ORIGINAL RESEARCH

Antibacterial Activity of *Thesium chinense* Turcz Extract Against Bacteria Associated with Upper Respiratory Tract Infections

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Purpose: The drug resistance of *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Haemophilus influenzae* has become more and more serious, and it is urgent to seek new antibacterial drugs. In this study, *Thesium chinense* Turcz. extracts were tested for its potential antibacterial activities.

Methods: *T. chinense* powder was extracted with 5 solvents of different polarity (ethyl alcohol, petroleum ether, ethyl acetate, n-butyl alcohol and double distilled water), and their antibacterial activities were tested. The Broth dilution method was used to evaluate the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of highly active plant extracts with a concentration of 1g/mL. The inhibitory activity of this extract on biofilm formation was investigated. Afterwards, we investigated its effect on the transcriptome of *S. aureus*.

Results: The ethanol extract coded as BRY, only inhibited *S. aureus*, whereas the ethyl acetate extract coded as BY2 showed inhibitory effect on all the tested bacteria. The MIC of BRY on *S. aureus* was 128 mg/mL, and the MBC was 512 mg/mL. The MIC of BY2 against *S. aureus*, *S. pneumoniae*, *S. pyogenes* and *H. influenzae* were 8 mg/mL, 4 mg/mL, 4 mg/mL, and 4 mg/mL, respectively. The MBC of BY2 for these four bacteria ranged from 4 to 256 mg/mL. Mechanism studies have shown that BRY and BY2 have an impact on anti-formation of biofilms at MIC concentrations. Transcriptome sequencing results showed that 531 genes were up-regulated and 340 genes showed down-regulated expression in *S. aureus* after BY2 treatment.

Conclusion: BY2 has a broader antibacterial spectrum than BRY. Meanwhile, the inhibitory effect of BY2 on *S. aureus* is better than BRY. The mechanism of BY2 against *S. aureus* may relate to its inhibition of ribosome synthesis, restriction of key enzymes of citric acid cycle, decrease of pathogenicity and influence on biofilm formation. The results confirmed that BY2 was the main antibacterial part of *T. chinense*, which can be used as a source of antibacterial agents.

Keywords: plant extract, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, antibacterial

Introduction

Upper respiratory tract infections (URTIs) are primarily caused by bacterial and viral pathogens, such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Streptococcus pyogenes*, influenza virus (IFV), and respiratory syncytial virus (RSV).[1–3] When infected with these pathogens, people may develop sinusitis, pharyngitis, tonsillitis, or otitis media.[4,5] Among the pathogens that cause UTRIs, bacteria is one of the most threatening to human health. A study of deaths from bacterial pathogen infections found that *S. aureus* infections were the leading cause of death from bacterial infections in 135 countries, and the deadliest bacteria in people over the age of 15 worldwide. Similarly, *S. pneumoniae* is the pathogen associated with the majority of deaths in children younger than 5 years of age.[6] In addition to infecting the respiratory tract alone, bacterial pathogens can cause secondary infections following viral

Graphical Abstract
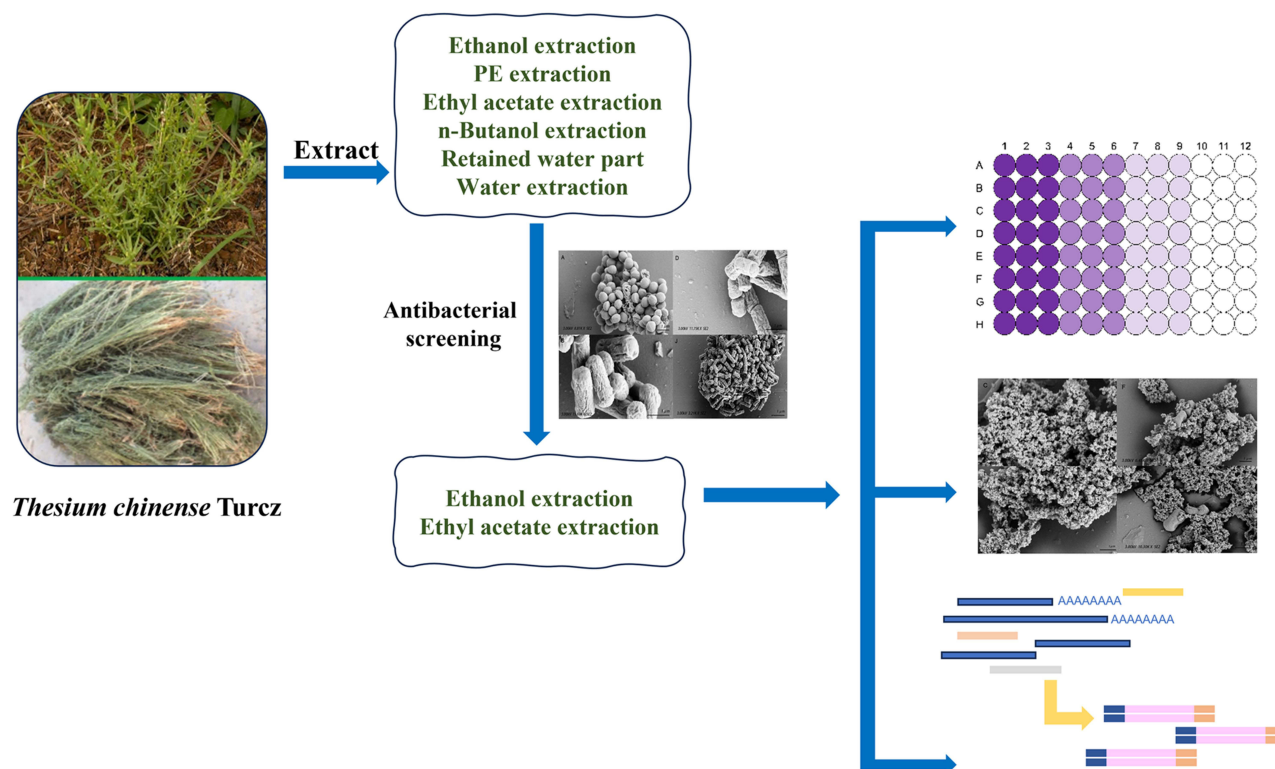
infection. About one-third of viral respiratory infections were caused by influenza viruses.[1] After influenza virus infection, approximately 50 to 90% of severe or fatal influenza infections were associated with bacterial co-infection or secondary infection.[7–9] At least 15 bacterial strains have been reported to be associated with viral respiratory tract infections. The main bacterial strains that increased the risk of death from viral infection were *S. aureus, S. pneumoniae, S. pyogenes*, and *H. influenzae*.[10–14]

It should be emphasized that antimicrobial resistance is rapidly emerging and spreading globally. There have been numerous reports of resistance in *S. aureus, S. pneumoniae, S. pyogenes*, and *H. influenzae*.[15–18] As a result, we need to develop new antimicrobial drugs. In previous studies, medicinal plant extracts have shown good antibacterial effects, which may offer some solutions to the drug resistance problem.[19,20]

China has a long history of using botanicals for antibacterial treatment. *Thesium chinense* Turcz. is a traditional Chinese medicine plant in the genus *Thesium* of family Santalaceae. It is a perennial herb and is commonly used to treat URTIs in traditional Chinese medicine (TCM),[21,22] which is also known as a "natural antibiotic".[23] *T. chinense* was first recorded about a thousand years ago in a TCM monograph called *Bencao Tujing*. According to *Bencao Tujing*, *T. chinense* promotes lactation and regulates bodily functions. This is the embodiment of the ancient use of *T. chinense* for acute mastitis. Bacterial infection is an important cause of acute mastitis. Most patients with acute mastitis are found to have pathogenic bacteria in their milk or pus.[24] Besides, according to *Guoyao Tiyao*, it can be used to treat cervical lymphadenitis which is often caused by infection with *S. pyogenes* or *S. aureus*.[25] In addition, phytochemical studies in recent years have shown that (12*E*) -heptadec-12-en -8,10-diynoic acid, dodec-9,11-diynoic acid and exocarpic acid in *T. chinense* have inhibitory effects on oral bacterial infection.[26] Aqueous extracts of *T. chinense* have also been shown to have antibacterial activity against some Gram-positive and Gram-negative bacteria. Similarly, Yuan et al[27] reported that water extracts of *T. chinense* showed inhibitory effects on *Escherichia coli, S. aureus*, and *Bacillus thuringiensis*. Therefore, *T. chinense* has a good antibacterial effect.[28]

In this paper, we studied the antibacterial effect of six extracts from *T. chinense* (ethanol, petroleum ether, ethyl acetate, n-butanol and double distilled water) against four bacteria closely related to respiratory tract infection (*Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes* and *Haemophilus influenzae*), in order to explore new natural drugs against respiratory tract infection-related bacteria and solve the urgent problem of antibiotic resistance. We hope that this study can provide new therapeutic ideas for the clinic.

Materials and Methods
Plant Materials
The whole dry herb of *T. chinense* was collected from Xiangyang city, Hubei Province, P. R. China in May 2019. The species was identified by Prof. Kai-Jin Wang of Anhui University, and a voucher specimen (No. 20190927) was deposited in the School of Pharmacy, Anhui Medical University.

Ethanol Extract Preparation
Plant materials (10 kg) were sequentially soaked in ethanol (95%, 85%, 75%, 1:10 w/v) at room temperature for 12 h, and ultrasonically extracted at 60°C for 4 h. The filtrate was combined, filtered, and evaporated with a rotary evaporator. The extract was named BRY.

Preparation of Other Extracts
A portion of BRY (23 kg) was suspended with 23L water, and then sequentially extracted 4 times (1:1 v/v, 30 min) with petroleum ether (PE), ethyl acetate (EA) and n-butanol. The organic phases were evaporated and freeze-dried to obtain BS1, BY2 and BZ3, respectively. The last remaining aqueous phase was collected and lyophilized and named as BS4.

The filter residue in "Ethanol extract preparation" was extracted with double distilled water (1:1 w/v, 4 h) at 60°C for 3 times. The filtrate was collected, and finally freeze-dried, named as BRS.

The prepared samples were dissolved in sterile PBS and filtered at 0.22 μm.

Bacterial Strains and Culture Conditions
S. aureus (ATCC 29213) was obtained from the First Affiliated Hospital of Anhui Medical University. *S. pneumoniae* (ATCC 49619), *S. pyogenes* (ATCC 19615) and *H. influenzae* (ATCC 49766) were purchased from Hefei MORE Bio.

S. aureus was cultured in Mueller–Hinton broth (MHB). *S. pneumoniae, S. pyogenes* and *H. influenzae* were cultured in Mueller–Hinton broth (MHFB) supplemented with 5% lysed horse blood and 20 mg/L β-NAD.[29] The strains were cultured at 37°C, 200 rpm.

Preliminary Screening of Antibacterial Activity
Bacterial suspension (10^6 CFU/mL) was prepared with sterile medium. An equal volume of bacterial suspension was added to agar medium and allowed to dry. Wells were made with a sterile borer in the inoculated agar plates. Extracts (100 μL) were propelled into the wells for each test plates.[30] The plates were incubated at 37°C, 5% CO_2 for 24 h, and then the diameter of the inhibition zone was measured with a vernier caliper.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)
MIC was measured by broth microdilution[31] in 96-well microtiter plates. A 100 μL of each bacterial suspension (2×10^6 CFU/mL) was incubated 24 h with 100 μL aliquot of test concentration of each extract prepared before.

Inoculum (100 μL) from wells without turbidity was spread onto the agar plate at 37 °C for 24 h. The number of the colony-forming units was counted. There will be no colony formation on the plate of the MBC.[32]

Determination of Time–Kill Curves
The 96-well microtiter plates were containing bacterial culture (1×10^6 CFU/mL, final concentration) and 100 μL extract prepared before. The final concentration of extract was $0.5 \times$ MIC and $1 \times$ MIC, respectively. The bacteria-containing PBS

without extract was set as the negative control group, and PBS was used to be solvent control. Then, the absorbance value was measured at 630 nm (Synergy™ HTX) at different time points (0, 2, 4, 8, 12 and 24 h), and a growth curve was drawn.

Biofilm Formation Inhibition Assay

Bacteria with or without extracts were cultured in 96-well plates at 37°C for 24 h. The supernatant was discarded to remove free bacteria. Bacteria were fixed with methanol for 20 min, stained with crystal violet (0.1%), washed with PBS after 20 min to remove unbound crystal violet solution. Finally, absolute ethanol was added to the 96-well plate. Plates were incubated at 37°C and 200 rpm for 30 minutes. The absorbance value was measured at 590 nm.

Determination of CFU of Bacteria in Biofilms

The treatment of biofilms was as described in "Biofilm formation inhibition assay". Free bacteria were discarded gently and 200 μL PBS was used to resuspend the bacteria on the biofilm. The bacterial suspension was collected and inoculated on agar plates. CFU was calculated after 24 h.

MTT Metabolism Detection

After treating the biofilm as described in "Biofilm formation inhibition assay", the free bacteria were discarded. A 20 μL MTT (5 mg/mL) was added to each well, and the plates were incubated in the dark for 4 h. After that, MTT was discarded, and DMSO was added. Plates were incubated at 37°C for 15 min. The absorbance value was measured at 570 nm.

SEM

The 0.5 × MIC extracts were co-cultured with bacterial suspensions with a final concentration of 10^6 CFU/mL. Bacterial suspension without drug was taken as negative control. The bacterial strains were cultivated at 37°C and 200 rpm for 24 h. After centrifugation, the bacteria were immobilized with 2.5% glutaraldehyde at 4°C for 8–12 h. The sample was dehydrated with different concentrations of ethanol (10%, 30%, 50%, 70%, 80%, 90%, 100%) and then dehydrated with HMDS. After coated with gold-palladium, the sample was observed and photographed with a scanning electron microscope (ZEISS Gemini SEM 300, Germany).

Pretreatment of Transcriptome Sequencing Samples

Transcriptome sequencing was performed on *S. aureus* treated with BY2.

The bacterial suspension was adjusted by 0.5 McIntosh units. The drug was added to a prepared bacterial suspension with a bacterial concentration of 10^6 CFU/mL and a final drug concentration of 0.5 × MIC. The bacterial suspension without drug was taken as the negative control. The suspensions were cultured at 37°C and 200 rpm for 24 h.

After centrifugation at low temperature, the supernatant was discarded, and the sample was cleaned with PBS 3 times to remove the medium. The sample was quickly frozen with liquid nitrogen at low temperature and stored in the refrigerator at −80°C. For each group, 3 biological samples were prepared for repeated testing. The above samples were transferred on dry ice to the high-throughput laboratory of Wuhan BGI Technology Service Co., LTD for transcriptome sequencing and follow-up analysis.

Transcriptome Sequencing and Analysis Table

The clean reads were compared to the reference genome for the mapped data. Then, quality assessments were performed, and differential expression genes (DEGs) were detected between groups, for bioinformatics analyses of functional annotation, Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways and functional enrichment.

Real-Time PCR Assay for Gene Expression Analysis

The bacteria were cultured for 24 hours according to the method described in "Pretreatment of transcriptome sequencing samples". After centrifugation, the total RNA of *S. aureus* was isolated using an RNA extraction kit as described. Reverse transcription of total RNA into cDNA.[33] RT-qPCR was used to detect gene expression. 16S RNA was used as housekeeper gene, and the results were expressed by $2^{-\Delta\Delta Ct}$. All primer sequences are shown in Table 1.

Table 1 List of Primer Sequences Used for Quantitative Real-Time PCR (qRT-PCR)

Genes	Primer Direction	5'- 3' Sequence
argJ	Forward	GTTGAGCAACCCATGTTTGTGTTTG
	Reverse	GCTGCACCACTTATTGTCACTGAAG
rpsL	Forward	TTAACGCATACATCCCTGGTATCGG
	Reverse	TGGTAACGCACACCTGGTAAGTC
sucC	Forward	TGAGATTGAAGAAGTTGCTGCGAAG
	Reverse	CGTCTTGCTTGGAATGGTGAAAGTC
gcvT	Forward	AGTCAGGTTACACAGGTGAAGATGG
	Reverse	CTTAATGTATCACGAGCGCCTAACC
clpP	Forward	TCACCAGGTGGAAGTGTAACAGC
	Reverse	CCATTGATGCAGCCATACCGATAC
dnaK	Forward	TGAAAGACGCTGGCTTAACAAACTC
	Reverse	GCTTCTTGTACTGCTGGAATACGAG
thrB	Forward	GGCAAGAGGGTTAGGTTCGTCAG
	Reverse	GTCGGCGCAACATTATCAGGATG
leuB	Forward	ATTGTTGCCCTACCTGGTGATGG
	Reverse	AGGCTCGCCGAATGTATCAATAGAG
icd	Forward	GGCAAGAAGCGGCAGATAAGATTAC
	Reverse	CACCATCCATTAAACGGGCAAAGTC
gltA	Forward	CGTTGTGCGGTATCATCATTGTCAG
	Reverse	AATGGCCCTTTCAGAGAACCTACAG
16s RNA	Forward	CACACCGCCCGTCACACC
	Reverse	CCAGCCGCACCTTCCGATAC

Statistical Analysis

All data were analyzed using GraphPad Prism 8. Significant differences were calculated using one-way ANOVA, and $P < 0.05$ was considered statistically significant.

Results

Antimicrobial Activity

The diameters of the inhibition zones of BRY, BS1, BY2, BZ3, BS4 and BRS against the four bacteria are shown in Table 2. BRY and BY2 have shown antibacterial activity against *S. aureus*. Moreover, BY2 also has an inhibitory effect on *S. pneumoniae, S. pyogenes*, and *H. influenzae*. No significant inhibitory zone was detected for other extracts. Therefore, BRY and BY2 were considered to be highly active extracts, and they were used to conduct subsequent studies.

The MICs and MBCs of BRY and BY2 against the four strains were determined by the broth microdilution method (Table 3, Figure 1), the MIC of BRY against *S. aureus* was 128 mg/mL, and the MBC was 512 mg/mL. BY2 inhibited the

Table 2 The Diameter of the Inhibition Zone

Plant Extracts	*S. aureus*	*S. pneumoniae*	*S. pyogenes*	*H. influenzae*
BRY	9.67±0.24mm	ND	ND	ND
BS1	ND	ND	ND	ND
BY2	13.27 ± 0.68mm	16.4 ± 1.78 mm	16.57 ± 0.54mm	17.23 ± 0.34mm
BZ3	ND	ND	ND	ND
BS4	ND	ND	ND	ND
BRS	ND	ND	ND	ND

Note: The data are expressed as mean ± standard deviation (n=3).
Abbreviations: mm, millimeter ND, not detected.

Table 3 Antibacterial Activities of BRY and BY2

Bacterial Strains	BRY		BY2	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
S. aureus	128	512	8	16
S. pneumoniae	NA	NA	4	4
S. pyogenes	NA	NA	4	128
H. influenzae	NA	NA	4	64

Abbreviation: NA, not applicable.

growth of *S. aureus, S. pneumoniae, S. pyogenes*, and *H. influenzae* at MICs of 8 mg/mL, 4 mg/mL, 4 mg/mL, and 4 mg/mL, respectively. The MBC values were 16 mg/mL, 4 mg/mL, 256 mg/mL, and 64 mg/mL, respectively.

Time–Killing Curves

The time–killing curves for BRY and BY2 against bacteria are shown in Figure 2. At a BRY concentration of 0.5 × MIC, *S. aureus* grows slowly in the range 0–12 h. However, bacterial growth became faster after 12 h, and the OD_{630nm} value at 24 h was somewhat similar with the negative control. As the concentration is increased to 1 × MIC, the OD_{630nm} value of the bacterial solution is very low, indicating that very few bacteria grow. Unlike BRY, BY2 also shows a good inhibitory effect on *S. aureus* at 0.5 × MIC concentration.

The OD_{630nm} values for *S. pneumoniae, S. pyogenes*, and *H. influenzae* demonstrate that bacterial growth is still significantly suppressed when the BY2 concentration is at 1 × MIC. However, when the concentration is 0.5 × MIC, the growth of the bacteria accelerates at 8h, and eventually the results are similar to those of the negative control.

Bacterial Biofilm Inhibition Results

Bacterial pathogenicity is associated with a number of factors, including biofilm formation. Therefore, we performed a biofilm formation disruption experiment. As shown in Figure 3, BRY and BY2 appear to have disrupted the formation of bacterial biofilms at MICs. However, the effect was less pronounced at a concentration of 0.5 × MIC.

According to the inhibitory effect of BRY and BY2 on bacterial biofilm formation, we further studied the effect of BRY and BY2 on the bacterial viability in biofilm (Figure 4) and the growth of bacteria on solid medium after treatment
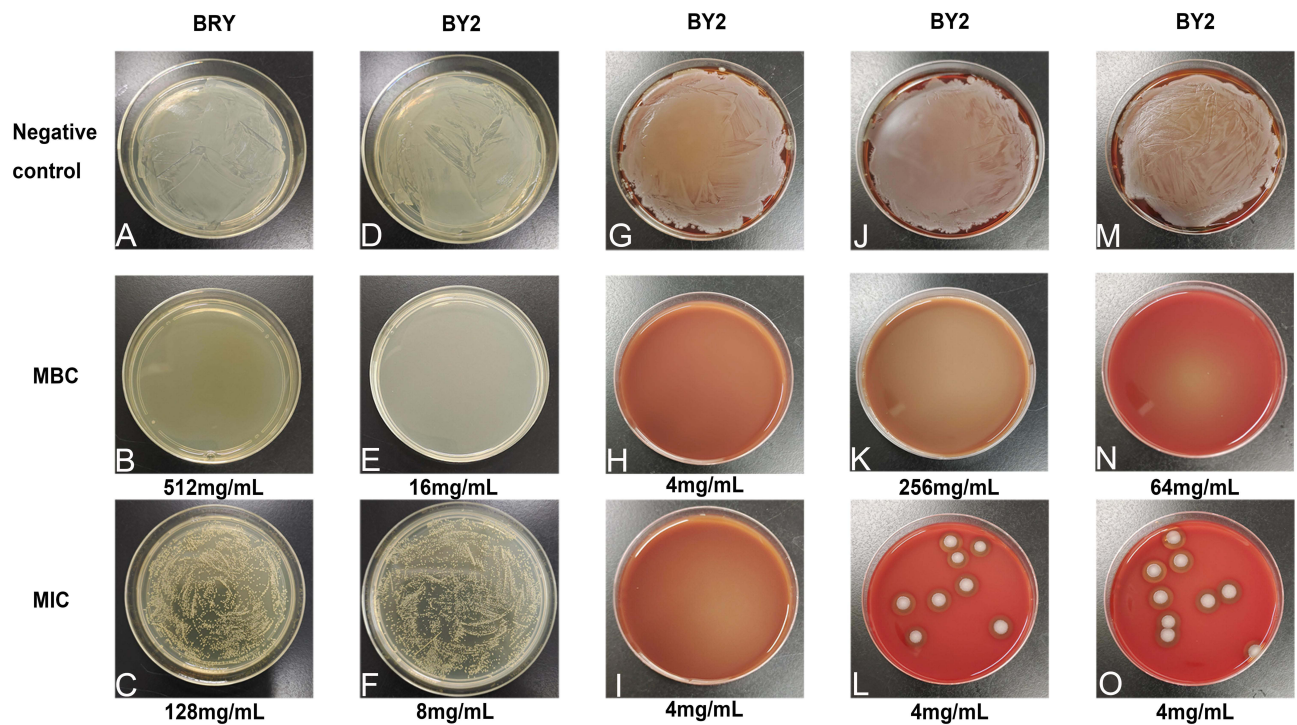
Figure 1 The growth of bacteria strains on solid medium (BRY and BY2 at MICs and MBCs).
Notes: (**A–F**) *S. aureus*, (**G–I**) *S. pneumoniae*, (**J–L**) *S. pyogenes*, (**M–O**) *H. influenzae*.
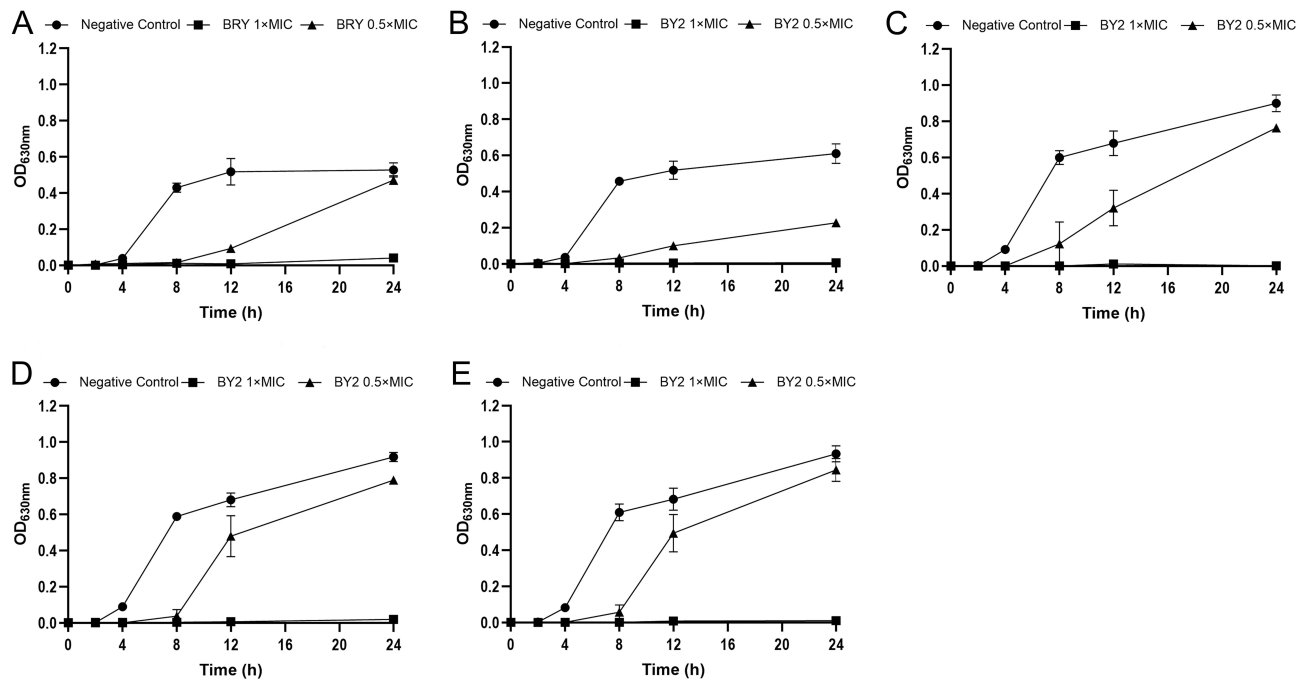

Figure 2 Time–killing curves for BRY and BY2 against tested bacteria at 1 × MIC and 0.5 × MIC.
Notes: (**A** and **B**) *S. aureus*, (**C**) *S. pneumoniae*, (**D**) *S. pyogenes*, (**E**) *H. influenzae*.

(Figure 5). BRY and BY2 (1 × MIC) significantly reduced the bacterial activity in biofilm of *S. aureus*, and the effect of BY2 was better than that of BRY. BY2 performed similarly on the other three tested bacteria, with a significant reduction in cell viability at 0.5 × MIC and 1 × MIC.
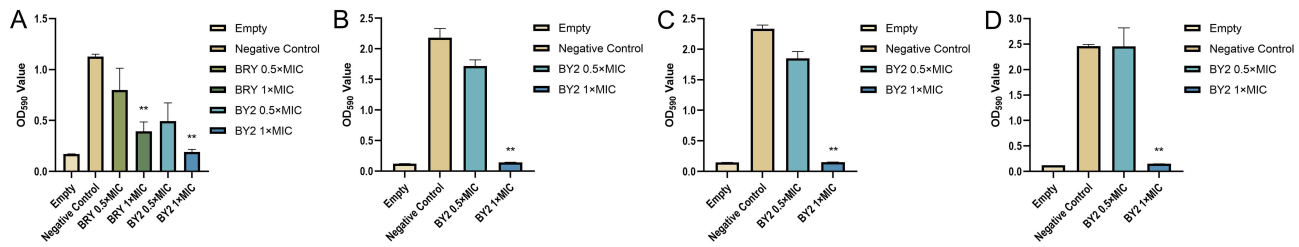
Figure 3 BRY and BY2 inhibits the biofilm formation of tested bacteria.
Notes: (**A**) *S. aureus*, (**B**) *S. pneumoniae*, (**C**) *S. pyogenes*, (**D**) *H. influenzae*. **Compared with "Negative Control", $P < 0.01$.
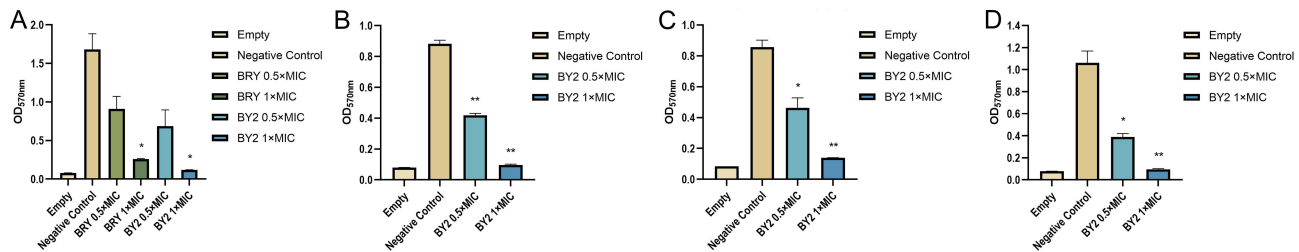

Figure 4 MTT metabolic assay results of bacteria.
Notes: (**A**) *S. aureus*, (**B**) *S. pneumoniae*, (**C**) *S. pyogenes*, (**D**) *H. influenzae*. *Compared with "Negative Control", $P<0.05$. **Compared with "Negative Control", $P<0.01$.
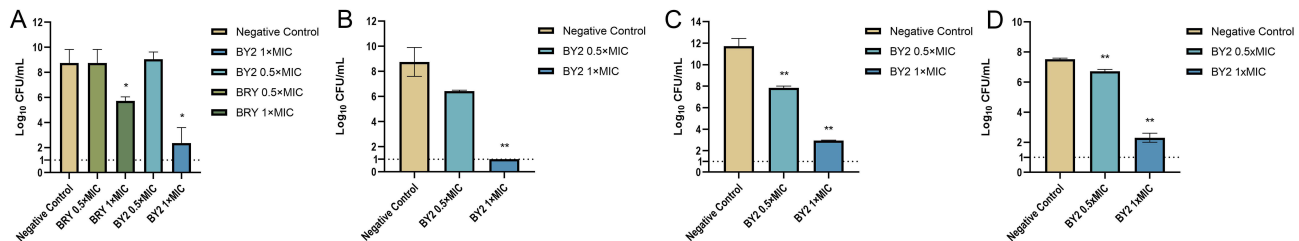

Figure 5 CFU of bacteria biofilm.
Notes: (**A**) *S. aureus*, (**B**) *S. pneumoniae*, (**C**) *S. pyogenes*, (**D**) *H. influenzae*. *Compared with "Negative Control", $P<0.05$. **Compared with "Negative Control", $P<0.01$.

Both BRY and BY2 were able to reduce the number of bacteria in the biofilm of the tested bacteria at $1 \times$ MIC. BY2 ($0.5 \times$ MIC) is also effective in reducing bacteria in biofilms of *S. pyogenes* and *H. influenzae*.

Observation of Bacteria

SEM results (Figure 6) showed that the bacteria without drug treatment (negative control) grew well. Bacteria were severely damaged after BRY or BY2 treatment ($1 \times$ MIC). The antimicrobial effect of BRY and BY2 can be evaluated by the fact that only a very small number of bacteria survive.

Analysis of Transcriptome Sequencing Results

RNA sequencing was used to analyze the transcriptome of *S. aureus*. The differentially expressed genes between the control group and the experimental group were analyzed. The results showed that a total of 1019 genes were changed in *S. aureus* treated with BY2 compared with the untreated sample control, of which 531 genes were up-regulated and 340 genes showed down-regulated expression (Q value < 0.05 and log2-fold-change > 1 or < −1). The volcano plot of differential genes is shown below (Figure 7), with upregulation in red and downregulation in blue. In order to verify the accuracy of the results of RNA-Seq, some differentially expressed genes were selected randomly for RT-qPCR, and 16S RNA was used as the housekeeping gene. The results of RT-qPCR were consistent with the trend of gene expression by RNA-seq (Figure 8). To analyze the antibacterial mechanism of BY2 against *S. aureus*, gene ontology (GO) analysis
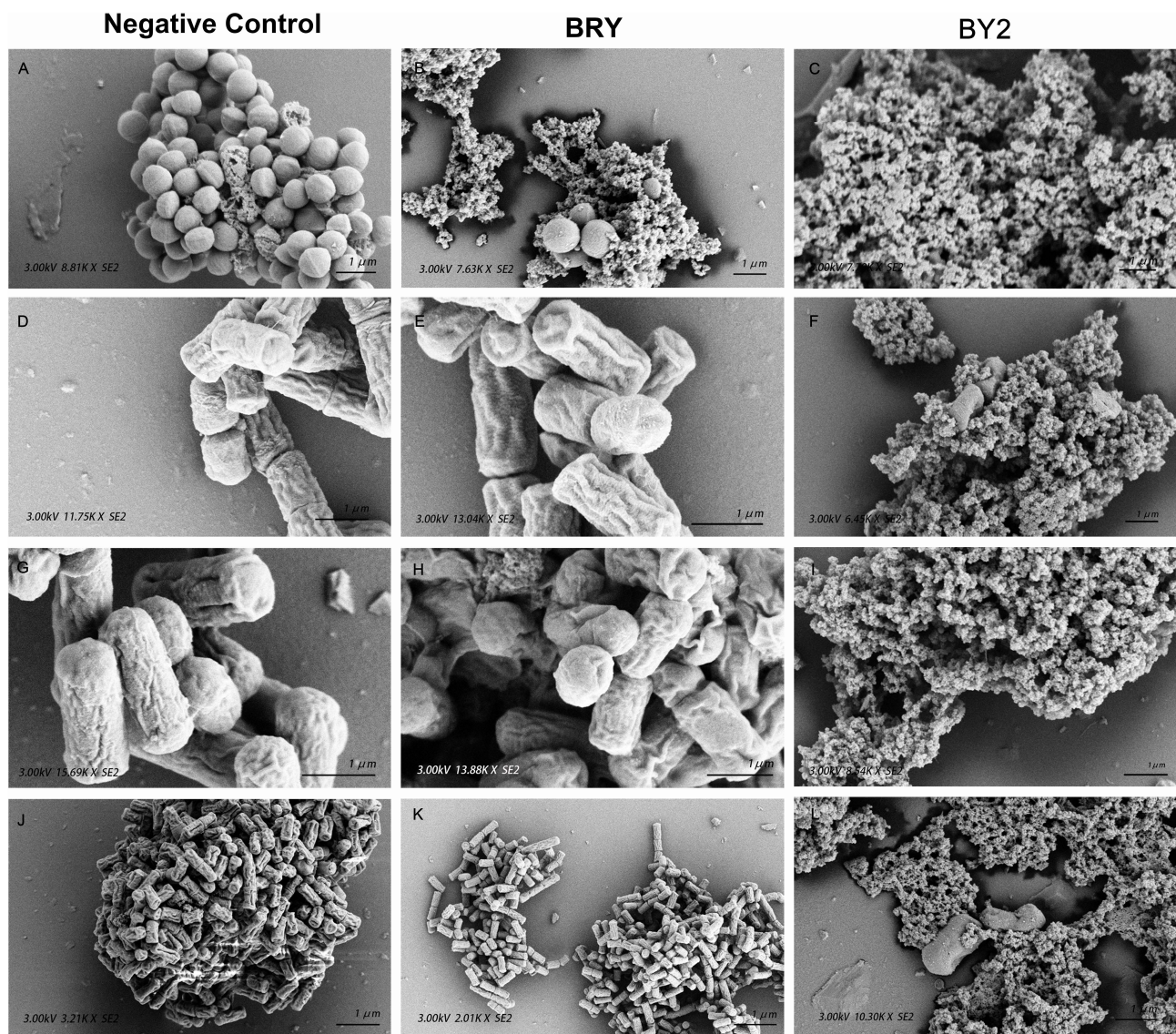
Figure 6 SEM visualization of bacteria.
Notes: (**A–C**) *S. aureus*, (**D–F**) *S. pneumoniae*, (**G–I**) *S. pyogenes*, (**J–L**) *H. influenzae*.

(Figure 9A) and KEGG analysis (Figure 9B) were performed, respectively. GO results showed that after BY2 treating, DEGs were mainly enriched in cellular process, metabolic process, localization and biological regulation. They contained 641, 580, 120 and 114 genes, respectively. The up-regulated genes involved 121 pathways, which were mainly enriched in 15 pathways, of which 3 pathways were significantly enriched (Q-value < 0.05), namely photosynthesis (8 genes), amino acid biosynthesis (53 genes) and ribosome (33 genes). Down-regulated genes were mainly involved in 131 pathways, and the main enrichment pathways included citric acid cycle (15 genes), carbon metabolism (40 genes) and life span regulation (6 genes) (p < 0.01), including 641, 580, 120 and 114 genes, respectively.

Discussion

T. chinense extract is believed to have a good inhibitory effect on Gram-positive bacteria. Additionally, it is sensitive to *Aeromonas hydrophila*, a gram-negative bacterium.[28] Another study reported that it showed antimicrobial activity against the proliferation of three oral pathogens, including *Porphyromonas gingivalis, Fusobacterium nucleatum* and *Streptococcus mutans*.[26] This suggests that *T. chinense* have a broad-spectrum antibacterial effect. Therefore, we conjecture that it also has an inhibitory effect on other bacteria.
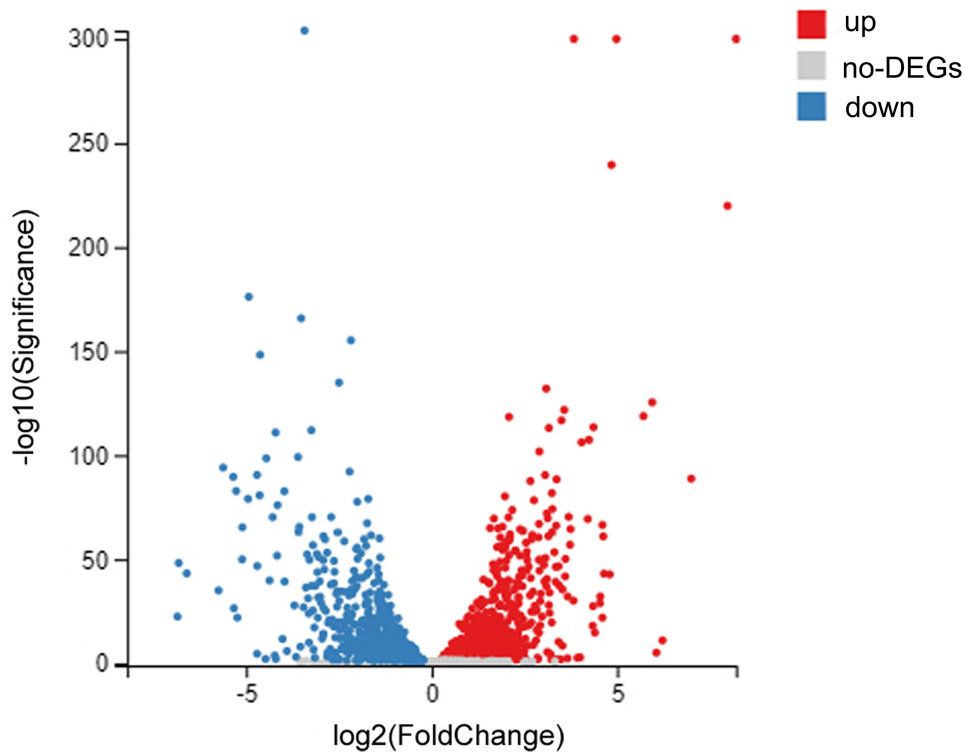
Figure 7 Volcano plot of differential gene expression in *S. aureus* treated with BY2.
Notes: The y-axis is the -log10(significance), and the x-axis is log2-fold-change. Positive values (red) indicate upregulated gene expression and negative values (blue) indicate downregulated gene expression.

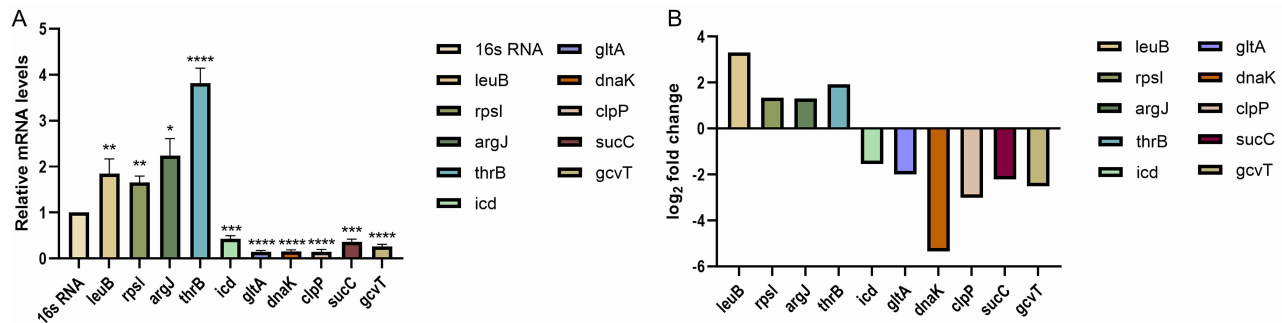**Figure 8** Real-time qPCR was used to verify the results of RNA-seq.
Notes: Bacteria were collected under the same conditions as used for transcriptome sequencing, and total RNA was extracted and reverse transcribed into cDNA. QPCR was used to detect changes in gene expression. (**A**) qPCR results of target genes (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$). (**B**) analysis of differential gene expression results by transcriptome sequencing ($Q<0.05$).

In this paper, we selected several bacteria that caused the most deaths due to co-infection or secondary infection in the influenza pandemic, which were *S. aureus, S. pneumoniae, S. pyogenes* and *H. influenzae,* and evaluated the antibacterial activity of each extract part of *T. chinense*. Different from the previous studies where only one or two solvents were used for extraction,[34] this time, ethanol extraction was performed first. The filtered and filtered residuals of the ethanol extract were then extracted using PE, EA, n-butanol and ddH2O, respectively. The final water section was retained. Eventually, six different fractions of the extract were obtained.

Clearly, the results of in vitro experiments using the punch method to measure the inhibitory zone suggested that BRY has an inhibitory effect on *S. aureus*, in agreement with the results of Liu et al.[27] However, the diameter of the suppression zone of BRY was not detected for other strains. The antibacterial zone diameters of BY2 against
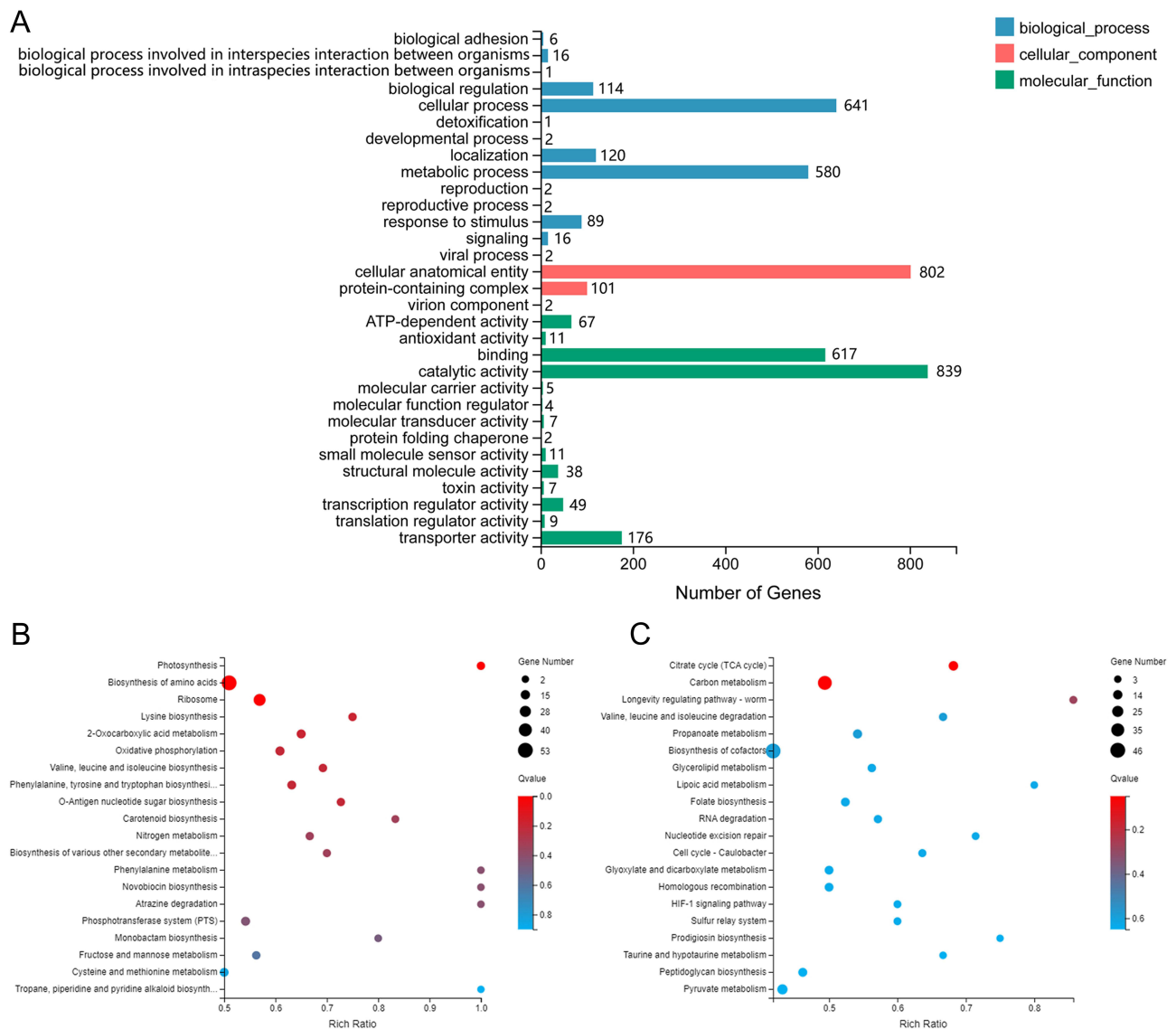
Figure 9 Differential gene expression involved in the BY2 treatment of *S. aureus* upon transcriptome sequencing.
Notes: (**A**) GO annotation classification map. The ordinate text represents the category of GO, to which the type is indicated in the legend. (**B**) KEGG pathway enrichment bubble map of upregulated genes. (**C**) KEGG pathway enrichment bubble map of downregulated genes. Larger bubbles in the figure indicate a greater number of DEGs. The color gradient indicates the significance of enrichment.

S. aureus, S. pneumoniae, S. pyogenes and *H. influenzae* were 13.27±0.68mm, 16.4±1.78mm, 16.57±0.54mm and 17.23 ±0.34mm, respectively. The inhibition of BS1, BZ3, BS4, and BRS were not available. Therefore, the next experiments were performed only on BRY and BY2. The effect of BRY and BY2 on bacterial growth could be visually observed through dynamic time–kill curves. Especially, when the concentration was 1 × MIC, the absorbance results indicated that there is almost no bacterial growth. On this basis, crystal violet staining detection (Figure 3) and scanning electron microscope observation (Figure 6) were carried out. Crystal violet is a basic dye that binds to nucleic acids in nuclei and stains them blue. Based on the results of crystal violet staining, it can be determined that BRY disrupts the biofilm formation of *S. aureus*. The biofilm destruction effect was strong for a concentration of 1 × MIC, which was also verified by the SEM results. Compared to BRY, BY2 disrupts the biofilm formation of more bacterial strains.

Biofilms are fixed microbial communities attached to a surface. In the respiratory tract, bacteria localize in communities of biofilms.[35] Biofilm formation is one of the main epidemiological factors in bacterial infection. The presence of biofilms can impede the entry of some immune defenses, for example, causing macrophages to incompletely penetrate

the biofilm matrix and appear to be "frustrated phagocytosis". Bacteria in biofilms are more resistant to current treatments than planktonic bacteria. In addition, biofilm formation has the potential to increase patient mortality. Severe infection with methicillin-resistant *S. aureus* has a mortality rate of approximately 20%–25%.[36–40]

BRY and BY2 not only disrupted bacterial biofilm formation, but also reduced bacterial viability in the biofilm (Figure 5). In other words, after treatment with two extracts, the number of bacteria in the biofilm decreased significantly. It means that *T. chinense* may have the potential to alleviate the pressure of antibiotic resistance.

By analyzing the transcriptome sequencing results of *S. aureus* treated and untreated with BY2, combined with GO functional annotation analysis and KEGG pathway enrichment analysis, we found significant enrichment of up-regulated genes in photosynthesis, amino acid biosynthesis, and ribosome. The photosynthetic pathway is mainly associated with the F-type H^+/Na^+ ATPase. The F-type ATPase gene is found primarily in the plasma membranes of plants and bacteria. It is powered by the ATP. A large number of genes were enriched in amino acid biosynthesis pathways, mostly in glycolysis and amino acid metabolism, such as gap, ilvd, leuB, aroK, trpD, etc. The expression of glyceraldehyde-3-phosphate dehydrogenase was up-regulated, which facilitates the conversion of three carbon compounds from glyceraldehyde-3p to pyruvate. At the same time, the expression of 3-isopropyl malate dehydrogenase is increased, which promotes the production of branched-chain amino acids. Genes enriched in the ribosomal pathway included rplE and rpsL, which encode ribosomal component proteins. The ribosome is an important organelle for bacteria to survive and consists of three rRNA chains, 16S, 23S and 5S. 23S rRNA, 5S rRNA, and more than 30 proteins together form the large 50S subunit, and the small 30S subunit and large 50S subunit combine to form the 70S complex. Ribosome is known as the "molecular machine" of protein synthesis, which converts the genetic code into amino acid sequences and constructs protein polymers from amino acid monomers, which is of great significance to cell life activities.[41] In this study, BY2 could cause a significant up-regulation of ribosome-related genes after acting on *S. aureus* (Figure 7B), which affected the process of bacterial ribosomal protein synthesis. In the enrichment analysis of down-regulated genes, we did not find significantly enriched pathways (Qvalue > 0.05). The analysis results indicate that the number of genes annotated as part of the KEGG pathway in this strain is relatively small, which may contribute to the distortion of statistical significance. Therefore, pathways with $P < 0.01$ were analyzed here. The down-regulated enrichment results for the differentially expressed genes indicate that the pathways primarily involved include citric acid cycle, carbon metabolism, and lifespan regulation. The citric acid cycle is an important aerobic pathway that is the final step in the metabolism of sugar, fat, and amino acids.[42] In a series of reactions, the two carbons in citrate are oxidized to carbon dioxide, and this reaction pathway provides NADH for oxidative phosphorylation and other metabolic processes. After treating *S. aureus* with BY2, the expression of 15 genes involved in the citric acid cycle was down-regulated. In particular, the expression of citrate synthase, isocitrate dehydrogenase, dihydrolipoamide succinyltransferase and dihydrolipoamide dehydrogenase, which are involved in the formation of the pyruvate dehydrogenase complex, were significantly down-regulated. Furthermore, we note that the expressions for clpP and dnaK are similarly suppressed. ClpP is one of the housekeeping genes regulating the virulence of *S. aureus*.[43] By inhibiting the expression of the clpP gene, bacterial pathogenicity can be reduced and thus antibacterial effects can be enhanced. In addition, it has been pointed out that clpP and its molecular chaperone dnaK can affect bacterial biofilm formation.[44] Therefore, we suggest that BY2 shows good antimicrobial activity against *S. aureus*, which may be related to its effect on ribosome synthesis, limiting key enzymes of the citric acid cycle, reducing pathogenicity and affecting biofilm formation.

However, the lack of in vivo experiments and the lack of further identification and characterization of the active compounds are limitations of this study. We will also continue to conduct research to provide insights into the next step.

Conclusion

This study demonstrated the antibacterial activity of *T. chinense* extracts against *S. aureus, S. pneumoniae, S. pyogenes* and *H. influenzae*. The results showed that ethanol extract and ethyl acetate extract inhibited in vitro growth of at least one or more of the tested bacteria. Ethyl acetate extract effectively inhibited the growth of four bacterial species, indicating that the metabolites in the extract have broad spectrum of antibacterial activity. It had low MIC values for all the tested bacteria. In addition, the growth of *S. aureus* was inhibited when ethyl acetate extract was at $0.5 \times$ MIC. The results of RNA-seq indicate that the inhibitory mechanism of ethyl acetate extract on *S. aureus* growth may be related to its inhibition of ribosomal synthesis, restriction of key enzymes in the citric acid cycle, reduction of pathogenicity, and

influence on biofilm formation. The overall results substantiate the use of *T. chinense* as an antibacterial agent. After a more in-depth analysis of isolated and characterized active compounds, this traditional plant could be the best candidate for further research in the development of new antimicrobial agents.

Abbreviations

BMD, broth microdilution; BRS, water extract of *Thesium Chinense* Turcz; BRY, ethanol extract of *T. chinense*; BS1, petroleum ether extract of *T. chinense*; BS4, the remaining aqueous phases of BZ3; BY2, ethyl acetate extract of *T. chinense*; BZ3, n-butanol extract of *T. chinense*; ddH$_2$O, double distilled water; EA, ethyl acetate; g, gramme; HMDS, Hexamethyldisilazane; MBC, minimum bactericidal concentration; mg, milligramme; MHB, Mueller–Hinton broth; MHFB, Mueller–Hinton broth supplemented with lysed horse blood and β-NAD; MIC, minimum inhibitory concentration; MTT, 3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl Tetrazolium Bromide; NA, not applicable; PBS, phosphate-buffered saline; PE, petroleum ether; SEM, scanning electron microscopy; URTIs, upper respiratory tract infections; β-NAD, β-Nicotinamide adenine dinucleotide.

Ethics Approval and Informed Consent

Research on plants, including the collection of plant material, is conducted in accordance with guidelines provided by the author's institution and national or international regulations.

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, or in all these areas; 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.

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Disclosure

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