



# Network Pharmacology and Experimental Validation to Explore the Molecular Mechanisms of Compound Huangbai Liquid for the Treatment of Acne

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**Background:** Acne is a highly prevalent skin disease, and inflammation plays an important role. Compound Huangbai Liquid (CHL) is a classical traditional Chinese medicine (TCM) with remarkable clinical therapeutic effects on acne. However, a holistic network pharmacological approach to explain the mechanism of CHL in the treatment of acne has not been explored.

**Methods:** In this study, active components and action targets of Compound Huangbai Liquid were assessed via BATMAN-TCM. The target genes related to acne were extracted from GeneCards, DisGeNet and OMIM databases. Venn diagrams to predict potential targets for the treatment of acne. Protein-Protein interaction (PPI) analysis was proceeded through String database to obtain the core protein, and the protein interaction network was constructed by Cytoscape 3.9.1. Gene Ontology (Go) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed on Metascape platforms and bioinformatics.com.cn. TCM-compound-target-disease network and disease-target pathway network were constructed using Cytoscape to give the visual result. Finally, the results were further verified by establishing the mouse acne animal model.

**Results:** This approach identified 165 active compounds, 1117 gene targets, 156 acne-related targets, and 34 potential target proteins for the treatment of acne with CHL. The biological processes were primarily related to cellular response to lipid, response to lipopolysaccharide, and regulation of secretion. The CHL was significantly associated with ten pathways including the Chagas disease and pathways in cancer. Animal experiments showed that CHL could significantly alleviate the levels of inflammatory factors and TLR4/NF- $\kappa$ B/p38 MAPK signaling pathway in acne.

**Conclusion:** This study revealed the multiple active components, multiple targets, and multiple pathways of CHL in the treatment of acne, which provided a new perspective for the study of the mechanism of traditional Chinese medicine in the treatment of acne.

**Keywords:** Compound Huangbai Liquid, acne, network pharmacology, inflammation

## Introduction

Acne is a highly prevalent chronic inflammatory skin disease,<sup>1</sup> which is a common problem affecting more than 85% of adolescents and usually lasts into adulthood, causing scarring and hyperpigmentation.<sup>2,3</sup> The occurrence of acne is closely related to many factors, including sebum secretion, hormone levels, bacterial infection and so on. Among them, the change of sebum secretion is considered to be one of the important factors of acne.<sup>4</sup> The pathogenesis of acne is complex, and inflammation is one of the important clinical characteristics.<sup>1,5</sup> Research has confirmed that inflammation plays a pivotal role in the onset, development and resolution of acne. The IL-1 family of cytokines is the initiator and key factor of acne.<sup>6</sup> Recent studies have found that sebaceous cells are affected by *P. acnes* significantly enhanced IL-1 $\beta$  release.<sup>7</sup> However, the up-regulated expression of IL-1 can stimulate local endothelial cells and blood vessels around follicles, leading to an inflammatory cascade.<sup>8</sup> IL-1 $\beta$  was found to induce the expression of proinflammatory cytokines IL-6 and IL-8 in sebocytes, suggesting that IL-1 $\beta$  plays an important role in acne.<sup>9</sup> Keratinocytes are also closely related to the development of acne. It was found that *P. acnes* is a powerful inducer of IL-1 $\beta$  release from keratinocytes in a dose-dependent manner.<sup>10</sup> In addition to IL-1, cytokines such as IL-6, tumor necrosis factor (TNF), and IL-10 are also

involved in the inflammatory response of acne.<sup>11</sup> In addition, *Cutibacterium acnes* is an important factor in the activation of toll-like receptors (TLR-2 and TLR-4), which leads to the activation of mitogen-activated protein kinase (MAPK) and transcription factor-nuclear factor-Kappa B (NF- $\kappa$ B) pathways.<sup>12</sup> Anti-acne medications usually include retinoids, benzoyl peroxide, antibiotics and hormonal preparations.<sup>13</sup> However, in recent years, traditional Chinese medicine (TCM) has also been very effective in anti-acne treatment.

Through thousands of years of development, TCM has been widely used to treat various diseases.<sup>14</sup> TCM has accumulated a lot of experience in the treatment of skin diseases, including psoriasis, vitiligo, eczema, acne and so on.<sup>15–18</sup> In addition, TCM is easy to obtain, economical, and has a good clinical effect on acne with few side effects.<sup>15,19</sup> Qing-Shang-Fang-Feng-Tang, Zhen-Ren-Huo-Ming-Yin, Jia-Wei-Xiao-Yao-San, Wu-Wei-Xiao-Du-Yin and Huang-Lian-Jie-Du-Tang were used to treat acne.<sup>15</sup> Different decoctions according to its own characteristics have played an effective role in acne treatment. Compound Huangbai Liquid (CHL), which is composed of five traditional Chinese herbs, Forsythiae Fructus (Lianqiao), Phellodendri chinensis Cortex (Huangbai), Honeysuckle (Jinyin Hua), dandelion (Pugong Ying) and centipede (Wugong), has shown superior efficacy in acne treatment in China. However, the mechanism is still unclear and needs further study.

Network pharmacology is a modern research method based on network to elucidate the relationship between drug, target and disease and the mechanism of action of drug.<sup>20</sup> It can predict the mechanism of a drug's action on a disease.<sup>21</sup> In this study, pharmacological components of CHL were used to identify its target, and then fused with acne target to obtain a potential target for acne treatment. The signal pathways related to the treatment of acne were obtained through the enrichment analysis of functions and pathways and verified by experiments, providing new ideas and directions for comprehensively and systematically explaining the mechanism of action of CHL in the treatment of acne. The flowchart of the study is shown in [Figure 1](#).

## Materials and Methods

### Drug and Disease Targets Identification

The traditional Chinese medicine (TCM) in the Bioinformatics Analysis Tool for molecular mechanism of Traditional Chinese Medicine (BATMAN-TCM) (<http://bionet.ncpsb.org.cn/batman-tcm/>) was searched with keywords “LIAN QIAO”, “HUANG BAI”, “JIN YIN HUA”, “PU GONG YING”, “WU GONG”. In BATMAN-TCM platform, active compounds and target genes were screened according to a score cutoff  $\geq 20$  and  $P < 0.05$ .

Potential target genes of acne were collected from GeneCards (<https://www.genecards.org>), DisgeNet (<https://www.disgenet.org/>) and Online Mendelian Inheritance in Man (OMIM) (<https://omim.org/>) databases with the keyword of “acne”.

### Prediction of Compound Huangbai Liquid Targets for Acne Treatment

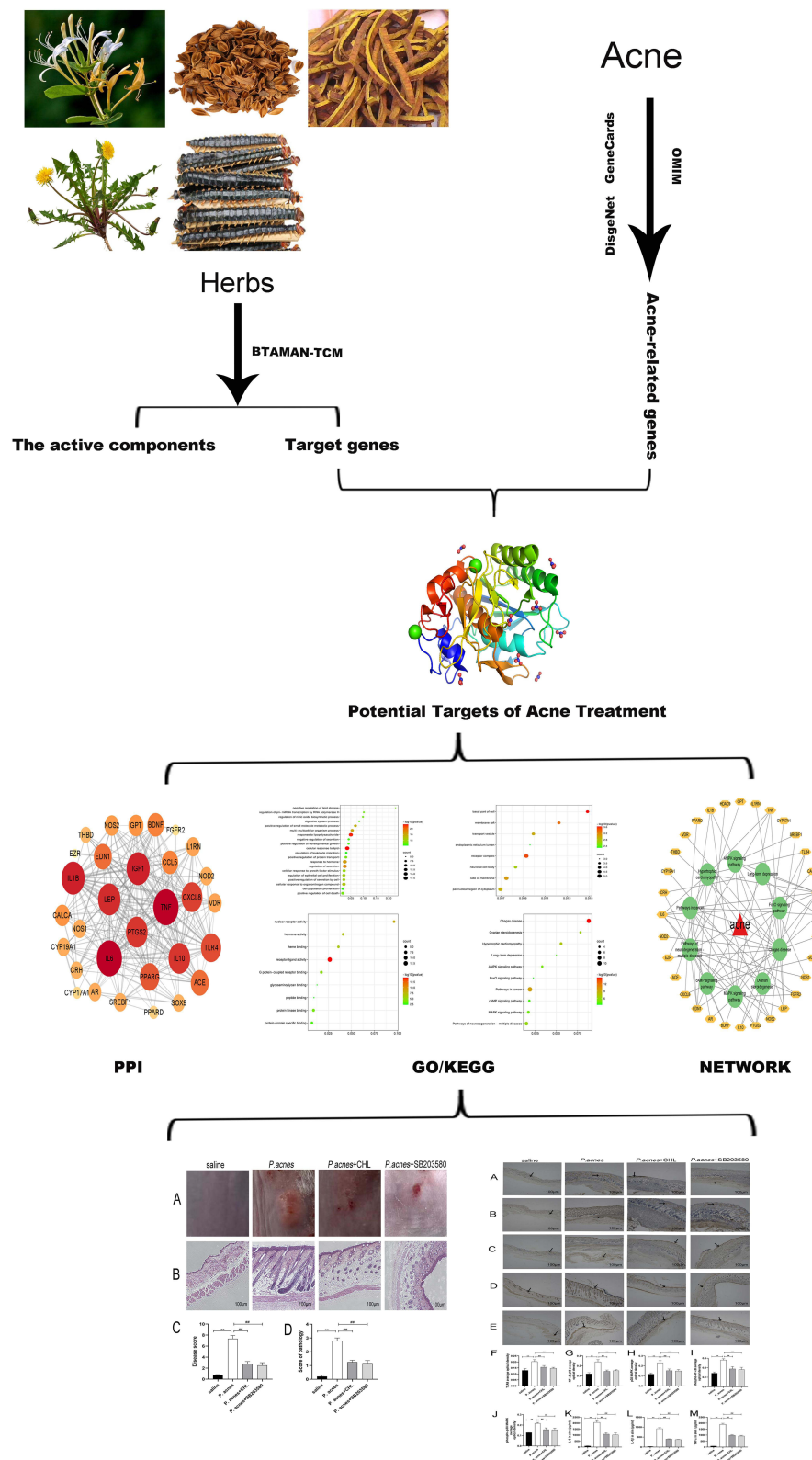
The Venn diagram (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) was drawn to predict the potential targets for the treatment of acne, and the results were derived.

### Construction and Analysis of Protein–Protein Interaction (PPI) Network

The common targets of drugs and diseases are potential therapeutic targets. Protein-protein interaction (PPI) network was constructed by String 11.0 (<https://string-db.org>) database and visualized by Cytoscape 3.9.1 software. The core target protein was identified through the construction of PPI network.

### Gene Function and Pathway Enrichment Analysis

The Gene Ontology (GO) analysis commonly describes three distinct aspects gene functions, including biological pathways (BP), cellular components (CC), and molecular function (MF). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (<http://www.genome.jp/kegg/>) can help us to find the core pathway. GO biological function analysis and KEGG enrichment analysis (<http://www.genome.jp/kegg/>) were performed by Metascape platforms and bioinformatics.com.cn. The cut-off criteria for significant enrichment of KEGG pathway were  $P < 0.05$ .



**Figure 1** Flow diagram of network pharmacological analysis of Compound Huangbai Liquid for acne treatment.

## Network Construction

To understand complex relational herbs, compounds, targets, pathway and diseases, we used Cytoscape 3.9.1 to build “TCM-compound-target-disease network and Disease-target pathway network”.

## Culture of *Propionibacterium acnes* (*P. acnes*) Strains

The *Propionibacterium acnes* (*P. acnes*, ATCC6919) were purchased from BeNa Culture Collection. *P. acnes* were cultured on blood agar plates, and isolated colonies were anaerobic cultured in BHI medium at 37°C. The *P. acnes* bacterial suspension was diluted with fresh BHI medium 1:100 until the bacterial suspension was re-cultured to OD 600 of 0.1–0.3. The cultures were centrifuged (3220g, 5 minutes) and washed by BHI for 3 times. The suspension concentration of the culture was about  $1 \times 10^7$  CFU on BHI medium.

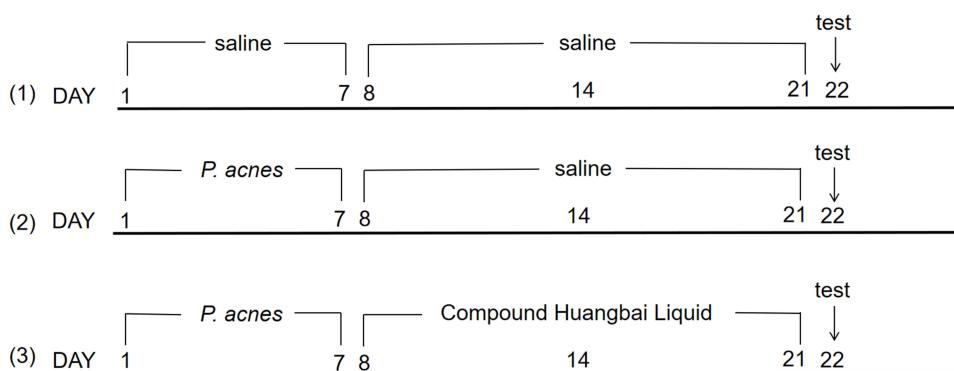
## Preparation of Synthetic Sebum

Synthetic sebum was composed of squalene, triolein, oleic acid, jojoba oil and Vitamin E ( $\pm$ a-tocopherol). We were all purchased from Macklin (St Huatuo, Shanghai, China). The purities of the squalene were 98% and 99% for oleic acid and triolein. The 90% pure jojoba oil and 95% pure Vitamin E ( $\pm$ a-tocopherol) were used as synthetic sebum. Each drug was dissolved in dichloromethane: methanol (2:1). On the day of the experiment, 17% oleic acid, 44.7% triolein, 25% jojoba oil 12.4% squalene and 0.9% Vitamin E were used to prepare sebum.

## Ethics Statement and Animal Experiments

The specific pathogen-free (SPF) male BALB/c mice (5–6 weeks; 20–22g) were purchased from the Peng Yue experimental animal center (Jinan, China) with the permission number SCXK 2022-0009. Approval for all animals used in this study in the experiment was received from Jinan city people’s hospital. Animals were housed in the Laboratory Animal Center of Jinan city people’s hospital. Strictly abide by the national laboratory animal-related laws, regulations and standards, including but not limited to 《Guidelines for ethical review of the welfare of laboratory animals》 (GB/T35892-2018), IGP 2012 and IAVE Guidelines 2010. The mice were housed at a temperature between 20°C and 25°C and humidity between 50% and 70% for one week. Food and drinking water were freely available.

The mice were randomly divided into 4 groups of eight each: (1) saline group (saline), (2) *P. acnes* group (*P. acnes*), (3) *P. acnes*+ Compound Huangbai Liquid group (*P. acnes*+CHL), (4) *P. acnes*+ SB203580 group (*P. acnes*+ SB203580). Animal models of acne were established by injecting intradermally with approximately  $1 \times 10^7$  CFU of *P. acnes* in 50  $\mu$ L BHI media. Immediately after injection, 20  $\mu$ L of fresh synthetic sebum was applied to the skin once a day. The saline group was injected intradermally with equal volume of saline, and the same volume of saline was applied to the skin. The establishment of animal models of acne lasted for 7 days. From day 8 to 21, apply 1.5 mL of Compound Huangbai Liquid to the *P. acne*+Huangbai group mice skin twice a day for 15–20 minutes. The drug was administered continuously for 14 days. 5 mg/kg/day SB203580 is injected intraperitoneally on days 11, 14, and 17. The particular grouping and protocol is shown in Figure 2.



**Figure 2** Experimental protocol.



## Histopathological and Immunohistochemistry Analysis of Skin Tissue

The skin of mice in each group was fixed with 4% paraformaldehyde, embedded in paraffin and stained with hematoxylin and eosin (H&E). The protein expression levels of TLR4, NF- $\kappa$ B p65, P38 MAPK, phospho-NF- $\kappa$ B p65 and phospho-p38 MAPK were detected by immunohistochemistry. In this experiment, anti-TLR4 (Abcam, MA, USA), anti-NF- $\kappa$ B p65 (Abcam, MA, USA), anti-p38 MAPK (Abcam, MA, USA), phospho-NF- $\kappa$ B p65 (Abcam, MA, USA) and phospho-p38 MAPK (Abcam, MA, USA) were used in the immunohistochemical staining. The experimental methods refer to Chen's research.<sup>22</sup> Histopathological changes and immunohistochemistry protein expression were observed under microscope. Image-pro Plus 6.0 (Media Cybernetics) was used to analyze the experimental results, and the average optical density was used to represent the protein expression level "Mean density=integrated optical density/area of interest".

## Preparation of Tissue Homogenate

The skin on the back of the mice was collected, the tissue was washed with ice PBS buffer, and the excess water was absorbed by filter paper and weighed. The tissue is placed in a glass homogenizer and ground on ice. A certain volume of pre-cooled PBS was added into the homogenizer to make its mass fraction 10% of tissue homogenate. After centrifugation (9400 g, 4°C, 10 min), the supernatant was collected and stored at -80°C until tested.

## Enzyme-Linked Immunosorbent Assay

The ELISA kits for IL-6, IL-1 $\beta$  and TNF $\alpha$  were purchased from eBioscience (San Diego, CA, USA). IL-6, IL-1 $\beta$  and TNF $\alpha$  were tested using ELISA kits according to the manufacturer's instructions. The sensitivities of the ELISA kits were 3 pg/mL for IL-6, 1.2 pg/mL for IL-1 $\beta$ , 3.7 pg/mL for TNF $\alpha$ .

## Statistical Analysis

All data of each group were expressed with mean  $\pm$  SEM. In this experiment, all analyses were carried out using SPSS 19.0, while statistical graphs were generated using GraphPad Prism 8.0 software. Differences were considered significant at \*P < 0.05 and \*\*P < 0.01 according to one-way ANOVA analysis.

## Result

### Compounds and Target Genes in Compound Huangbai Liquid

We searched Forsythiae Fructus (Lianqiao), Phellodendri chinensis Cortex (Huangbai), Honeysuckle (Jinyin Hua), dandelion (Pugong Ying) and centipede (Wugong) in BATMAN-TCM database and collected the ingredients and potential targets. Then, the parameters score cutoff  $\geq 20$  and P < 0.05 were used as conditions to screen the active components of Compound Huangbai Liquid, and the number of compounds and target genes were obtained as shown in Table 1. Using the BATMAN-TCM database, 165 compounds were retrieved: 47 in Forsythiae Fructus, 37 in Phellodendri chinensis Cortex, 72 in Honeysuckle, 8 in Dandelion, and 7 in Centipede (5 herbs shared 6 compounds). Of the 47 compounds in Forsythiae Fructus, 32 satisfied the criterion of cutoff  $\geq 20$  and P < 0.05. Of the 37 compounds in Phellodendri chinensis Cortex, 28 satisfied the criterion of cutoff  $\geq 20$  and P < 0.05. Of the 72 compounds in Honeysuckle, 46 satisfied the criterion of cutoff

**Table 1** The Number of Compounds and Target Genes in Compound Huangbai Liquid

Herbs	Total of Compounds	Number of Compounds (Cutoff $\geq 20$ , p < 0.05)	Number of Target Genes
Forsythiae Fructus (Lianqiao)	47	32	543
Phellodendri chinensis Cortex (Huangbai)	37	28	325
Honeysuckle (Jinyin Hua)	72	46	853
Dandelion (Pugong Ying)	8	8	123
Centipede (Wugong)	7	5	212

$\geq 20$  and  $P < 0.05$ . Of the 8 compounds in Dandelion, 8 satisfied the criterion of cutoff  $\geq 20$  and  $P < 0.05$ . Of the 7 compounds in Centipede, 5 satisfied the criterion of cutoff  $\geq 20$  and  $P < 0.05$ . Among the 32 compounds obtained in Forsythiae Fructus, 543 target genes were obtained. Among the 28 compounds obtained in Phellodendri chinensis Cortex, 325 target genes were obtained. Among the 46 compounds obtained in Honeysuckle, 853 target genes were obtained. Among the 8 compounds obtained in Dandelion, 123 target genes were obtained. Among the 5 compounds obtained in Centipede, 212 target genes were obtained. More detailed drug compounds and target information were available in the [Supplementary Data \(Tables S1–S5\)](#).

## Prediction of Potential Targets

In [Table 2](#), a total of 156 acne-related targets were screened from GeneCards, DisgeNet and OMIM databases. The related targets of active components of Compound Huangbai Liquid and those of acne were plotted by Venn diagram ([Figure 3](#)), and 34 overlapping targets were obtained, which were potential targets for the treatment of acne by Compound Huangbai Liquid ([Table 3](#)), including IL1RN, TNF, UROD, SOX9, etc.

**Table 2** Acne-Related Target Genes

Number	Gene Symbol	Number	Gene Symbol	Number	Gene Symbol
1	VDR	53	TIMP2	105	SEMA4B
2	ACE	54	Clorf49	106	IGFBP7
3	IL17A	55	XLI	107	PEN2
4	CYP17A1	56	ATOD1	108	ARCI5
5	Clorf112	57	EEF1D	109	EZR
6	TLR4	58	NCSTN	110	DEFB4B
7	LYNX1	59	S100A7	111	CHI3LI
8	PSTPIP1	60	CD83	112	DGS
9	MEFV	61	AFND	113	LOC102723407
10	EDN1	62	PSTPIP	114	RDCCAS
11	CARD14	63	TTD1	115	FOXO1
12	LGALS7B	64	CD79A	116	GPT
13	IL36RN	65	ANGPTL3	117	PINX1
14	CYP19A1	66	PSORS11	118	RHOA
15	PPNAD4	67	CTLA4	119	CCL7
16	IGF1	68	SMIM10L2A	120	CALCA
17	MAPK14	69	CORTRD1	121	ADCY10
18	SOX9	70	S100A7A	122	HGF
19	TRAF3IP2	71	LCN2	123	ESTRR
20	DEFB4A	72	RNASE7	124	SERPINA1
21	CCL5	73	BDNF	125	HDAC9
22	IL1RAPL2	74	CDH1	126	ORI6
23	PDGFRB	75	LOC102724971	127	FTHS
24	NAP1L2	76	CAMP	128	CDK2
25	ATOD2	77	RETN	129	TNFSF12
26	UROD	78	APH1A	130	DSG3
27	IL20	79	SREBF1	131	TNFSF12-TNFSF13
28	LPL	80	NOD2	132	IL1RN
29	PLXNA2	81	PAGR1	133	LYPLAL1-DT
30	ANGPTL4	82	ACNIN3	134	PSORS4
31	NLRP3	83	PAPAS	135	ABCD2
32	LEP	84	XLP2	136	CD2BP1
33	LGALS7	85	PSORS1	137	DCD
34	LYZ	86	FOXO3	138	ADIPOQ

(Continued)

**Table 2** (Continued).

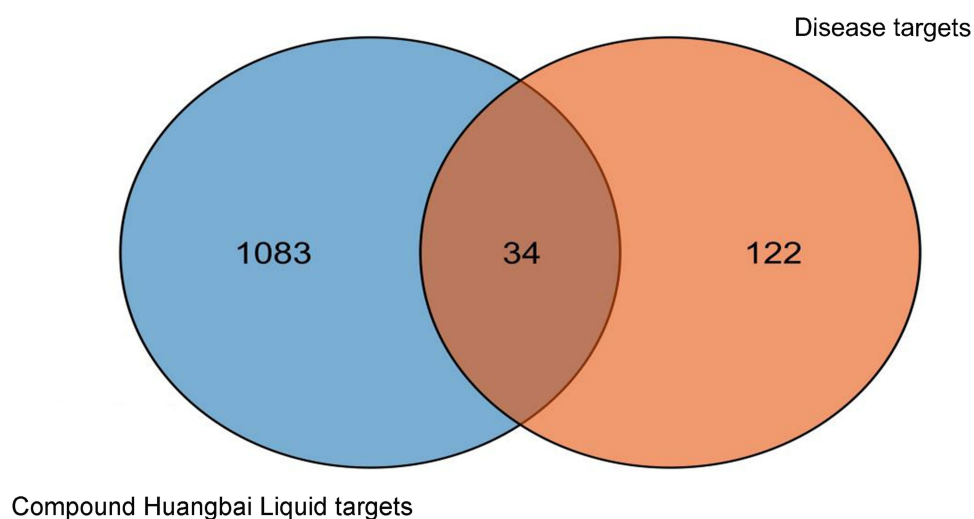
Number	Gene Symbol	Number	Gene Symbol	Number	Gene Symbol
35	FGFR2	87	DSGI	139	PPARD
36	SUGCT	88	CGD5	140	MMP2
37	SFPQ	89	PSORS14	141	NOS2
38	HSD3BI	90	PCNX3	142	TNF
39	ABOLM	91	PSENI	143	EGF
40	RBP4	92	TLR2	144	IL1A
41	STGDI	93	WNT10A	145	AKT1
42	DEFBI	94	GALNS	146	IL10
43	CRH	95	SETBPI	147	PTGS2
44	NOS1	96	SMIM10L2B	148	EGFR
45	HSD17B3	97	UBE2E1	149	IL1B
46	IL22	98	HDAC8	150	SOD1
47	DNMT1	99	GAST	151	AR
48	SRXY1	100	CANDFI	152	PPARG
49	BCYM4	101	TNFRSF1B	153	IL6
50	PSENI	102	AD3	154	CXCL8
51	ADORA2A-AS1	103	IGHA1	155	MMP9
52	APOC3	104	MCOPCB10	156	THBD

### Protein–Protein Interaction (PPI) Network Construction

Thirty-four potential targets were imported into the multiple proteins of the String database to obtain the protein interaction network data, which was then imported into the Cytoscape 3.9.1 software. Then, the network was further optimized to obtain PPI network (Figure 4A and Figure 4B). In Figure 4A, there are 31 nodes, 221 edges, and the average degree value is 14.258. The redder the color and the larger the circle, the more core the protein plays, such as IL-6, TNF and IL-1B, which are the core nodes of the network.

### GO and Pathway Analysis

Thirty-four overlapping targets were performed for enrichment analysis using bioinformatics.com.cn. The top 20 items of biological process (BP), cell component (CC) and molecular function (MF) were screened by  $P < 0.05$ , as shown in



**Figure 3** A Venn diagram of the Compound Huangbai Liquid related potential targets and acne related targets.

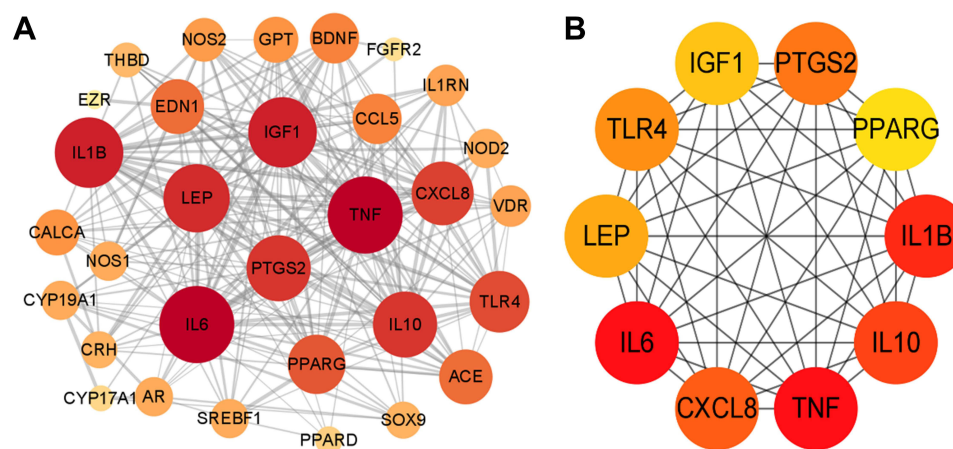
**Table 3** Potential Targets of Acne Treatment

Number	Gene Symbol	Number	Gene Symbol	Number	Gene Symbol
1	IL1RN	13	ACE	25	THBD
2	TNF	14	VDR	26	NOS1
3	UROD	15	BDNF	27	IL10
4	SOX9	16	IGF1	28	SREBF1
5	AR	17	IL6	29	EDN1
6	EZR	18	FGFR2	30	IL1B
7	CALCA	19	CXCL8	31	CRH
8	CYP19A1	20	GPT	32	PPARG
9	TLR4	21	HDAC9	33	SUGCT
10	PPARD	22	CYP17A1	34	LEP
11	NOD2	23	CCL5		
12	PTGS2	24	NOS2		

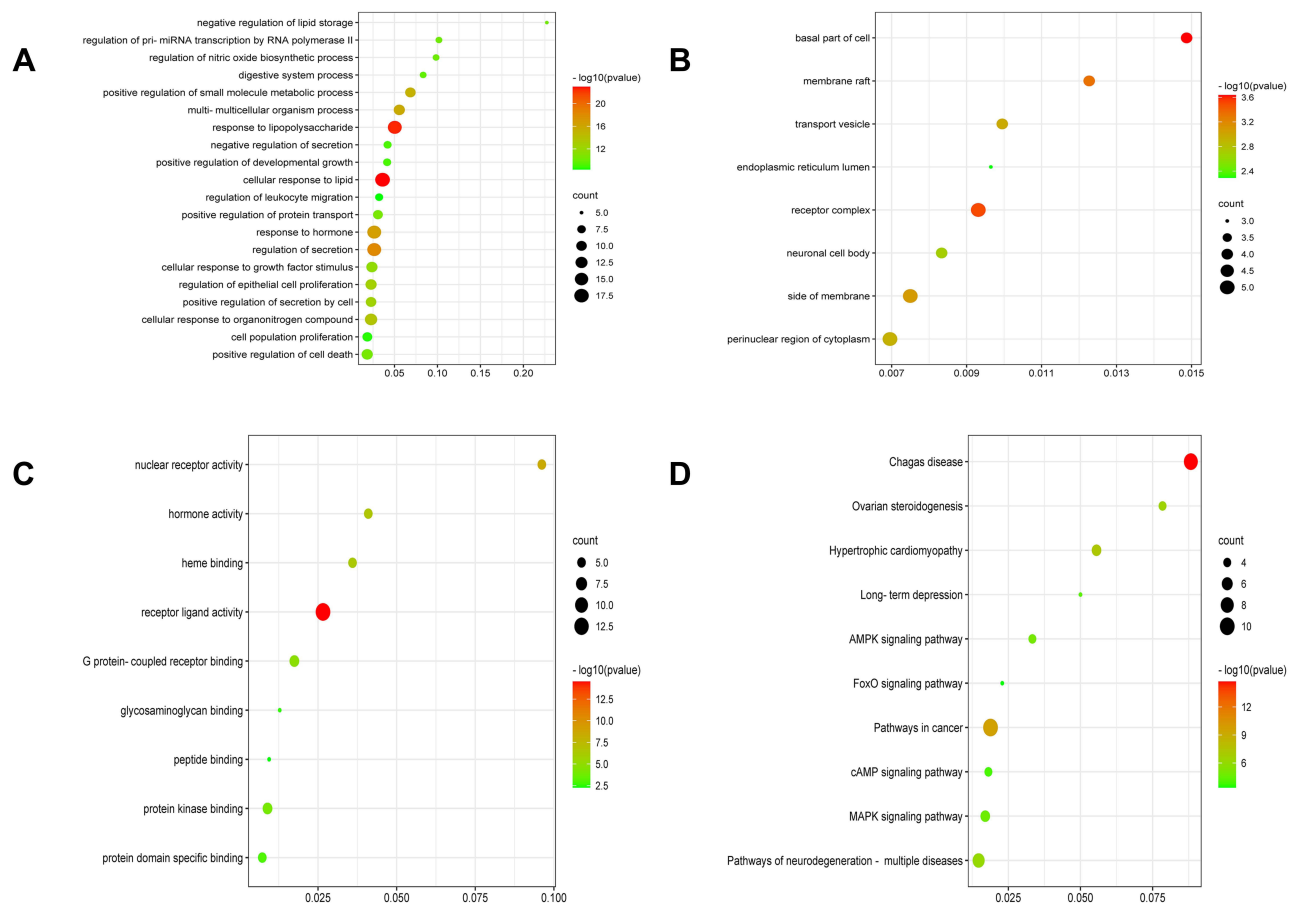
**Figure 5A.** The results showed that BP targets were mainly enriched in cellular response to lipid, response to lipopolysaccharide, regulation of secretion, response to hormone, multi-multicellular organism process, etc. The targets of CC were mainly enriched in basal part of cell, receptor complex, raft and side of membrane, transport vesicle, etc. The targets of MF were mainly enriched in receptor ligand activity, nuclear receptor activity, hormone activity, heme binding, G protein-coupled receptor binding, etc. Based on the results of enriched biological process ontologies, the anti-acne effect of the CHL may result from a complex multibiological process synergetic effect.

In terms of pathway analysis, 34 targets are involved in 10 KEGG Pathways with significant P values ( $P < 0.05$ ), including Chagas disease and pathways in cancer, etc. As can be seen from the bubble diagram (**Figure 5D**), the larger the bubble in the diagram, the more genes are enriched in the pathway, and the redder the color, the smaller the P value.

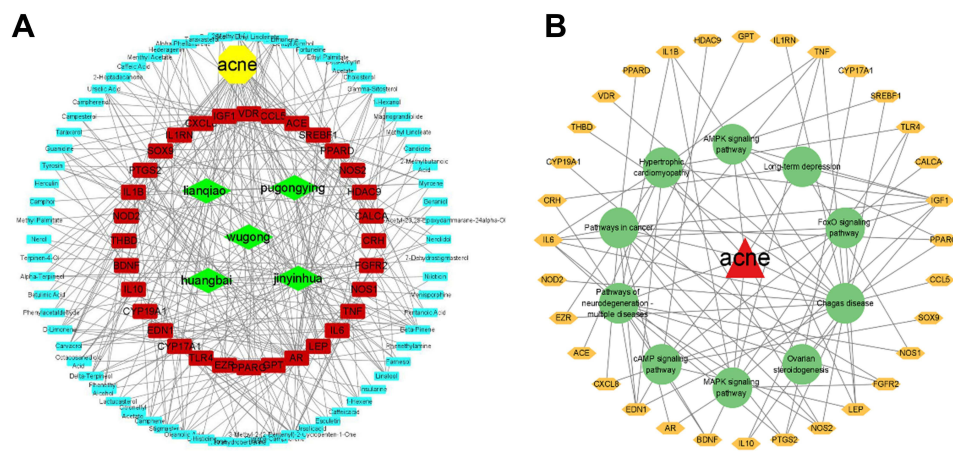
Cytoscape 3.9.1 software was employed to draw the TCM-compound-target-disease and disease-target-pathway networks (**Figure 6A** and **Figure 6B**). The TCM-compound-target-disease network comprised a total of 106 nodes (1 disease node, 5 herb nodes, 68 compound nodes, 32 target nodes) and 308 edges. According to the node degree parameters of the nodes in the network (shown in **Table 2**), the top five compounds in the network were 3-Methyl-2-(2-Pentenyl)-2-Cyclopenten-1-One, beta-Pinene, carvacrol, ursolic acid, D-Limonene, and could interact with 13, 12, 9, 7, 7 targets, respectively. From the perspective of the target, the top 5 were AR, VDR, PPARD, PTGS2 and IL1B, which could interact with 35, 20, 15, 14, 13 compounds, respectively.



**Figure 4** The PPI network map of target in Compound Huangbai Liquid for acne treatment. **(A)** The PPI network of 34 therapeutic targets. **(B)** The PPI network of top 10 targets of degree. The changes of color from red to orange reflect the changes of degree value from high to low.



**Figure 5** GO and KEGG pathway enrichment analysis. (A) Biological process categories. (B) Cellular component categories. (C) Molecular function categories. (D) KEGG pathway analysis.



**Figure 6** Network construction. (A) TCM-compound-target-disease network of core targets of Compound Huangbai Liquid and acne. The green represents five TCM in Compound Huangbai Liquid, and the blue diamond represents the main active compounds contained in five TCM. The red represents the core targets, while the yellow represents acne. (B) Disease-target pathway network of core targets. The orange represents the core targets, and the red triangle represents acne. The green circle represents the top 10 GO annotation results.



Based on targets and top 10 KEGG pathway analyses, disease-target-pathway network was constructed using Cytoscape 3.9.1. As shown in Figure 6B, the interaction network has 43 nodes and 86 edges. The pathway with the most enriched genes was Chagas disease, with 19 genes, followed by the pathways in cancer and Hypertrophic cardiomyopathy with 10 genes and 8 genes, respectively. KEGG Pathway Maps for Chagas Disease as is shown in Figure 7. Therefore, based on the outcomes of PPI analysis, GO-BP analysis, KEGG analysis and disease-target-pathway network, we determined that the CHL might reverse the pathological changes associated with acne via six essential targets, namely, the TLR4, NF-κB, p38 MAPK, TNFα, IL-6 and IL-1β.

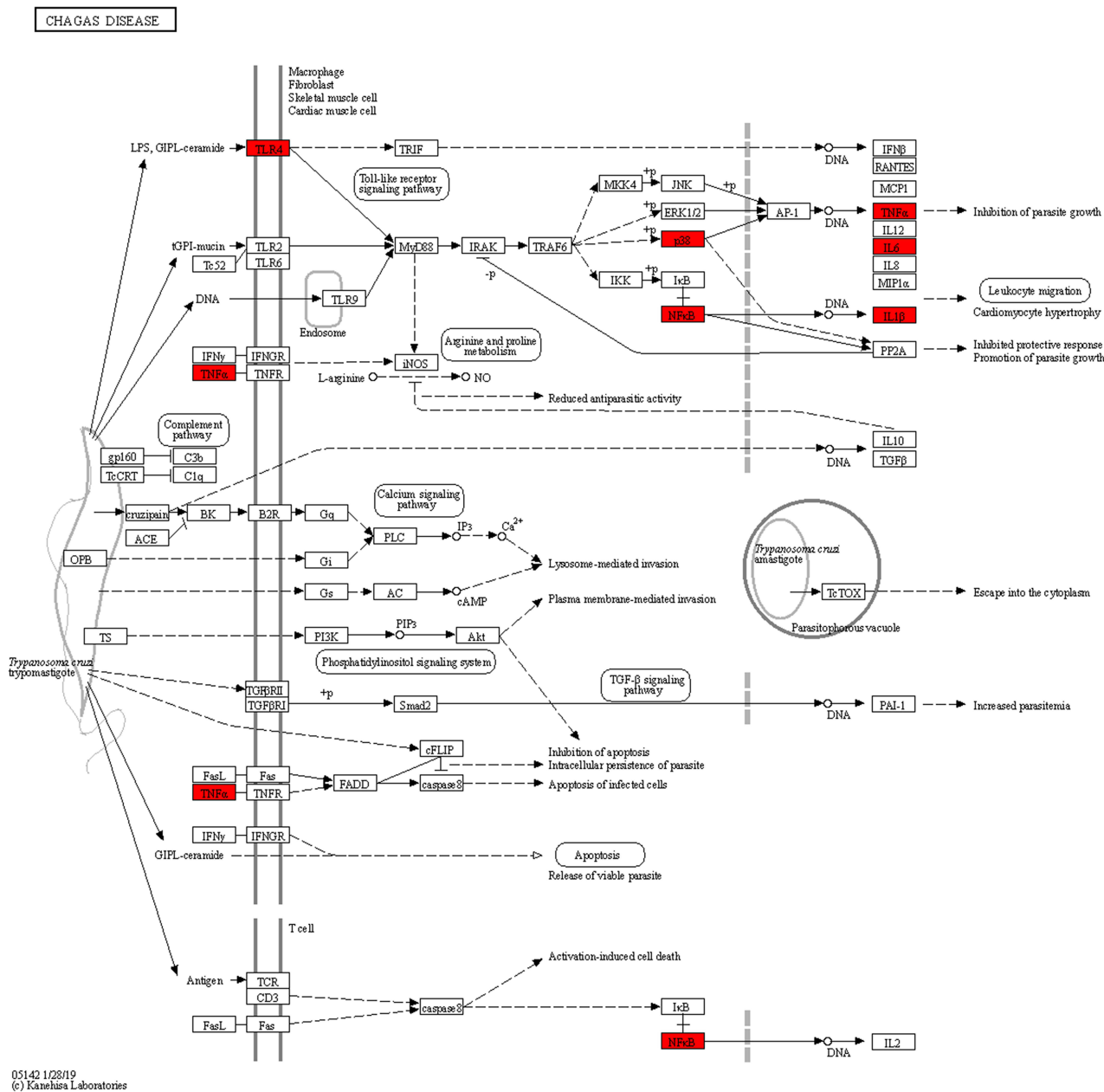
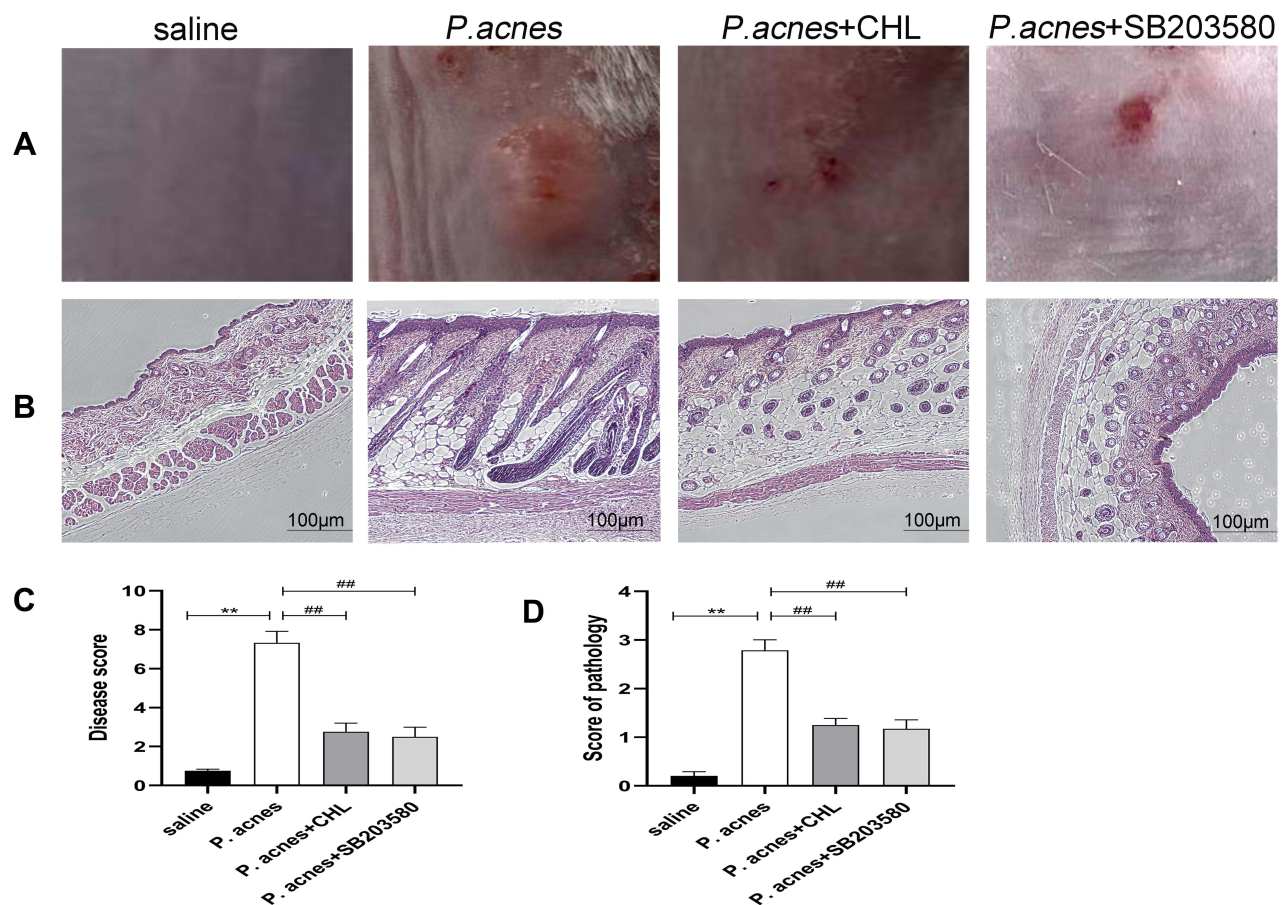


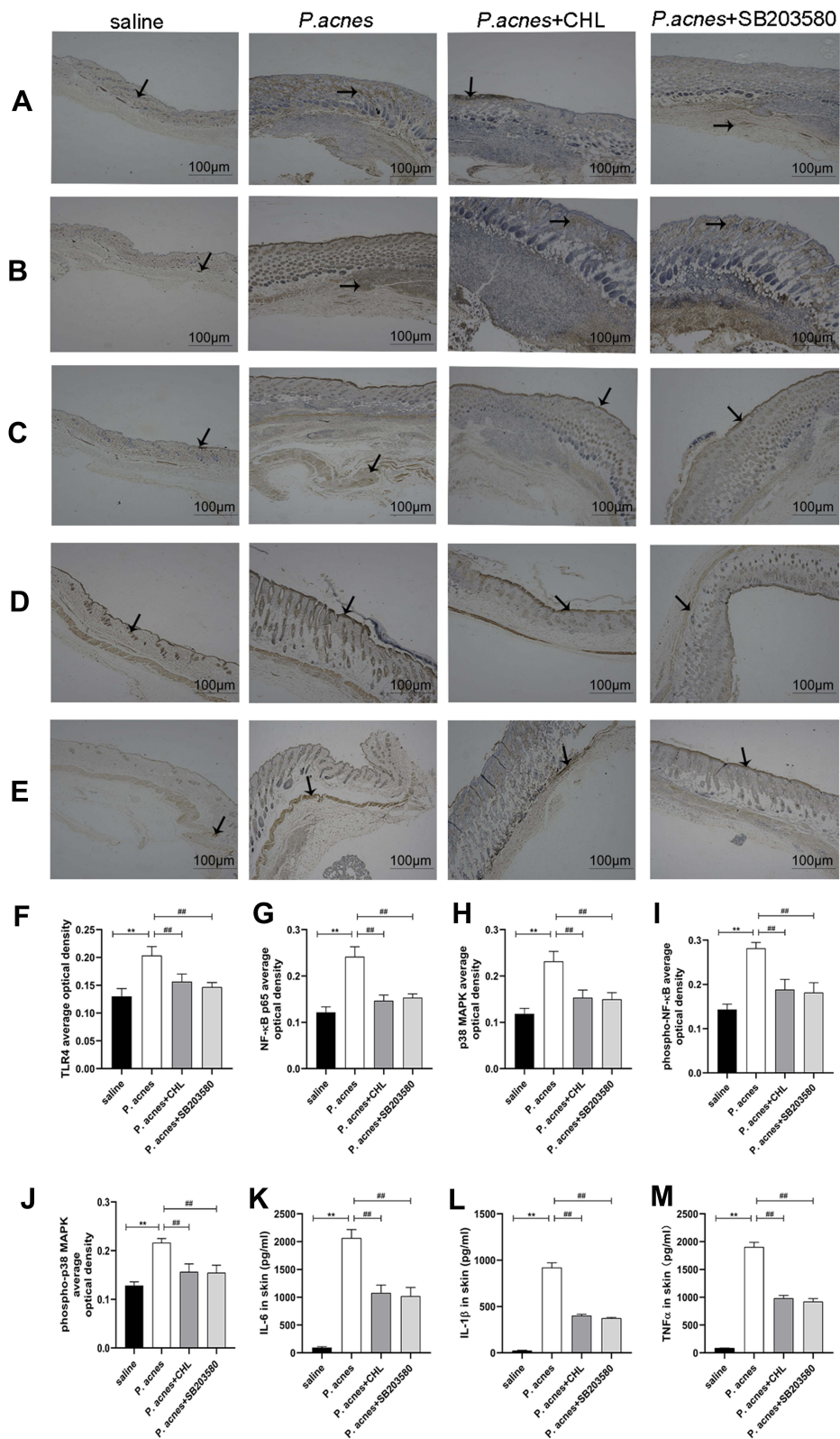
Figure 7 The anti-acne pathways of the Compound Huangbai Liquid (CHL). The red squares represent the core therapeutic targets in the pathway.

## Compound Huangbai Liquid Alleviated Acne-Like Skin Lesions Induced by *P. acnes*

As shown in Figure 8A, status of back skin in each group on day 22. The saline group presented a healthy skin condition. Mice in the *P. acnes* group skin is red and swollen, with raised papules and hemorrhagic spots. After CHL treatment, the raised papules have almost disappeared. Similarly, after SB203580 treatment, the raised papules almost disappeared and the skin was scabbed. In addition, the skin was stained with H&E to investigate any histopathological changes (Figure 8B). The acne-like skin lesions were found in the *P. acnes* group, but not in the saline group. As seen in Figure 9B, the sebaceous glands of the epidermis and subcutaneous hair follicles were clear in the saline group. In the *P. acnes* group, the epidermis was thickened, the granular layer and spinous layer of hair follicle epithelium were obviously thickened, and the keratinization was excessive. A large number of keratinized material accumulation can be seen at the mouth of the hair follicle and infundibular part, and the infundibular part is expanded into a pot. The number of dermal hair follicles increases, and the adjacent hair follicles fuse with each other. After CHL treatment, the degree of hair follicle orifice dilation and epidermis thickening were alleviated, the number of hair follicles was significantly reduced, and keratinization was obviously loose. SB203580 and CHL have the same therapeutic effect on acne lesions. We did a disease score (Figure 8C) and a pathology score (Figure 8D) based on the scoring criteria of previous studies.<sup>23</sup> CHL can significantly relieve acne-like lesions in mice by disease score and pathology score.



**Figure 8** Compound Huangbai Liquid alleviated acne-like skin lesions induced by *P. acnes*. **(A)** After 21 days, representative lesions induced by health, disease-associated *P. acnes*, CHL treatment, and SB203580 treatment are shown. **(B)** Histological analysis of skin tissue by hematoxylin and eosin staining. Pictures taken at 10x. **(C)** Disease scoring. **(D)** Score of pathology. Group data were expressed as mean  $\pm$  SEM. \* $p < 0.05$  and \*\* $p < 0.01$ , significant difference compared with the saline group; # $p < 0.05$  and ### $p < 0.01$ , significant difference compared with the *P. acnes* group (n=6).



**Figure 9** The Compound Huangbai Liquid alleviated the expression of TLR4/NF-κB/p38 MAPK and Cytokines. **(A)** Immunohistochemistry for TLR4 in the skin. **(B)** Immunohistochemistry for NF-κB p65 in the skin. **(C)** Immunohistochemistry for p38 MAPK in the skin. **(D)** Immunohistochemistry for phospho-NF-κB p65 in the skin. **(E)** Immunohistochemistry for phospho-p38 MAPK in the skin. **(F)** The expression scores of TLR4. **(G)** The expression scores of NF-κB p65. **(H)** The expression scores of p38 MAPK. **(I)** The expression scores of phospho-NF-κB p65. **(J)** The expression scores of phospho-p38 MAPK. **(K)** The level of IL-6 in the skin. **(L)** The level of IL-1β in the skin. **(M)** The level of TNFα in the skin. Group data were expressed as mean ± SEM. \**p* < 0.05 and \*\**p* < 0.01, significant difference compared with the saline group; #*p* < 0.05 and ###*p* < 0.01, significant difference compared with the *P. acnes* group. Pictures taken at 10×(n=6).

## Compound Huangbai Liquid Alleviated the Expression of TLR4/NF- $\kappa$ B/p38 MAPK and Cytokines

To clarify that CHL may reverse acne-related pathological changes via TLR4, NF- $\kappa$ B and p38 MAPK, mice were treated with CHL. Immunohistochemistry for TLR4 (Figure 9A), NF- $\kappa$ B p65 (Figure 9B), p38 MAPK (Figure 9C), phospho-NF- $\kappa$ B p65 (Figure 9D) and phospho-p38 MAPK (Figure 9E) in the skin tissue showed that the expression of TLR4 (Figure 9F), NF- $\kappa$ B p65 (Figure 9G), p38 MAPK (Figure 9H), phospho-NF- $\kappa$ B p65 (Figure 9I) and phospho-p38 MAPK (Figure 9J) in the *P. acnes* group increased significantly ( $p < 0.01$ ), compared to the saline group. The expression of TLR4, NF- $\kappa$ B p65, p38 MAPK, phospho-NF- $\kappa$ B p65 and phospho-p38 MAPK in the *P. acnes*+CHL group and *P. acnes*+SB203580 group significantly reduced after treatment with CHL and SB203580 ( $p < 0.05$  or  $0.01$ ), compared to the *P. acnes* group.

The ELISA test showed that the expression of IL-6 (Figure 9K), IL-1 $\beta$  (Figure 9L) and TNF $\alpha$  (Figure 9M) in the *P. acnes* group increased significantly ( $p < 0.01$ ), compared to the saline group. The production of IL-6, IL-1 $\beta$  and TNF $\alpha$  in the *P. acnes*+CHL group and *P. acnes*+SB203580 significantly reduced after treatment with CHL and SB203580 ( $p < 0.01$ ), compared to the *P. acnes* group.

## Discussion

Acne is a cutaneous chronic inflammatory disorder with complex pathogenesis. Hyperseborrhea and dysseborrhea, altered keratinization of the pilosebaceous duct, *Cutibacterium acnes* and inflammation play crucial roles in the pathophysiology of acne.<sup>13</sup> The doctors and patients in China are showing an increasing interest in the Compound Huangbai Liquid, which is a more natural and generally safer therapeutic option for acne. In the past, because Traditional Chinese medicine (TCM) cannot be understood at the molecular level and systemically, it is not well accepted.<sup>24,25</sup> In recent years, especially after the COVID-19 epidemic, TCM has attracted worldwide attention. “Network pharmacology” was proposed in 2007 and 2008 and has since quickly become the frontier of current drug research and new drug research model.<sup>26,27</sup> Network pharmacology can discover the active ingredients and target genes of traditional Chinese medicine, and reveal the underlying mechanism of action, which may increase the global acceptance of Chinese medicine. The mechanism of Compound Huangbai Liquid in the treatment of acne is still unclear which inhibits the development of Compound Huangbai Liquid. Therefore, network pharmacology provides a new way to explore the pharmacological mechanism of Compound Huangbai Liquid.

In this study, 165 effective active ingredients of Compound Huangbai Liquid were obtained through BATMAN-TCM database, and the core anti-inflammatory ingredients of Compound Huangbai Liquid were screened out as 3-Methyl-2-(2-Pentenyl)-2-Cyclopenten-1-One, beta-Pinene, carvacrol, ursolic acid, D-Limonene, etc. For example, 3-Methyl-2-(2-Pentenyl)-2-Cyclopenten-1-One mediated the downregulation of Vitamin D receptor (VDR) expression and exhibited an inhibitory effect on excessive sebaceous gland activity.<sup>28</sup> At the same time, we found a compound that modulates multiple targets. For instance, ursolic acid suppressed the expression of the TNF $\alpha$  in vitro and inhibited the production of inflammatory cytokine IL-1 $\beta$  and IL-6.<sup>29</sup> In conclusion, the 165 active compounds in the Compound Huangbai Liquid showed synergistic interaction in the treatment of acne. Therefore, based on the outcomes of PPI analysis, GO analysis, KEGG analysis and network analysis, TLR4/NF- $\kappa$ B/p38 MAPK pathway, and inflammatory cytokines IL-6, IL-1 $\beta$  and TNF $\alpha$  played a key role, which were closely related to inflammation response of acne.

Immunohistochemical and ELISA results showed that the expression of TLR4, NF- $\kappa$ B, p38 MAPK, IL-6, IL-1 $\beta$  and TNF $\alpha$  in *P. acnes* groups was significantly higher than that in saline group. In vivo studies have shown that *P. acnes* affects the activation of TLR4 in keratinocytes and increases TLR4 expression in acne-like lesions.<sup>30</sup> Overexpression of TLR4 leads to the activation of NF- $\kappa$ B and MAPK signaling pathways most are activated by TLR4.<sup>31</sup> This causes keratinocytes to produce IL-1 $\beta$ , IL-6 and TNF $\alpha$ .<sup>12</sup> This is consistent with the results of this study. It was reported that proinflammatory cytokine IL-1 $\beta$  is not only involved in the immune response but also played a potential role in the disease of pilosebaceous unit.<sup>32,33</sup> Clinical studies have found that IL-1 $\beta$  expression levels are upregulated around the hair follicles of patients with inflammatory acne lesions.<sup>31</sup> In addition, IL-1 $\beta$  may be responsible for skin inflammation and the resulting keratinocyte proliferation, and may play an important role in plugging of the follicular ostia and microcomedone formation.<sup>33</sup> IL-6, a key cytokine in a variety of inflammatory and immune responses.



IL-6 overexpression is associated not only with NF- $\kappa$ B and p38 MAPK but also with the induction of IL-1 $\beta$ . IL-1 $\beta$  has been shown to be a strong IL-6 inducer in sebocytes.<sup>31,34</sup> IL-6 is an agonist and catalyst of inflammatory response and high expression of IL-6 mediates and intensifies inflammatory response of acne-like lesions. TNF is produced mainly by monocytes and lymphocytes and has been shown to be involved in a variety of inflammatory diseases.<sup>35</sup> There is evidence that TNF $\alpha$  is involved not only in sebaceous lipogenesis but also in the formation of lipid droplets in cells in an in vitro model of inflammatory acne.<sup>36,37</sup> Therefore, acne-related pathological changes were related to the expression of the IL-6, IL-1 $\beta$  and TNF $\alpha$ .

In this study, the expression of TLR4, NF- $\kappa$ B, p38 MAPK, IL-6, IL-1 $\beta$  and TNF $\alpha$  in the *P. acnes*+CHL group decreased significantly compared with the *P. acnes* group. PPI results suggest that TLR4, IL-6, IL-1 $\beta$  and TNF were the potential target of compound CHL in the treatment of acne. KEGG results suggest that CHL treats acne by Chagas Disease signaling pathway. However, NF- $\kappa$ B and p38 MAPK have been involved in the Chagas Disease signaling pathway. There is evidence that inhibition of TLR4, NF- $\kappa$ B, p38 MAPK pathways and inhibiting secretion of IL-6, IL-1 $\beta$  and TNF $\alpha$  can relieve acne-like lesions.<sup>29</sup> Therefore, in this study, CHL may act as a potent anti-inflammatory by inhibiting *P. acnes*-mediated IL-6, IL-1 $\beta$  and TNF $\alpha$  expression via suppressing the TLR4/NF- $\kappa$ B/p38 MAPK activation pathways.

In summary, in this study, the molecular mechanism of Compound Huangbai Liquid in the treatment of acne was predicted by network pharmacology. In addition, the experimental results suggest that Compound Huangbai Liquid plays a good role in the treatment of acne by regulating TLR4/NF- $\kappa$ B/p38 MAPK inflammatory signaling pathway. This study not only provides clinical theoretical basis for the treatment of acne with Compound Huangbai Liquid but also suggests that the production and secretion of pro-inflammatory cytokines may be an effective choice for the treatment of acne.

## Data Sharing Statement

The datasets generated or analyzed in the current study can be found in the databases mentioned in this study. All data generated or analyzed during this study are included in the article published herein or in the [Supplementary Data](#) file.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, All authors took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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