RA and IL-17RC chains with the TRAF3IP2 linker. This leads to downstream TRAF6-mediated activation of NF-kappa-B and MAP kinase pathways, ultimately resulting in transcriptional activation of cytokines, chemokines, antimicrobial peptides, and interferon matrix metalloproteinases, accompanied by potentially strong immune inflammation\(^{18-23}\) studies confirming that IL-17 expression levels are increased in infected tissues and sera of MRSA rats infected with skin soft tissue infections, inducing inflammatory responses and the release of other inflammatory factors (IL-1β, IL-4, IL-5, IL-6, TNF-α, and IFN-γ) in rat skin soft tissue, thereby imbalance the host inflammatory response\(^{24}\). NF-κB has pleiotropic regulatory functions that bind to multiple promoters and participate in the regulation of multiple inflammatory genes\(^{25}\). When inflammatory changes occur in the skin and soft tissue, serum IL-17 levels rise, which induces NF-κB activity and promotes inflammation in the skin and soft tissue, and activated NF-κB in turn can reverse promote inflammatory factor expression and lead to skin and soft tissue injury\(^{26}\).

TAB1 is an adaptor protein related to the N-terminal kinase domain of transforming growth factor β-activated kinase 1 (TAK1) and is an essential binding protein for sustained TAK1 activation\(^{27}\). TAK1 is a key molecule in the IL-17/NF-κB signaling pathway that phosphorylates IkB kinase (IKK) upon binding to TAB1, ultimately leading to nuclear translocation of the transcription factor NF-κB and promoting downstream inflammatory factor production\(^{28}\). Mammalian NF-κB is a family of five related proteins that bind to form dimers, including p50 and p65, which contain the major NF-κB transcriptional activity, thus it is responsible for the expression of a large number of proinflammatory mediators and promotes innate immune cell leukocyte recruitment\(^{26}\). When the body is infected by microorganisms, the inflammatory response initiates gene expression of a large number of pro-inflammatory cytokines and chemokines through the activation of NF-κB\(^{29}\). These pro-inflammatory cytokines TNFα and IL-1β can also directly activate the NF-κB pathway, and this positive feedback effect contributes to the amplification of the inflammatory response, which persists at the site of infection and helps clear invading pathogens. Whereas recruited leukocytes, neutrophils are quite important for regulating the inflammatory response because neutrophils function in an unfavorable microenvironment, NF-κB regulates the survival of these neutrophils. NF-κB regulates the transcription of many genes, and post-transcriptionally expressed proteins are widely involved in cell
adhesion, differentiation, proliferation, angiogenesis, and apoptosis in addition to immune responses, inflammatory responses\textsuperscript{30}.

A rat model of MRSA infection in the skin and soft tissue was used to observe the effect of external application of Sanhuang ointment on infected soft tissue, and the anti-inflammatory mechanism of Sanhuang ointment was explored from the pathological changes and the expression of key molecules of signaling pathways in serum and infected tissues. The results showed that compared with the blank group, the contents of IL-1\(\beta\), IL-4, IL-5, IL-6, TNF-\(\alpha\), IFN-\(\gamma\), and IL-17 downstream inflammatory factors of IL-17/NF-\(\kappa\)B signaling pathway in the serum and skin tissues of rats in the model group were significantly increased, and the protein and mRNA expressions of TRAF6, TAK1, TAB1, IKK, and NF-\(\kappa\)B p65 were significantly increased, suggesting that the skin tissues were significantly infected and the inflammatory response was aggravated. Compared with the model group, after Sanhuang ointment external application treatment, the general condition and pathological changes of rats were significantly improved, and the expression of the above factors was reduced to varying degrees, indicating that Sanhuang ointment external application can reduce MRSA-induced skin and soft tissue inflammation in rats, which may play an anti-inflammatory role by inhibiting the expression of key factors of IL-17/NF-\(\kappa\)B signaling pathway, thereby reducing the release of downstream pro-inflammatory factors. In this study, the target and signal transduction pathway of Sanhuang ointment were screened by network pharmacology and verified by animal experiments to reveal its mechanism of action in the treatment of MRSA in the skin and soft tissue infections, providing a scientific basis and ideas for future research. In summary, Sanhuang ointment may inhibit the inflammatory response of MRSA in the skin and soft tissue infections by targeting the IL-17/NF-\(\kappa\)B signaling pathway. In addition, other mechanisms of Sanhuang ointment in the treatment of skin and soft tissue infections remain to be further investigated.

4. Material And Methods

4.1 Collection, Screening, and Target Prediction of the Chemical Composition of Sanhuang Ointment

By consulting the Systematic Pharmacology Database of Traditional Chinese Medicine (TCMSP, https://tcmspw.com/TCMSP.php)\textsuperscript{31}, the active ingredient of Sanhuang ointment is predicted. The active ingredient of Sanhuang ointment was identified using a drug similarity (DL) of $\geq 0.18$ as the screening threshold. Because Sanhuang ointment is a topical formulation, its first-pass effect is not related to liver and kidney metabolism but acts directly on target organs. Therefore, oral bioavailability cannot be used as a screening condition. Finally, all target names were converted into standard gene symbols using the UniProt database (https://www.UniProt.org/).

4.2 MRSA Infections Target Search

The keyword 'Methicillin-resistant Staphylococcus aureus infection' was used to screen for relevant targets. In the Gene Cards database (https://www.genecards.org/), Drug Bank database (https://go.drugbank.com/), TTD database (http://db.idrblab.net/ttd/), the Disgenet database
4.3 Network Construction and Analysis

The active ingredient of Sanhuang ointment and the corresponding target was introduced into Cytoscape 3.7.2 (https://Cytoscape.org/index.html), software, to construct an "active component-target" network diagram. Using the Venny platform (https://bioinfogp.cnb.csic.es/tools/venny/index.html), the target genes of Sanhuang ointment and disease were intersected. Intersection genes were selected as potential targets for Sanhuang ointment intervention during MRSA infection.

4.4 Construction and Analysis of Interaction Network

Introducing Sanhuang ointment and common targets of disease into the STRING database (https://string-db.org/cgi/input.pl), to construct protein interaction networks. The species was selected as 'Homo sapiens' with a minimum interaction score greater than '0.900' and PPI network maps were exported after hiding free points. The active ingredients and corresponding targets of Sanhuang ointment were imported into Cytoscape 3.7.2 software to construct the "active component-target" network diagram.

4.5 Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis

To explore the pathway and biological process of the network, Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analysis were performed using the cluster analysis package in the DAVID database platform. Gene Ontology enrichment analysis included cellular components, biological processes, and molecular functions. An enrichment analysis statistical significance threshold of $p \leq 0.01$ was established.
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Table 1 Main chemical constituents of Sanhuang ointment

4.6 Preparation of Sanhuang Ointment

Sanhuang ointment is composed of Coptidis Rhizoma, Scutellariae Radix, and Phellodendri Chinnsis Cortex according to the Chinese Pharmacopoeia 2020 edition. All medicinal materials were purchased from Lanzhou Fuci Pharmaceutical Industry Development Group Co., Ltd. These medicinal materials were identified by Professor Guotai Wu of Gansu University of Traditional Chinese Medicine and stored in the pharmacy of the Affiliated Hospital of Gansu University of Traditional Chinese Medicine. Preparation of Sanhuang plaster powder: 60 g of Coptidis Rhizoma, 30 g of Phellodendri Chinnsis Cortex, and 30 g of
Scutellariae Radix were washed with water and dried. The above drugs were mixed, crushed, passed through a 100-mesh sieve, dispensed, and sterilized. Preparation of Sanhuang ointment: 36 g sesame oil was placed in a stainless steel pot and heated to boiling; 36 g petrolatum was added, and after melting, Sanhuang ointment powder was added and mixed thoroughly by stirring until completely solidified. Dispense, then.

4.7 Phytochemical Analysis of Sanhuang ointment

4.7.1 Sample preparation and extraction

Take 50 mg of the mixed sample and place it in a 2 mL EP tube. Add 1200 μL 70% methanol internal standard extract solution, vortex for 15 min. Centrifuge (12000 r/min, 4 °C) for 10 min, the supernatant was filtered with a microporous filter membrane (0.22 μm) and store in the injection bottle for LC-MS/MS test.

4.7.2 UPLC Conditions

The sample extracts were analyzed using an UPLC-ESI-MS/MS system (UPLC, ExionLC™ AD https://sciex.com.cn/; MS, Applied Biosystems 6500 Q TRAP, https://sciex.com.cn/). The analytical conditions were as follows, UPLC: column, Agilent SB-C18 (1.8 μm, 2.1 mm * 100 mm); The mobile phase was consisted of solvent A, pure water with 0.1% formic acid, and solvent B, acetonitrile with 0.1% formic acid. Sample measurements were performed with a gradient program that employed the starting conditions of 95% A, 5% B. Within 9 min, a linear gradient to 5% A, 95% B was programmed, and a composition of 5% A, 95% B was kept for 1 min. Subsequently, a composition of 95% A, 5.0% B was adjusted within 1.1 min and kept for 2.9 min. The flow velocity was set as 0.35 mL per minute; The column oven was set to 40°C; The injection volume was 2μL. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS.

4.7.3 ESI-Q TRAP-MS/MS

The ESI source operation parameters were as follows: source temperature 500°C; ion spray voltage (IS) 5500 V (positive ion mode)/-4500 V (negative ion mode); ion source gas I (GS1), gas II(GSII), curtain gas (CUR) were set at 50, 60, and 25 psi, respectively; the collision-activated dissociation(CAD) was high. QQQ scans were acquired as MRM experiments with collision gas (nitrogen) set to medium. DP (declustering potential) and CE (collision energy) for individual MRM transitions was done with further DP and CE optimization. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period.

4.8 Experimental Animals

Ninety-six SPF healthy Wistar rats, half male and half female, weighing (280 ± 10) g, were provided by the Medical Experimental Center of Gansu University of Traditional Chinese Medicine with animal license number SCXK (Gan) 2015-0002. They were housed in the SPF laboratory of the Scientific Research
Experimental Center, Gansu University of Traditional Chinese Medicine. Experimental animals were handled by animal ethical principles. The study was approved by Ethics Committee of Institutional Committee for the Protection and Use of Animals at Gansu University of Chinese Medicine(2022-087) all methods were carried out in accordance with relevant guidelines and regulations. This study was carried out in compliance with the ARRIVE guidelines.

4.9 Main Reagents

Sanhuang Ointment, provided by Affiliated Hospital of Gansu University of Traditional Chinese Medicine, batch number (210329); Mupirocin Ointment, Sino-American Tianjin Shi Ke Pharmaceutical Co., Ltd., batch number 3L4K; MRSA (ATCC 25923), a gift from Clinical Medical Translation Center of Gansu Provincial People's Hospital; IL-4, IL-5, IL-17, TNF-α, IL-1β, IL-6, IFN-γ ELISA kits (article numbers JL13252, JL13268, JL13282, JL13202, JL20884, JL20897, JL207308, Shanghai Future Industrial Co., Ltd.; TRAF6, TAK1, TAB1, IKKβ, NF-κB p65 antibodies (batch numbers GR3277367-3, GR190324-31, GR3273233-1, GR117080-30, GR32932611), GeneTex, USA; TRlzol (batch No. 152104), Ambion, USA; reverse transcription kit, RT-qPCR kit (batch numbers AI40704A, AI61180A, respectively), TaKaRa, Japan; GAPDH antibody (batch No. B1501), ImmunoWay, USA.

4.10 Animal Grouping, Model Making, and Intervention

Ninety-six Wistar rats were divided into blank group, model group, Mupirocin Ointment group, and Sanhuang ointment high, medium, and low dose groups according to a random number table after 1 week of adaptive feeding, with 16 rats in each group. According to the method designed by Malachowa et al.32, each rat was depilated in a 2 cm × 2 cm area marked with a signature pen on the near cervical side of the back using the spine as the midline, the blank group did not receive any treatment, and the other groups of rats were subcutaneously injected with 1 mL MRSA bacterial suspension at a concentration of 6 × 10⁸ CFU/mL, with purulent infection foci in the depilated area showing successful modeling. On the next day after model establishment, the external application was started, Mupirocin Ointment was applied at 0.5 g/mouse in the Mupirocin Ointment group (the total amount of ointment adjusted with vaseline was 1 g/mouse), 1, 0.5, and 0.25 g/mouse in the Sanhuang Ointment high, medium, and low dose groups (the total amount of ointment adjusted with vaseline was 1 g/mouse), and the same amount of vaseline was applied twice daily for 7 days in the blank and model groups. The general condition and soft tissue infection of rats were observed daily. The changes in skin tissue before and after modeling are shown in Figure 12.

4.11 HE Staining for Detection of Infected Histopathology

Infected tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, deparaffinized in xylene, dehydrated in graded ethanol, stained with hematoxylin, differentiated in hydrochloric acid alcohol, dehydrated in graded ethanol, stained with eosin, cleared in xylene, mounted in neutral gum, and observed under a light microscope.
4.12 Detection of Infected Tissue and Serum by Enzyme-linked Immunosorbent assay

According to the instructions of the ELISA kit, the absorbance was measured at 450 nm of the microplate reader, the standard curve was drawn, and the contents of IL-1β, IL-4, IL-5, IL-6, IL-17, TNF-α, and IFN-γ in serum and infected tissues were calculated.

4.13 Detection of IL-1β, IL-4, IL-5, IL-6, IL-17, TNF-α, and IFN-γ mRNA Levels in Serum and Infected Tissues by qRT-PCR

Total RNA was extracted from infected tissues by the Trizol method, cDNA was synthesized by reverse transcription using RNA as a template, and the reaction system and parameters were set according to the qRT-PCR kit instructions for PCR. Relative mRNA expression was calculated by the $2^{-\Delta\Delta C_t}$ method using β-actin as an internal reference. Primers were synthesized by Bao Biological Engineering (Dalian) Co., Ltd. Primers sequences are shown in Table 3.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer sequences (5’-3’)</th>
<th>Length of output/bp</th>
</tr>
</thead>
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<tr>
<td>β-actin</td>
<td>F: GGAGATTACTGCCCTGGCTCCTA</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>R: GACTCATCGTACTCCTGCTTGC</td>
<td></td>
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<tr>
<td>IL-1β</td>
<td>F: CCTGAACTCACTGTGAAATAGCA</td>
<td>123</td>
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<tr>
<td></td>
<td>R: CCCAAGTCAAGGGCTTGGAA</td>
<td></td>
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<tr>
<td>IL-4</td>
<td>F: TGCACCAGAGATTTGTACCAGA</td>
<td>123</td>
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<td></td>
<td>R: TTGCGAAGCACCTGGGAAG</td>
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<tr>
<td>IL-5</td>
<td>F: CCTTGATACAGCTGTCCACTCAC</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>R: CCCTCGGACAGTTTGATTCTT</td>
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<tr>
<td>IL-6</td>
<td>F: TTGTATGACAGCGATGCAC</td>
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<tr>
<td></td>
<td>R: CCAGGTAGAAAACGGAACCTCCAGA</td>
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</tr>
<tr>
<td>IL-17</td>
<td>F: AGCGTGCTCAAACACTGAGG</td>
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<tr>
<td></td>
<td>R: ACGTGAACCGGTGAGGTAG</td>
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</tr>
<tr>
<td>TNF-α</td>
<td>F: TTCCAATGGGCTTTCGGAAC</td>
<td>118</td>
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<tr>
<td></td>
<td>R: AGACATCTTCAGCGCCTTTGGAG</td>
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<tr>
<td>IFN-γ</td>
<td>F: TCCTGCAGCATAGCAGATGTA</td>
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<td></td>
<td>R: CCAGGATTTAAACGGGATTC</td>
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<tr>
<td>TRAF6</td>
<td>F: CAGTGTCGATCGTGCACTTAA</td>
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<td></td>
<td>R: CCTTATGTTTCTTGAGTTC</td>
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<tr>
<td>TAK1</td>
<td>F: TATGCTGAAGGAGGCTCGTGTTA</td>
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<td></td>
<td>R: AGGCTTGAGTCCCTATGAATG</td>
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<tr>
<td>TAB1</td>
<td>F: CTGGAGAGGTTGGAGGACGA</td>
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<td></td>
<td>R: TCGCAAGAACCAGAATAAGAATG</td>
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<td>IKKβ</td>
<td>F: GCACCCCTGGCCTTTGAATG</td>
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<td>R: TCCGTTCAAGTCTCGCTAACA</td>
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<td>NF-κB p65</td>
<td>F: TCTTCGACTACGGGTGACCTAAGC</td>
<td>133</td>
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<tr>
<td></td>
<td>R: CTCACGGGCTGAGCATGAAGG</td>
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</table>
Table 3 PCR primer sequences of each gene.

4.14 Detection of TRAF6, TAK1, TAB1, IKKβ, and NF-κB p65 Protein Expression in Rat Infection Tissue by Immunocytochemistry

The infected paraffin sections were placed in a constant temperature oven at 72°C for 1 hour, then placed in a machine for deparaffinization, and then washed three times with PBS buffer at pH 7.5 for 5 minutes each time. The slices were laid flat in an incubation box, 80 μl of endogenous peroxidase blocking agent was added dropwise to each slice, incubated at room temperature for 15 minutes, and then washed three times in PBS buffer for 5 minutes each time. Antigen retrieval and blocking Sections were placed in citrate buffer with pH 6.0 and placed in a microwave oven for antigen retrieval. After the repair is completed, it is placed on the incubation box and plated with goat serum, and incubated at room temperature for 30 minutes to block the endogenous antibodies of the tissue. Antibody Incubation Decant the blocking solution on the sections and add approximately 80 μl of the corresponding primary antibodies (TRAF6, TAK1, TAB1, IKKβ, and NF-κB p65) dropwise to each section. Add a small amount of deionized water to the incubator to prevent dry slices, and close the lid. Incubate for 1 hour at room temperature, then incubate overnight (14-16 hours) in a 4°C refrigerator. After the incubation, the incubation box was taken out of the refrigerator and rewarmed at room temperature for 30 minutes. The primary antibody was poured off, and the sections were gently rinsed with PBS buffer. After each section was rinsed once, it was placed in the same staining tank, and then immersed in PBS buffer three times for 5 minutes each time. PBS was removed, and 80 μl of secondary antibody conjugated to horseradish peroxidase (HRP) was added dropwise, and incubated at room temperature for 30 minutes. Color development, counterstaining and mounting After incubation, the secondary antibody was discarded and washed three times in PBS buffer for 5 minutes each time. Prepare fresh DAB chromogenic solution (850μl deionized water + 150μl working solution). When developing the color, place the slide under the microscope, add about 60 μl of color developing solution dropwise, observe the color change under the light microscope and control the reaction time according to the color. Immediately place the sections in tap water to terminate the reaction. All sections were rinsed with tap water for 10 minutes after color development, and then counterstained in hematoxylin for 10-20 seconds (staining time is determined according to the freshness of the hematoxylin preparation, and overstaining is not allowed). After counterstaining, the sections were quickly placed in tap water to terminate, and rinsed for 10 minutes. The sections were naturally dried, a drop of 10% neutral gum was added dropwise to cover the slides, and the coverslips were covered to remove air bubbles..

4.15 Statistical Analysis

Comparison analysis was performed using one-way analysis of variance (ANOVA) using GraphPad Prism 9 software to compare differences between groups and to compare the least significant difference (LSD). Significantly different at the two-sided α = 0.05 and p < 0.05 test levels.

Declarations
Author Contributions Statement

Tianming Wang, Ying Che, Qi Fu, Qian Chen, and Xiaoli Li conducted the experiments, and Tianming Wang drafted the manuscript. Yan Cui, Quanxin Chen, Zhihang Wu, Richeng Li, and Mei Liu collated and analyzed the experimental data, and the experiments were developed by Jianfeng Yi, Bo Wang, and Haibang Pan. All authors discussed the results, reviewed the manuscript, and agreed to the final version for publication.

Data Availability Statement

The original data supporting the conclusions of this paper will be provided by the authors without unnecessary reservations.

Additional Information

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References


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**Tables**

Table 2 is available in the Supplemental Files section.

**Figures**
Figure 1

Flow chart of network pharmacological analysis and validation of Sanhuang ointment against MRSA infection.
Figure 2

Network diagram of active ingredients and targets of Sanhuang ointment.
Figure 3

The intersection of target genes of Sanhuang ointment and disease target genes using the Venn diagram.
To comprehensively explore the core pharmacological mechanism of Elian granules in the treatment of PLGC. We constructed a PPI network using the first 50 overlapping genes. Among them, the top three bases are IL-6, IL-1β, and TNF-α. The size of the gene node is linked to the degree value. The larger the node, the more prominent the node is in the network.
Figure 5

GO enrichment analysis of 34 crosspoint targets. The X-axis represents the top 10 significantly enriched biological processes, cellular composition, and molecular function categories. Y-axis represents -log p-values.
Figure 6

KEGG enrichment analysis of 34 cross-targets. Y-axis represents the top 20 significantly enriched pathways. The X-axis shows the ratio of enriching target genes to background genes. The size of the dots indicates the number of target genes in the pathway, and their color range reflects the different p-values.
Figure 7

The intersection targets were shown in the IL-17/NF-κB signaling pathway in KEGG.
Figure 8

Histomorphology of the skin of rats in each group (HE staining, ×40).

Note: A. Blank group; B. Model group; C. Mupirocin Ointment group; D. Sanhuang Ointment high dose group; E. Sanhuang Ointment medium dose group; F. Sanhuang Ointment low dose group
Figure 9

Comparison of IL-1β, IL-4, IL-5, IL-6, IL-17, TNF-α, and IFN-γ contents in serum and skin tissue of rats in each group (x±s).

Note: Compared with the blank group, **P<0.01; compared with the model group, #P<0.05, ##P<0.01
Figure 10

Comparison of IL-1β, IL-4, IL-5, IL-6, IL-17, TNF-α, IFN-γ, TRAF6, TAK1, TAB1, IKKβ, and NF-κB p65 mRNA expression in skin tissue of rats in each group (x±s).

Note: Compared with the blank group, **P<0.01; compared with the model group, #P<0.05, ##P<0.01
Figure 11

Comparison of TRAF6, TAK1, TAB1, IKKβ, and NF-κB p65 protein expression in skin tissue of rats in each group (x±s).

Note: Compared with the blank group, **P<0.01; compared with the model group, #P<0.05, ##P<0.01

Figure 12

Before Modeling

After Modeling
Comparison chart before and after modeling.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Tabe2.xlsx
- supplementaryfiles1.zip