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Analysis and Experimental Validation of the Anti-Tuberculosis Mechanism of NiuBeiXiaoHe, a Traditional Chinese Medicine

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Abstract

NiuBeiXiaoHe (NBXH), a traditional Chinese medicine, is used to treat tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB), but its mechanism of action remains unclear. In this study, we screened 26 active compounds in NBXH linked to 119 potential anti-TB targets using network pharmacology and the TCMSP database, combined with GeneCards. The binding affinity of these compounds to the target proteins was evaluated using the LibDock module of Discovery Studio 2019. The results of the DAVID database analysis indicated that the therapeutic targets of NBXH are involved in 41 KEGG pathways related to the TB treatment. Experimental validation in a mouse TB model treated with NBXH showed significant therapeutic effects through gene expression analysis, quantitative bacterial culture, and pathological examination. Compared to the model group, NBXH-treated mice showed altered gene expression, affecting KEGG pathways, reduced lung tissue colony counts, and mild pathological changes. This study preliminarily elucidates the anti-TB mechanism of NBXH, paving the way for future clinical trials and drug development.

Keywords: NBXH; Tuberculosis; Mechanism; Network Pharmacology; Gene Expression Profile; Bioinformatics Analysis

Introduction

Tuberculosis remains a global health problem. According to the World Health Organization (WHO) report, the number of new active TB cases and deaths worldwide in 2023 was approximately 10.8 million and 1.25 million. Additionally, the number of new TB cases in China accounted for 6.8% of the global total [1]. The emergence of drug-resistant TB, particularly multidrug-resistant (MDR) TB, poses a significant challenge to global TB control efforts.

Currently, the mainstay of TB treatment is chemotherapy with a prolonged treatment cycle. Patients with MDR-TB or severe liver and kidney damage face the dilemma of limited treatment options. Traditional Chinese Medicine (TCM) believes that the external cause of TB is infection with MTB, while the internal cause is weakness of the body's vital energy (qi), insufficient qi and blood, and depletion of yin and essence. Therefore, MTB is a factor in the occurrence of the disease, deficiency of vital Qi is an important basis for the occurrence of the disease, and infection with MTB due to body deficiency is the key to the formation of this disease. For thousands of years, TCM has played an extremely important role in the treatment and control of TB, particularly before the invention of chemotherapy drugs, accumulating rich clinical experience and establishing the treatment principle of "tonifying deficiency to restore true vitality and eliminating pathogens to eradicate the root cause". tonifying deficiency and restoring its essence" means strengthening the body's anti-TB immunity, balancing the disorder of yin and yang in the internal organs during the occurrence and development of diseases, and emphasizing the purpose of treating TB by adjusting the internal factors of the body and enhancing its own repair ability. “Eliminating pathogens” refers to mobilizing the body's anti-TB immunity to inhibit or remove MTB. TCM offers advantages such as rich compounds, multiple targets, multiple pathways, and extensive effects. It acts on the body's target networks, tonifying deficiencies and strengthening vitality, nourishing yin and clearing heat, regulating and enhancing the patient's immune function, and improving TB symptoms, but its antibacterial effect is weaker than that of chemical drugs. On the other hand, chemotherapy has a robust bactericidal ability, but it is generally a single effective component with a relatively single target, which can easily lead to drug resistance. Therefore, the combination of TCM and chemotherapy for the treatment of TB offers complementary advantages, significantly improving clinical symptoms, increasing the negative rate of sputum bacteria, promoting lesion absorption and cavity closure, improving treatment efficacy, and reducing the incidence of adverse reactions [2].

Through long-term clinical practice and considering the clinical characteristics of TB, our group has developed a practical prescription called NBXH, mainly composed of *Fritiliariae Cirrhosae Bulbus (Chuan Bei Mu), Houttuyniae Herba, Bletilla Striata [Bletilla Striata (Thunb.Ex A.Murray) Rchb.F.], Platycodon Grandiforus [Platycodon grandiflorum (Jacq.) DC.]*, and *Fructus Arctii*. NBXH has the effects of moistening the lungs, relieving cough, clearing heat, resolving phlegm, eliminating carbuncles, and expelling pus, demonstrating improved outcomes in the treatment of TB [3-12]. This study analyzed the interactions among drug active ingredients, effective targets, and diseases through network pharmacology, and validated the key genes and their regulatory pathways through gene expression profiles of a mouse TB model treated with NBXH, to explore the potential targets and mechanisms of action of NBXH in the treatment of pulmonary TB.

Materials and Methods

Network Pharmacology Analysis of NBXH

Identification of Active Compounds and their Targets in NBXH

The bioactive compounds in NBXH were identified by querying the TCMSP (https://old.tcmsp-e.com/tcmsp.php) database with the following TCM herbs: "*Fritillariae Cirrhosae Bulbus*", "*Houttuyniae Herba*", "*Bletilla Striata*", "*Platycodon Grandiflorus*", and "*Fructus Arctii*". To ensure the selection of high-quality, potentially effective compounds, screening filters for oral bioavailability (OB) ≥ 30% and drug-likeness(DL) ≥ 0.18 were applied, as recommended in the literature [13]. The targets for the identified compounds of NBXH were obtained from the TCMSP database and then standardized using UniProt (https://www.uniprot.org/).

Therapeutic Targets Prediction

Tuberculosis-related targets were derived from the GeneCards (https://www.genecards.org/) using the keyword "tuberculosis" and then standardized with UniProt. The potential therapeutic targets of NBXH for TB were identified by inter-

secting the targets of the active NBXH compounds and the tuberculosis-related targets.

Molecular Docking

The NBXH active compounds and targets with higher degree values in the C-T network were selected for molecular docking to verify their affinity. The molecular structure files in mol2 format for the active compounds were retrieved from the TCMSP platform, and the target protein structures in PDB format were obtained from the PDB database (https://www.rcsb.org/). The binding affinities between these compounds and proteins were evaluated using the LibDock module of Discovery Studio 2019, with LibDock scores indicating the predicted binding strength, where higher scores suggest stronger affinities.

KEGG Pathway Analyses

The DAVID platform (https://david.ncifcrf.gov/, last updated October 12, 2023) was used to conduct Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses on the identified therapeutic targets. The analyses were confined to the human species (Homo sapiens) and adhered to a significance threshold of $p \leq 0.05$. The pathways related to TB treatment were screened.

Experimental Verification

Animals and Ethics Statement

BALB/c mice aged 6-8 weeks and weighing 17-19 grams were obtained from Vital River Laboratories (Beijing, China) for the experiments. All procedures involving the animals were reviewed and approved by the Animal Ethical Committee of the 8th Medical Center of the Chinese PLA General Hospital (Approved Number: 309201909270913). The experiments were conducted strictly following the 'Guide for the Care and Use of Laboratory Animals'.

Preparation and Drug Treatment of Tuberculosis Animal Models

Thirty BALB/c mice were randomly assigned to three groups: (1) Normal control group, which was not subjected to MTB infection and did not receive NBXH; (2) TB model group, which was infected with 5×10^5 colony-forming units (CFUs) of MTB H37Rv and later treated with distilled water; and (3) NBXH treatment group, which was infected similarly but then received daily doses of 333 mg/kg of the NBXH, prepared by Guangdong Qifang Pharmaceutical Co., Ltd., for three months. After treatment, mice were humanely euthanized for subsequent pharmacodynamic studies.

Differential Gene Screening and Analyses

Three peripheral blood samples from the Normal control group, TB model group, and NBXH treatment group were randomly selected. Mononuclear cells were isolated from the samples, and total RNA was extracted. The gene expression profiles of the peripheral blood were analyzed by Kang Cheng Biotechnology Co., Ltd. (Shanghai, China) using the Agilent Array platform. The R package limma was used to identify the significant differentially expressed genes (DEGs) between the TB model and NBXH treatment groups.

Quantitative Bacterial Culture and Pathological Analysis of the Lungs

The whole lungs and left lung lobes from mice in both the TB model group and NBXH treatment group were weighed. Following homogenization and digestion, the left lung lobe samples were diluted 100 times using phosphate-buffered saline. Subsequently, a 100μL aliquot of each diluted sample was inoculated onto Löwenstein-Jensen medium and incubated at 37℃ for 4 weeks. The total number of colonies in the whole lung was then determined by colony counting.

The right lung lobes were fixed using a 10% neutral buffered formalin solution, followed by dehydration and embedding in paraffin. Subsequently, tissue sections were prepared and subjected to hematoxylin and eosin (H&E) staining for the observation of pathological changes.

Results

Network Pharmacological Analysis

The Active Compounds and the Targets of NBXH

After conducting an exhaustive search and analysis of the TCMSP database, this study has identified that NBXH contains a total of 26 active compounds. Specifically, Fritillariae Cirrhosae Bulbus contains 13 active compounds, Houttuyniae Herba contains 7, Bletilla Striata contains 9, Platycodon Grandiflorus contains 7, and Fructus Arctii contains 8. These

compounds relate to 211 targets.

Furthermore, by searching the GeneCards database, this study collected 2,636 potential targets related to tuberculosis. By comparing these targets with the 211 targets, the study successfully identified 119 targets that may be associated with anti-tuberculosis activity (Figure 1A). These potential anti-tuberculosis targets are associated with 26 active compounds of NBXH (Figure 1B).

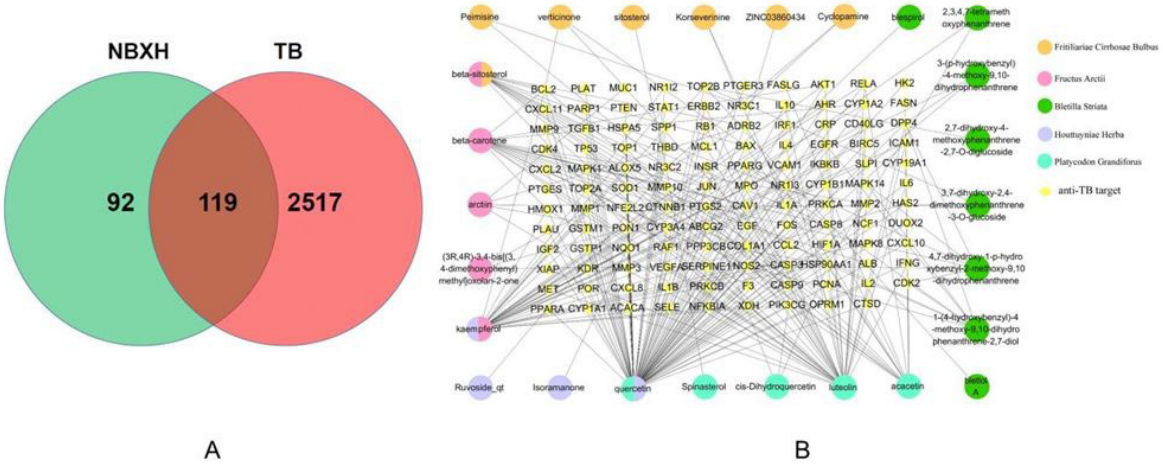

Figure 1: The tuberculosis-related targets, NBXH targets and the network of NBXH active compounds and anti-TB targets (C-T) plot.

Table 1: The active compounds of NBXH

category	compounds
phenanthrenes	2,7-dihydroxy-4-methoxyphenanthrene-2,7-o-diglucoside 3-(p-hydroxybenzyl)-4-methoxy-9,10-dihydrophenanthrene 1-(4-hydroxybenzyl)-4-methoxy-9,10-dihydrophenanthrene-2,7-diol 4,7-dihydroxy-1-p-hydroxybenzyl-2-methoxy-9,10-dihydrophenanthrene 2,3,4,7-tetramethoxyphenanthrene blespirol 3,7-dihydroxy-2,4-dimethoxyphenanthrene-3-o-glucoside bletlol A
flavonoids	quercetin acacetin cis-dihydroquercetin kaempferol luteolin
steroids	sitosterol isoramanone cyclopamine korseverinine beta-sitosterol spinasterol
others	arctiin beta-carotene verticinone ZINC03860434 Ruvoside_qt (3R,4R)-3,4-bis[(3,4-dimethoxyphenyl)methyl]oxolan-2-one peimisine

Table 2: The biological functions of the potential anti-tuberculosis targets

molecules	functions
IL1A,BIRC5,IFNG,CASP9,XDH,CASP3,IL4,TNFAIP3,BCL2,CXCL8, CXCL11,CASP8,VEGFA,NFKBIA,AHR,FASLG,IL10,AKT1,IL2,CD40LG,ICAM1, IL1B,IL6,BAX,CXCL2,CXCL10	immune modulation
AKT1,CASP3,CASP8,CCL2,CXCL10,CXCL11,CXCL2,CXCL8, IL1A,IL1B,IL2,IL4,IL6,IFNG,TNF,CRP,PTGS2,NOS2,AHR,BIRC5,BAX,BCL2, MMP1, MMP10, MMP2, MMP3, MMP9, NFKBIA, IL10, VCAM1, ICAM1, VEGFA	inflammatory response
CD40LG,FASLG,TGFB1,JUN,IL1A,MAPK8,VEGFA,ICAM1,AKT1,EGF,NOS2,PRKCB, MAPK1,IL4,PIK3CG,RAF1,PPARG,RELA,PRKCA,EGFR,FOS,INSR,ACACA,IL10, BAX,IL6,STAT1,HIF1A,MAPK14,HSP90AA1,MET,CASP9, ERBB2,CASP8,XDH,BIRC5,IL2,KDR,IFNG, HK2,CASP3,CDK2, AHR,PTEN,NFE2L2,HSPA5,BCL2,CDK4,IL1B,NFKBIA	signal transduction
AKT1,BAX,BCL2,BIRC5,CASP3,CASP8,CASP9,FASLG,MAPK1, MAPK14,MAPK8,PARP1,TP53,XIAP	apoptosis
AHR,FOS,HIF1A,JUN,NFE2L2,NFKBIA,NR1I2,NR1I3,NR3C1, NR3C2,PPARA,PPARG,RELA,STAT1,TP53,CTNNB1	transcription factors
CCL2,CXCL10,CXCL11,CXCL2,CXCL8,EGF,FASLG, IFNG,IL10,IL1A,IL1B,IL2,IL4,IL6,TGFB1,VEGFA	cytokines
CCL2,CXCL10,CXCL11,CXCL2,CXCL8,EGF,EGFR,ICAM1,IL6, KDR,MET,MMP1,MMP10,MMP2,MMP3,MMP9,PTEN,RAC1, RHOA,SERPINE1,TGFB1,VEGFA	cell migration
ACACA,AKT1,CYP1A1,CYP1A2,CYP1B1,CYP3A4,FASN,GSTM1, GSTP1,HK2,HMOX1,NFE2L2,NQO1,PPARA,PPARG,PTGES,PTGS2,XDH	metabolism
ACACA,EGF,EGFR,ERBB2,FOS,HIF1A,INSR,KDR,MAPK1,MAPK14, MAPK8,MET,MMP2,NFE2L2,NR1I2,NR1I3,NR3C1,PPARA,PPARG,TGFB1,TP53	cell differentiation
AKT1,CDK2,CDK4,EGF,EGFR,ERBB2,FOS,HK2,INSR,JUN,MAPK1, MAPK14,MAPK8,MET,PCNA,PIK3CG,PRKCA,PRKCB,PTEN, RAF1,RELA,RB1,STAT1,TP53,VEGFA	cell cycle and proliferation
HSP90AA1,HSPA5,NFE2L2,NQO1,XDH,BAX,BCL2,CASP3, FOS,JUN,MAPK1,MAPK14,MAPK8,NFKBIA,RELA,STAT1,TP53,IRE1	cellular stress
AKT1,CYP1A1,CYP1A2,CYP1B1,CYP3A4,GSTM1,GSTP1, HMOX1,NQO1,NFE2L2,PRKCA,PRKCB,PTGES,PTGS2,SOD1,XDH	oxidative stress
ICAM1,VCAM1,SELE,MUC1,CTNNB1,CAV1,ALOX5,F3, SERPINE1,THBD	cell adhesion
COL1A1,MMP1,MMP2,MMP3,MMP9,MMP10,SERPINE1,THBD	extracellular matrix
BIRC5,CASP9,VEGFA,KDR,MMP1,HIF1A,EGF,PDGF,FGF, ANGPT1,TIE1,NR1I3,EGFR,PDGFR,ANGPT2,TIE2,MMP2,MMP3,MMP9	angiogenesis

These active compounds can be categorized based on their distinct chemical structures into phenanthrenes, flavonoids, steroids, and others (Table 1). The potential anti-tuberculosis targets are significantly involved in critical biological processes, including cell signaling transduction, immune modulation, transcription factor regulation, apoptosis, and cell differentiation (Table 2).

Molecular Docking of Active Compounds and Key Target Proteins

In this study, 16 NBXH active compounds and 55 key targets with higher degree values in the C-T network were selected for molecular docking to verify their affinity. Generally, stronger binding affinity between the active compounds and targets is indicated by higher molecular docking scores. Out

of these pairs, 152 (15.64%) exhibited docking score values above 120, 557 (57.30%) displayed docking score values ranging from 60 to 120, 32 (3.29%) showed docking score values from 0 to 60, and 231 (23.77%) had docking score of zero, implying that they lacked any interaction (Figure 2A). The docking results for the 13 pairs of active compounds and targets with the highest scores were shown in Figure 2B.

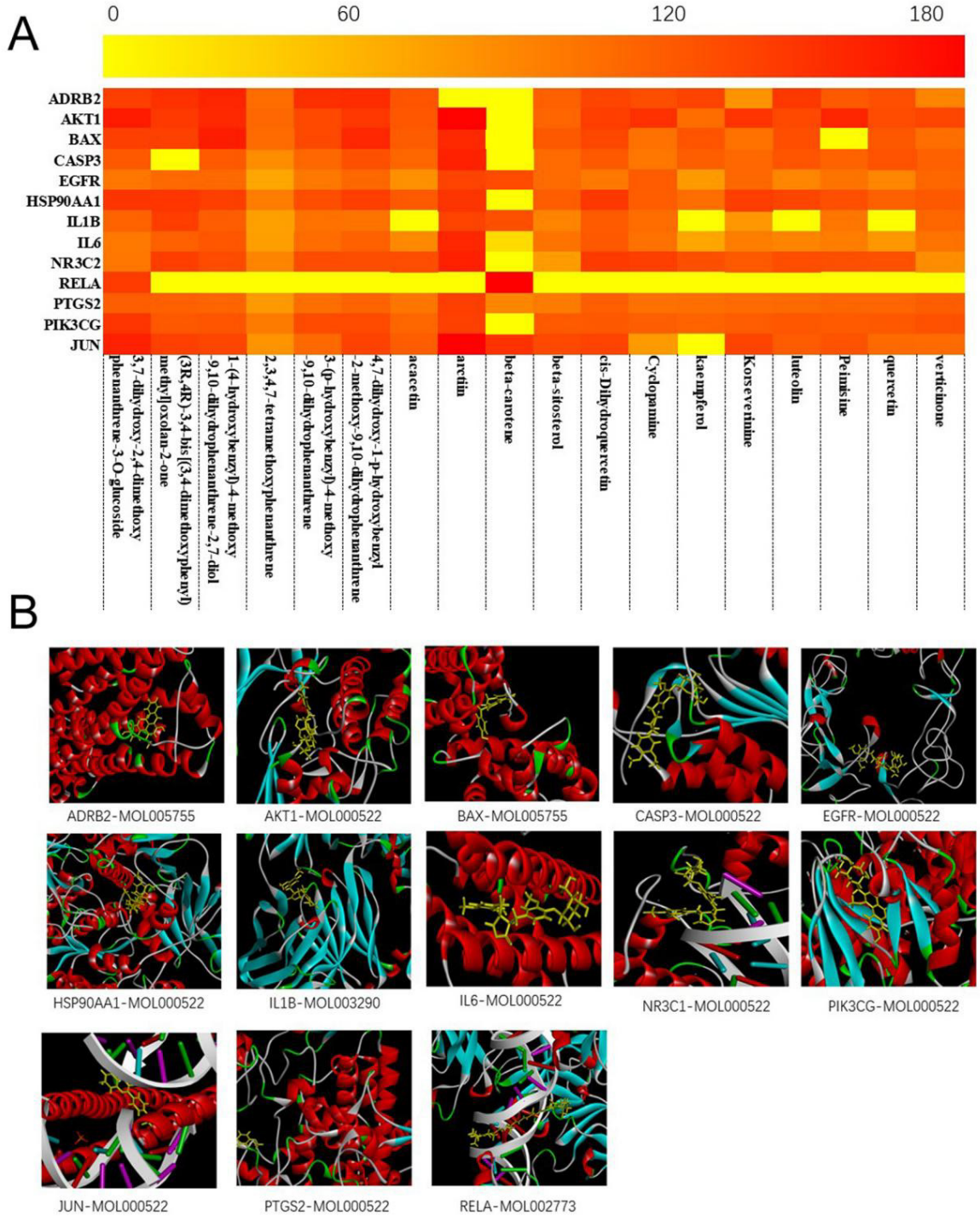

Figure 2: The molecular docking of NBXH active compounds and targets.

(A) Heatmap of compound-target docking scores. The vertical axis represents 13 target proteins, while the horizontal axis represents 16 active compounds. The color gradually deepening from yellow to red indicates a gradual increase in the docking score. (B) Molecular docking results of active compounds and 13 key targets show that the active compounds (yellow molecule) are embedded in the cavities formed by the targets.

KEGG Pathways Analysis of the Potential Anti-tuberculosis Targets

The KEGG enrichment analysis identified a total of 166 signaling pathways, of which 158 had $p < 0.05$. After careful screening, a total of 41 signaling pathways were found to be related to the treatment of tuberculosis, such as hsa04657: IL-17 signaling pathway, hsa04668: TNF signaling pathway, hsa04210: Apoptosis, hsa04659: Th17 cell differentiation (Figure 3).

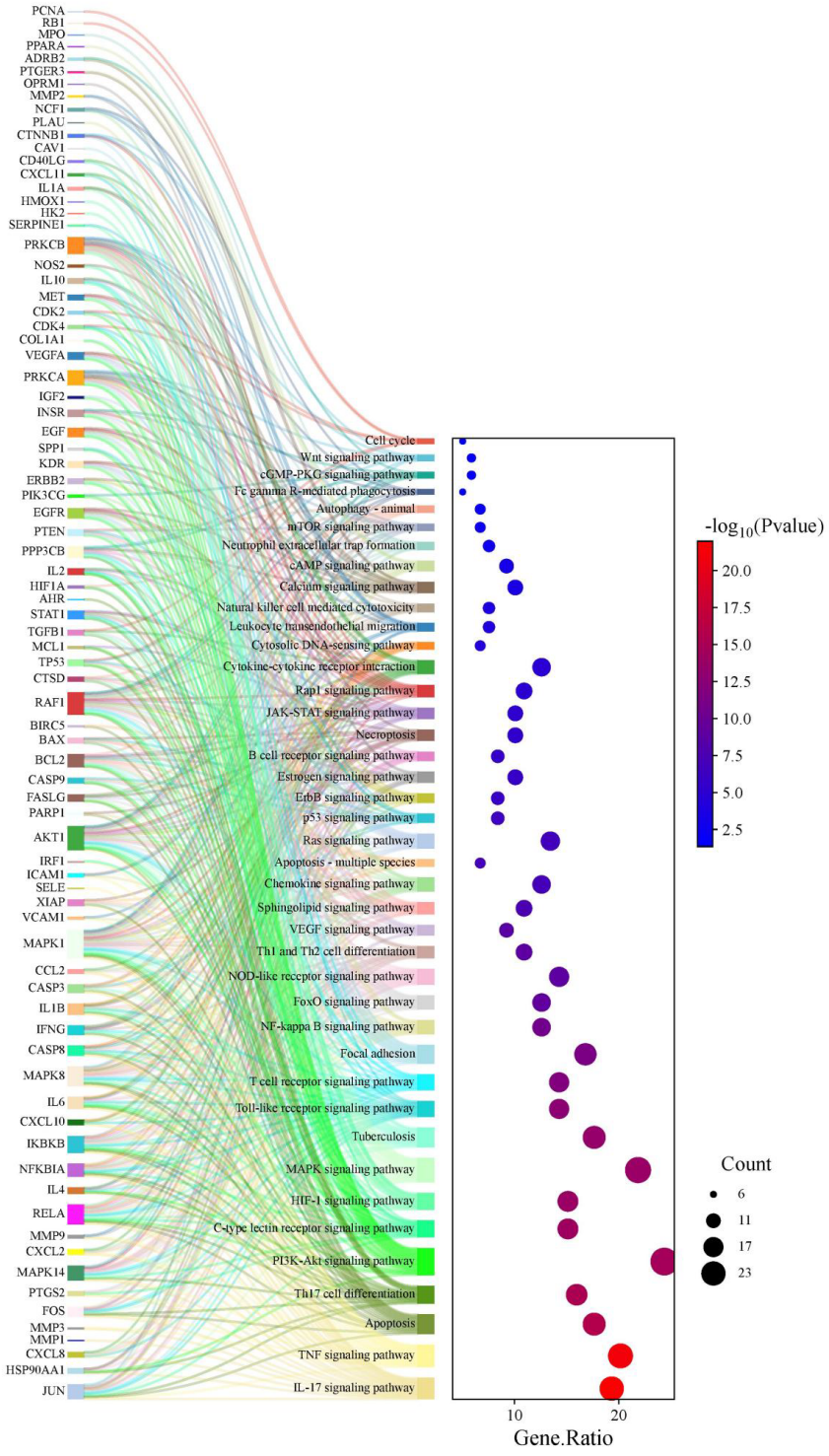

Figure 3: potential anti-TB targets and pathways related to the treatment of TB

The Sankey diagram shows that 96 potential anti-TB targets are involved in 41 KEGG pathways related to TB treatment. The scatter plot shows the ratio of the number of potential anti-TB targets on each pathway to the total number of potential anti-TB targets associated with NBXH, as well as the p-values for each enriched pathway.

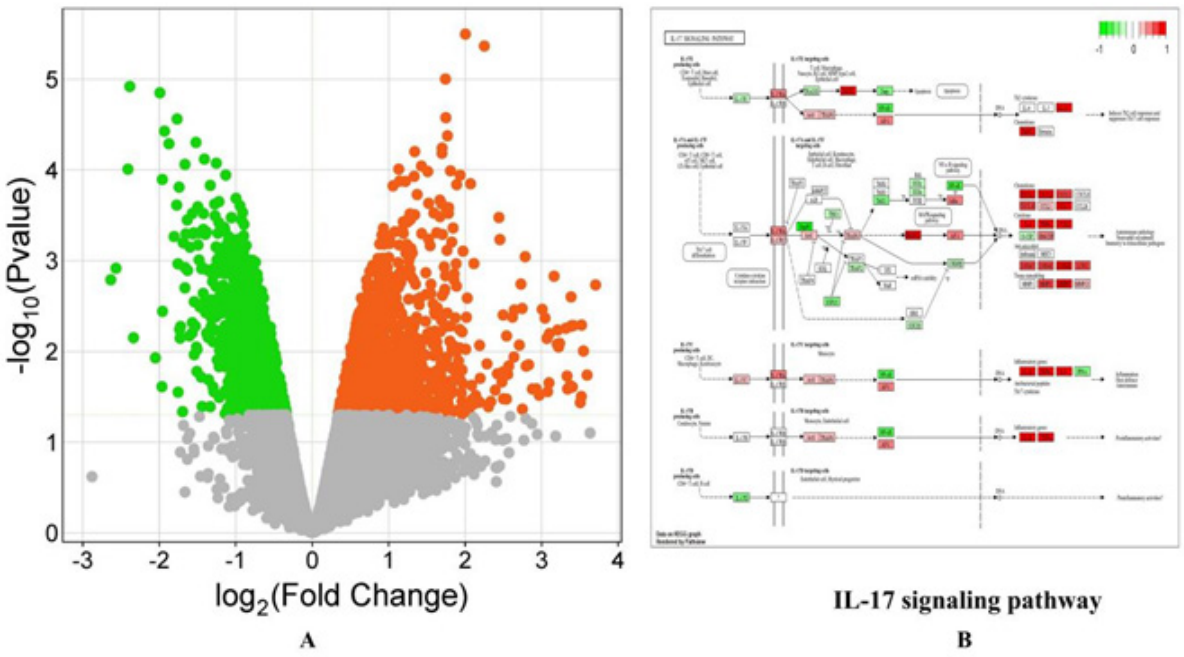

Figure 4: DEGs following NBXH treatment and visualization of KEGG pathways (with the IL-17 signaling pathway as an example, red represents upregulation, green represents downregulation, and the deeper the color, the greater the absolute value of log2FC)

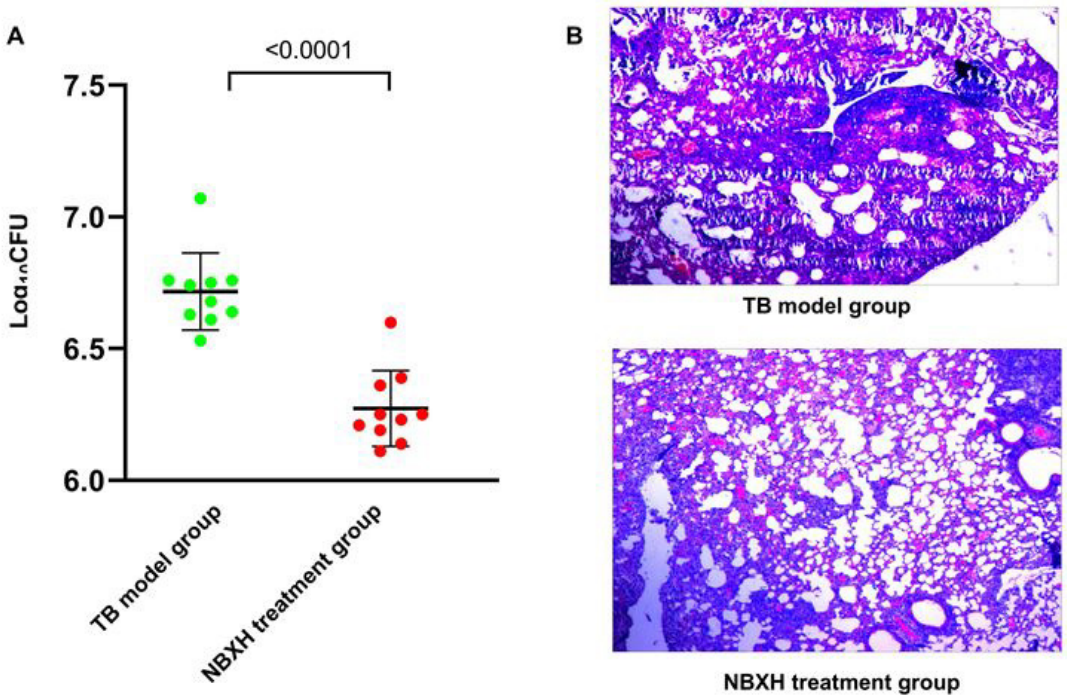

Figure 5: Pulmonary bacterial burden and histopathological analysis

Experimental Verification

Gene Expression Analysis

With the criteria of $p < 0.05$ and an absolute log2FC value > 1, a total of 2,126 upregulated differentially expressed genes (DEGs) and 2,049 downregulated DEGs were identified in the gene expression profile of NBXH-treated TB mice (Figure 4A). Comparative analysis of these DEGs with the results from Section 3.1.3 revealed that NBXH can cause alterations in the expression levels of molecules within these signaling pathways, thereby affecting the body's resistance to Mycobacterium tuberculosis. We have visualized the changes in molecular levels within the top-ranked IL-17 signaling pathway (Figure 4B).

Quantitative Bacterial Culture and Pathological Analysis of the Lungs

After culturing the lung tissue homogenate in Löwenstein--Jensen medium for four weeks, the bacterial count analysis revealed that the bacterial load in the lung tissue of mice in the NBXH treatment group was significantly lower than that in the TB model group ($p<0.0001$,Figure 5A). Furthermore, histological examination of the TB model group revealed severe disruption of alveolar structure, characterized by extensive lymphocyte and monocyte infiltration, as well as alveolar hemorrhage. Conversely, the NBXH treatment group demonstrated markedly preserved alveolar architecture, with notably reduced lymphocyte and monocyte infiltration (Figure 5B).

Discussion

NBXH is a traditional Chinese medicine formula composed of five herbs: *Fritillariae Cirrhosae Bulbus, Houttuyniae Herba, Bletilla Striata, Platycodon Grandiflorus,* and *Fructus Arctii*. Through bioinformatics analysis, we found that at least 26 active compounds related to tuberculosis treatment exist in five traditional Chinese medicines from NBXH. These compounds are mainly classified into phenanthrene derivatives, flavonoids, and steroids. Phenanthrene derivatives are known for their diverse effects, including anti-inflammatory and antioxidant activities, promotion of wound healing, antibacterial properties, and the ability to reverse drug resistance [14-17]. On the other hand, flavonoids exhibit anti-inflammatory and antioxidant activities, regulate immune responses, and possess liver-protective activities [18-20]. Meanwhile, steroidal compounds are primarily known for their anti-inflammatory and antibacterial properties [20,21]. These active compounds can interact with 119 intracellular molecules that are involved in cell signaling, immune regulation, transcription factor activity, cell death, and cell differentiation. Furthermore, the results of molecular docking simulations indicate that most of the active components in NBXH can effectively bind to therapeutic targets, suggesting their strong biological activity and the ability to elicit pharmacological effects through targeted interactions. We conducted 880 docking simulation experiments by pairing 16 NBXH active compounds with 55 key targets in a 16×55 matrix. Some pairs scored highly, while others scored zero. For instance, as shown in Figure 2, arctiin achieved a high score when paired with AKT1, but scored zero when paired with ADRB2. This illustrates how TCM exerts its effects by acting on different targets through active compounds, which is consistent with whether there is a connection between NBXH active compounds and targets in the C-T network.

The regulation of signaling pathways by traditional Chinese medicines plays a crucial role in the treatment of various diseases [22-24]. Through KEGG pathways analysis, we enriched 41 KEGG pathways that may be related to TB treatment, some of which have been well validated in animal experiments. For example, Figure 3 indicated that compounds in NBXH can specifically interact with 23 anti-TB targets in the IL-17 signaling pathway. These 16 active components are derived from 5 herbs in NBXH (shown in Figure 1). Existing studies have shown that the upregulation of the IL-17 signaling pathway plays a protective role in resistance to bacterial and fungal infections [25,26]. The results of the expression profiling experiment revealed that in mice infected with Mycobacterium tuberculosis and treated with NBXH, a total of 50 genes were upregulated and 35 genes were downregulated in the IL-17 signaling pathway. During the early stages of infection, the high expression of chemokines such as CXCL1, CXCL2, CXCL3, CXCL5, and CXCL10 contributes to the recruitment of immune cells to the site of infection, thereby enhancing local immune responses and facilitating the clearance of pathogens [27,28]. The high expression of cytokines such as IL-6 and TNF promotes inflammatory responses and the activation of immune cells, thereby enhancing the immune response against Mycobacterium tuberculosis and facilitating the clearance of infected cells [28-30]. The high expression of antimicrobial molecules such as S100A8, S100A9, and

LCN2 plays a crucial role in facilitating the host's clearance of pathogens. S100A8/A9 is capable of activating the NF-*K*B signaling pathway through binding to RAGE and TLR4 receptors, leading to the production of inflammatory cytokines and subsequently recruiting immune cells to the site of infection [31,32].

Additionally, the S100A8/A9 heterodimer can enhance the expression of β2 integrin CD11b, thereby improving the adhesion ability of phagocytic cells [33]. LCN2 exerts its antibacterial effect by chelating iron carriers, thereby interfering with the bacterium's acquisition of iron [34]. Iron plays a pivotal role in the growth, reproduction, and pathogenicity of Mycobacterium tuberculosis [35].

In addition to the IL-17 signaling pathway, through KEGG pathways analysis, we have identified that 26 compounds within NBXH exert an influence on 41 KEGG pathways that are intimately associated with the treatment of tuberculosis. These include pathways such as the TNF signaling pathway, apoptosis, and Th17 cell differentiation. This finding is consistent with the multi-target, multi-pathway therapeutic concept of traditional Chinese medicine for the treatment of tuberculosis, reflecting the complexity and multifaceted nature of the therapeutic effects of traditional Chinese medicine. Quantitative bacterial culture results and histopathological findings from lung tissue demonstrated that after treatment with NBXH, the bacterial load in the lung tissue of mice was significantly lower than that in the control group, and the degree of lung tissue pathology was also markedly less severe than that of the control group. These results substantiate the therapeutic efficacy of NBXH in tuberculosis.

This study still has some limitations. The accuracy of network pharmacology and bioinformatics analysis results depends on the integrity of large-scale datasets. Moreover, mouse models may not fully replicate the complexity of human tuberculosis, including immune responses and disease progression. To overcome these limitations, we conducted comparative analyses of different results, such as comparing network pharmacology analysis results with molecular docking results. Additionally, we validated these predicted results with experimental data, thereby enhancing the credibility of the research. Our study has preliminarily elucidated the anti-TB mechanism of NBXH, which is important for the development of new anti--tuberculosis drugs and therapeutic methods. To our knowledge, this is the first systematic attempt to dissect the anti-TB mechanism of NBXH using network pharmacology and bioinformatics approaches, complemented by experimental data for validation.

Conclusions

NBXH comprises numerous bioactive compounds that interact with diverse therapeutic targets, elicit a wide range of biological responses, and exhibit anti-TB properties via various pathways. This study has preliminarily clarified the anti-TB mechanism of NBXH. In the future, we will further study the key targets and signaling pathways of NBXH in the treatment of TB through clinical trials, in order to lay a foundation for improving tuberculosis treatment strategies and developing new drugs.

Funding

This research received no external funding.

Ethics Approval and Consent to Participate

All procedures involving the animals were reviewed and approved by the Animal Ethical Committee of the 8th Medical Center of the Chinese PLA General Hospital (Approved Number: 309201909270913), strictly following the 'Guide for the Care and Use of Laboratory Animals'.

Consent for Publication

Not applicable

Availability of Data and Materials

The original contributions presented in the study are included in the article and Supplementary Material. Further inquiries can be directed to the corresponding author.

Acknowledgments

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Competing Interests

The authors declare no conflicts of interest.

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