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ORIGINAL RESEARCH

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Exploring the mechanism of Bu Zhong Yi Qi Tang in the treatment of chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) based on network pharmacology and molecular docking

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Abstract

Background: Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) is a common and persistent condition affecting the male urinary system, with a global prevalence ranging from 2% to 10%. This study aims to investigate the therapeutic mechanisms of Bu Zhong Yi Qi Tang (BZYQT) in treating CP/CPPS using network pharmacology and molecular docking techniques. **Methods**: The active compounds and their corresponding target proteins of BZYQT were identified and screened using the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP). Targets associated with CP/CPPS were determined through GeneCards, the Therapeutic Target Database (TTD), Online Mendelian Inheritance in Man (OMIM), and the Pharmacogenomics Knowledgebase (PharmGKB). Overlapping targets between BZYQT and CP/CPPS were analyzed using the STRING database to construct a protein-protein interaction (PPI) network. Key targets were further subjected to Gene Ontology (GO) functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Molecular docking studies were conducted to validate the interactions between core active compounds and key targets. In vitro experiments were performed to confirm the therapeutic efficacy of BZYQT in treating CP/CPPS. **Results**: The study identified 103 active compounds in BZYQT and 2064 potential target proteins. A total of 1020 CP/CPPS-related targets were retrieved from GeneCards, OMIM, TTD, and PharmGKB, leading to the identification of 73 overlapping targets and 5 core targets. GO enrichment analysis revealed that these targets are involved in inflammatory responses, apoptosis, oxidative stress, and cell proliferation. KEGG pathway analysis highlighted associations with pathways such as the ErbB signaling pathway. Molecular docking results suggested that kaempferol, an active compound in BZYQT, exhibited the highest binding affinity with the target proteins. Experimental validation demonstrated that kaempferol effectively inhibited the expression of EGFR, MMP9, TNF-α, and IL-6. **Conclusions**: BZYQT exhibits significant therapeutic potential in the treatment of CP/CPPS through its multi-target and multi-pathway mechanisms. The active compound kaempferol plays a crucial role in alleviating pathological changes in CP/CPPS by downregulating the expression of MMP9, EGFR, IL-6, and TNF-α. These findings provide a molecular basis for the application of traditional Chinese medicine in the management of CP/CPPS.

Keywords

BZYQT; Chronic prostatitis; Chronic pelvic pain syndrome; Network pharmacology

1. Introduction

Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) is a prevalent long-term ailment that impacts the male urinary system [1]. Epidemiological studies indicate that the global prevalence of chronic prostatitis ranges between 2% and 10%, making it a widespread health issue [2]. CP/CPPS is the most common form of prostatitis [3] and is considered a diagnosis of exclusion, with no standardized treatment available. Patients frequently experience chronic or recurring pain in the pelvic or perineal region, along with urinary issues, sexual dysfunction, and additional symptoms [4]. While the precise cause of CP/CPPS is not yet fully understood, studies indicate that its development may be closely linked to factors including

infection, inflammation and autoimmune reactions [5], and neuroendocrine dysfunction [6].

Bu Zhong Yi Qi Tang (BZYQT), a traditional Chinese herbal formula composed of Astragalus, Codonopsis, Atractylodes, Chenpi, Angelica, Cizhi, Bupleurum, Licorice, Ginger and Jujube [7], has been increasingly utilized in recent years to treat chronic prostatitis, complicated urinary tract infections, benign prostatic hyperplasia [8, 9], and other conditions. Research has indicated that BZYQT exerts a notable regulating influence on immune cells, impacting the activities of both T cells and B cells. This ultimately boosts the body's capacity to fend off infections [10]. Moreover, studies indicate that BZYQT has the potential to enhance immune function through regulating the composition of gut microbiota [11]. In conclusion, BZYQT holds great potential as an adjunctive therapy for CP/CPPS.

2. Reagents and instruments

RWPE-1 cells: Purchased from the Shanghai Cell Bank. Lipopolysaccharide (LPS): Catalog number L2630, sourced from Sigma, St. Louis, MO, USA. Kaempferol: Catalog number A10018, sourced from Yuanye, Shanghai, China. MMP9 antibody: Catalog number ab76003, sourced from Abcam, Cambridge, UK. Interleukin-6 (IL-6) antibody: Catalog number ab233706, sourced from Abcam, Cambridge, UK. Tumor Necrosis Factor-alpha (TNF-α) Polyclonal antibody: Catalog number 17590-1-AP, sourced from Proteintech, Rosemont, IL, USA. MMP9 Enzyme-Linked Immunosorbent Assay (ELISA) kit: Catalog number ab24653, sourced from Abcam, Cambridge, UK. Epidermal Growth Factor (EGF) Receptor antibody: Catalog number 4267, sourced from Cell Signaling Technology (CST), Danvers, MA, USA. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) antibody: Catalog number 5174, sourced from Cell Signaling Technology (CST), Danvers, MA, USA. EGFR ELISA kit: Catalog number ab100505, sourced from Abcam, Cambridge, UK.

3. Methods

3.1 Identification of active components and targets in BZYQT

The key ingredients of BZYQT, such as Astragalus, Codonopsis, Atractylodes, Tangerine Peel, Angelica, Cimicifuga, Bupleurum, Licorice, Ginger and Jujube, were entered into the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP). Potential active components and their targets were screened based on oral bioavailability (OB $\geq$30%) and drug-likeness (DL $\geq$0.18).

3.2 Selection of CP/CPPS-related targets

Targets related to the pathogenesis of CP/CPPS were obtained from several databases, including GeneCards (https://www.genecards.org/), Online Mendelian Inheritance in Man (OMIM, https://www.omim.org/), Therapeutic Target Database (TTD, http://db.idrblab.net/ttd/), and Pharmacogenomics Knowledge Base (PharmGKB, https://www.pharmgkb.org/).

3.3 Construction of drug-disease target network and Venn analysis

R language was used to identify intersecting targets between CP/CPPS and the active components of BZYQT.

3.4 Construction of protein-protein interaction network and core protein screening

Intersecting targets between CP/CPPS and the active components of BZYQT were input into the STRING platform (https://string-db.org/). Cytoscape 3.9.0 was employed to conduct an in-depth analysis of the generated PPI network, aiming to pinpoint crucial proteins associated with the overlap between drug and disease targets.

3.5 Enrichment analysis

GO functional enrichment analysis and KEGG pathway enrichment analysis were conducted on essential proteins identified from the CP/CPPS-BZYQT target intersections using R language. The analysis of the results was performed with Cytoscape 3.9.0, which encompassed three GO categories: molecular function (MF), biological process (BP), and cellular component (CC).

3.6 Molecular docking analysis and visualization

Core target proteins and their related active chemical components were determined based on network node degree values. The three-dimensional (3D) structures of the proteins were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) (https://www.rcsb.org/), and the 3D structures of the compounds were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/). The 3D structures were adjusted using AutoDock tools by adding hydrogen atoms and protonation. Molecular docking interactions between the core proteins and chemical components were validated using AutoDock Vina software, with docking binding free energies used to assess and score docking affinity. Pymol was utilized to visualize the 3D docking models.

3.7 Cell culture

RWPE-1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin. Cells were incubated at 37 °C in a 5% Carbon Dioxide (CO_2) environment.

3.8 Cell Counting Kit-8 (CCK8) assay for determining drug concentration

RWPE-1 cells were seeded in 96-well plates at 8000 cells per well. The cells were treated with varying concentrations of kaempferol, and cell viability was observed to determine the appropriate drug concentration.

3.9 ELISA for MMP9 and EGFR levels

Cells were divided into four groups: control group, model group (LPS 10 μg/mL), low-dose kaempferol group (LPS + 20 μM kaempferol), and high-dose kaempferol group (LPS + 40 μM kaempferol). MMP9 and EGFR levels were measured according to the instructions provided with the ELISA kits.

3.10 Immunofluorescence detection of MMP9 levels

The cells were grouped following the same pattern as in the ELISA experiment. Following the blocking and permeabilization steps, the cells were left to incubate with the MMP9 antibody overnight. Subsequently, a staining process with Hoechst was conducted for 8 minutes to highlight the nuclei before visualization using a laser scanning confocal microscope.

3.11 Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RNA was extracted using the Trizol method, and reverse transcription was performed according to the instructions of the First-strand cDNA Synthesis Mix with gDNA Remover kit. The expression of IL-6 mRNA was detected using SYBR Green fluorescence dye in PCR. Primer sequences were as follows:

IL-6: F: CCTGAACCTTCCAAAGATGGC, R: TTCACCAGGCAAGTCTCCTCA;

GAPDH: F: AGTAGAGGCAGGGATGATG, R: AGTAGAGGCAGGGATGATG.

3.12 Western blot

IL-6, TNF-α, EGFR, MMP9, GAPDH protein expression was assessed using 12% pre-cast gels, followed by 45 minutes of electrophoresis at 180 V and membrane transfer at 350 mA for 30 minutes. The membrane was blocked with milk for 1 hour and incubated overnight at 4 °C with the primary antibody. After washing the membrane with Tris-Buffered Saline with Tween 20 (TBST) for 5 minutes, repeated 3 times, the membrane was incubated with the secondary antibody at room temperature for 1 hour. Detection was performed using Enhanced Chemiluminescence (ECL) reagent.

3.13 Statistical analysis

All data were obtained from three independent experiments. Statistical analysis was performed using SPSS 25, IBM, Armonk, NY, USA, with Analysis of Variance used to evaluate differences among multiple groups. A p-value < 0.05 was considered statistically significant.

4. Results

4.1 Identification of active components and targets in BZYQT

BZYQT is composed of 10 traditional Chinese medicinal herbs. Through screening in the TCMSP database, a total of 103 active compounds were identified, which are associated with 2064 target proteins.

4.2 Identification of common targets between disease and drug

A total of 1020 disease-related targets were retrieved from the GeneCards, OMIM, TTD and PharmGKB databases. Analysis using R language identified 73 common targets between the drug and the disease (Fig. 1).

4.3 Drug-disease interaction network analysis

Utilizing information extracted from the STRING database, a dynamic protein-protein interaction (PPI) network linking the drug and the disease was established, setting a threshold of 0.9 for the minimum interaction score (Fig. 2A). The network was further analyzed using Cytoscape 3.9.0, revealing effective compounds in the drug and their corresponding disease targets (Fig. 2B). Core targets were screened based on multiple scoring criteria (Fig. 2C), and the top five genes ranked by degree value were identified (Fig. 2D).

4.4 Enrichment analysis elucidates functional pathways and potential anti-CP/CPPS gene associations of BZYQT

The 73 intersecting targets were subjected to GO and KEGG enrichment analysis using R language. A total of 2301 GO terms were identified and categorized into three distinct groups: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). The top 10 terms in each category are shown in Fig. 3. Subsequently, KEGG pathway enrichment analysis identified 158 pathways, with the top 30 displayed in Fig. 4. The results indicate that BZYQT may exert its therapeutic effects on CP/CPPS by modulating functional processes such as inflammation, apoptosis, oxidative stress, and cell proliferation, as well as affecting signaling pathways like the ErbB signaling pathway, TNF signaling pathway, and Toll-like receptor signaling pathway, which are crucial in the pathogenesis of CP/CPPS.

4.5 Molecular docking results analysis

Using network pharmacology as a guide, we conducted molecular docking studies involving the active ingredients quercetin, beta-sitosterol, kaempferol and stigmasterol interacting with the target proteins Albumin (ALB), MMP9, Estrogen Receptor 1 (ESR1), IL-6 and EGFR. The docking visualizations and chemical bonds are shown in Fig. 5. In general, a binding energy below −5 kcal/mol is indicative of strong binding affinity between the ligand and the receptor. As shown in Fig. 6, kaempferol exhibited the highest binding energy with MMP9, at −10.9 kcal/mol.

4.6 Inhibitory effect of kaempferol on MMP9

Cells were exposed to different doses of kaempferol to investigate its impact on MMP9 (1 μM, 5 μM, 10 μM, 20 μM, 40 μM, 60 μM, 80 μM). Based on the results (Fig. 7A), concentrations of 20 μM and 40 μM were selected for further experiments. ELISA results showed that MMP9 levels

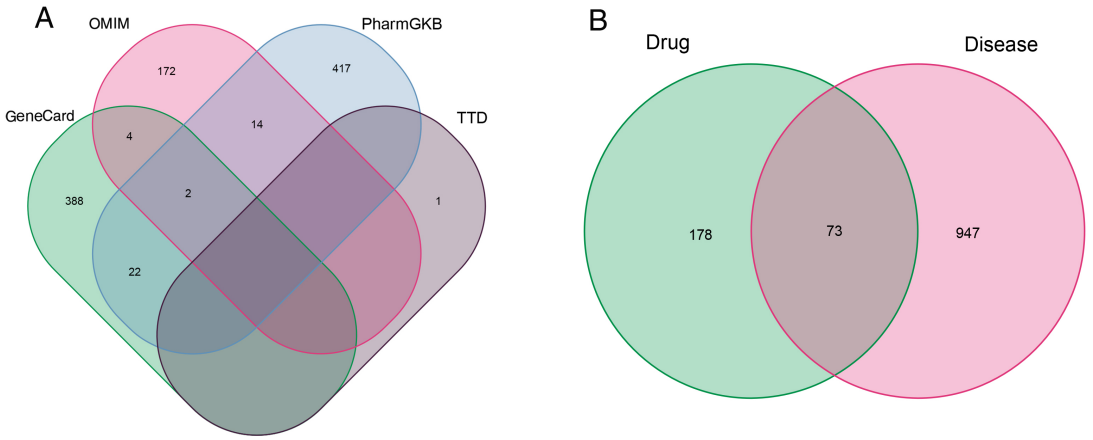

FIGURE 1. Identification of common targets between disease and drug. (A) Targets related to CP/CPPS disease; (B) Drug-disease intersection targets. OMIM: Online Mendelian Inheritance in Man; TTD: Therapeutic Target Database; PharmGKB: Pharmacogenomics Knowledge Base.

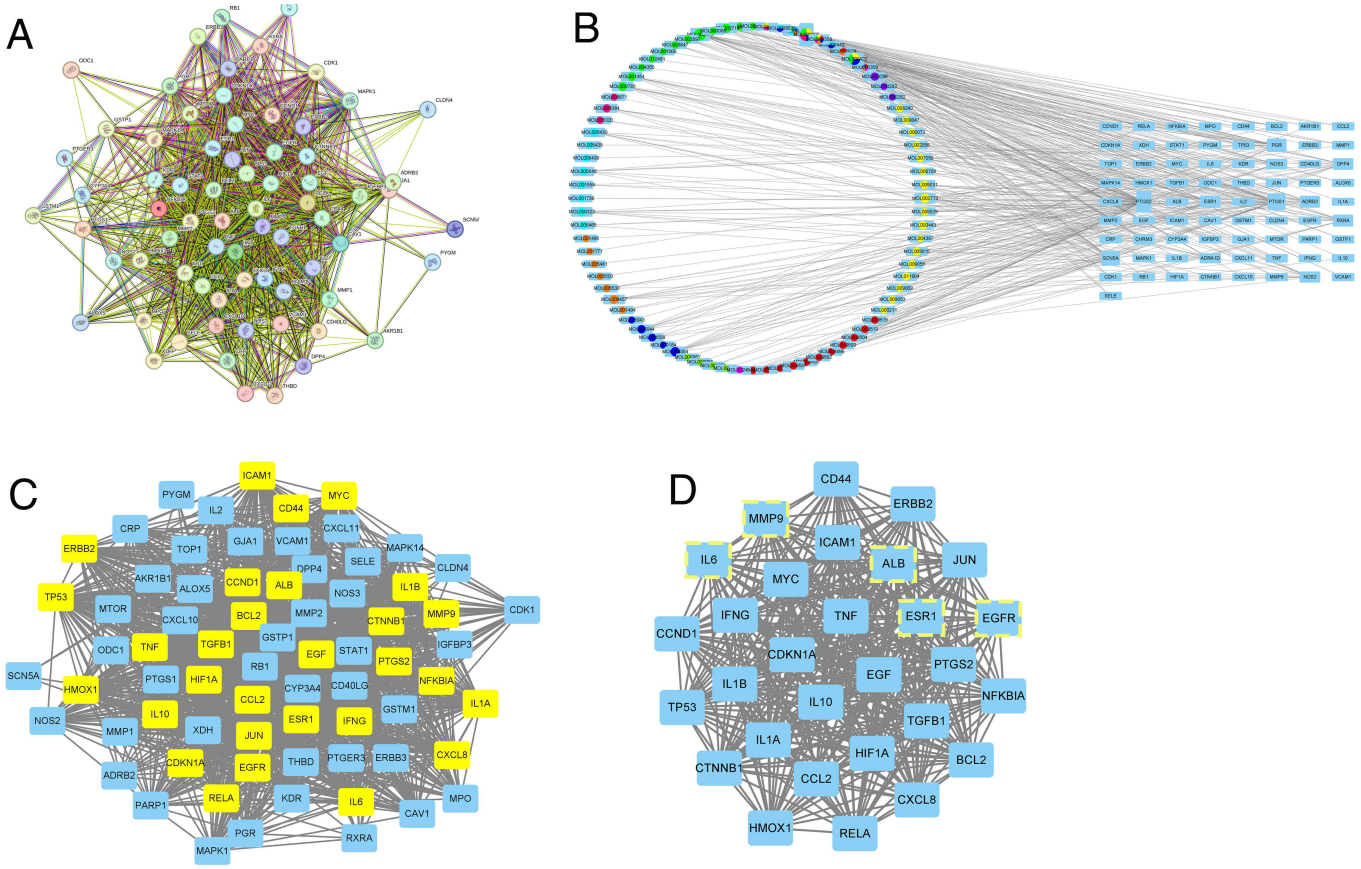

FIGURE 2. Drug-disease interaction network analysis. (A) Drug-Active Ingredient-Target Network Diagram; (B) Core Target PPI Network Diagram; (C) Target Screening; (D) Core Target Screening. ICAM1: Intercellular Adhesion Molecule 1; CD44: CD44 Molecule (Indian Blood Group); MYC: MYC Proto-Oncogene, BHLH Transcription Factor; ERBB2: Erb-B2 Receptor Tyrosine Kinase 2; TP53: Tumor Protein P53; CCND1: Cyclin D1; ALB: Albumin; IL1B: Interleukin 1 Beta; BCL2: BCL2, Apoptosis Regulator; CTNNB1: Catenin Beta 1; MMP9: Matrix Metallopeptidase 9; TNF: Tumor Necrosis Factor; HMOX1: Heme Oxygenase 1; HIF1A: Hypoxia Inducible Factor 1 Subunit Alpha; EGF: Epidermal Growth Factor; PTGS2: Prostaglandin-Endoperoxide Synthase 2 (also known as COX-2); NFKBIA: NFKB Inhibitor Alpha; IL1A: Interleukin 1 Alpha; CXCL8: C-X-C Motif Chemokine Ligand 8 (also known as IL-8); IFNG: Interferon Gamma; ESR1: Estrogen Receptor 1; JUN: Jun Proto-Oncogene; AP-1: Transcription Factor Subunit; CDKN1A: Cyclin Dependent Kinase Inhibitor 1A (also known as p21); RELA: RELA Proto-Oncogene NF-κB Subunit; IL-6: Interleukin 6; CCL2: C-C Motif Chemokine Ligand 2 (also known as MCP-1).

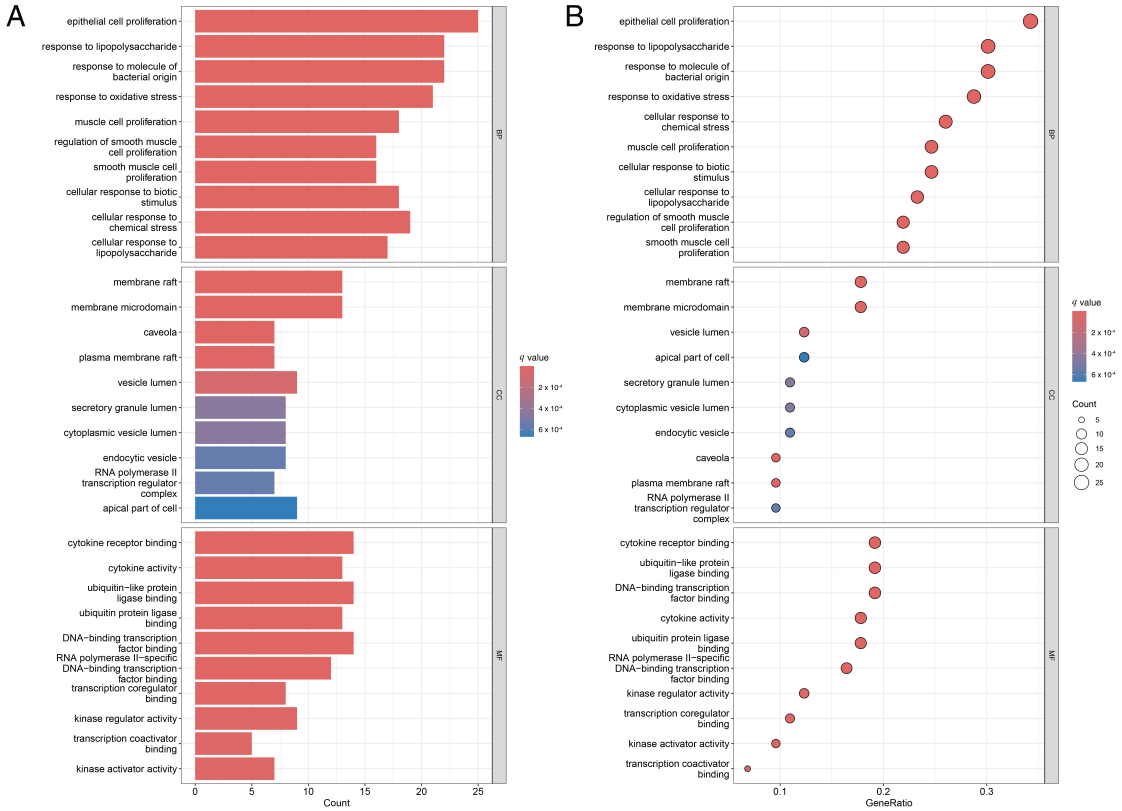

FIGURE 3. GO Function Enrichment Analysis. (A) GO Function Enrichment Analysis Bar Chart; (B) GO Function Enrichment Analysis Bubble Chart. BP: Biological Process; CC: Cellular Component; MF: Molecular Function.

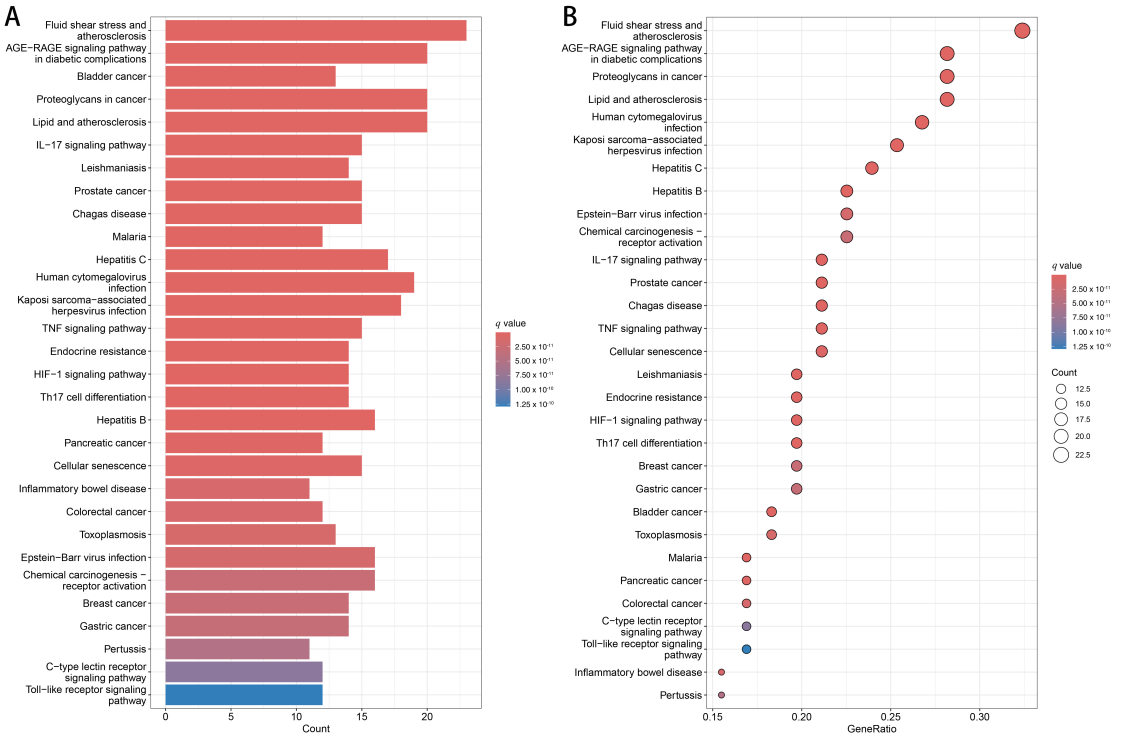

FIGURE 4. KEGG Pathway Enrichment Analysis. (A) KEGG Pathway Enrichment Analysis Bar Chart; (B) KEGG Pathway Enrichment Analysis Bubble Chart. AGE-RAGE signaling pathway: Advanced Glycation End Products-Receptor for Advanced Glycation End Products signaling pathway; IL-17 signaling pathway: Interleukin 17 signaling pathway; TNF signaling pathway: Tumor Necrosis Factor signaling pathway; HIF-1 signaling pathway: Hypoxia-Inducible Factor 1 signaling pathway; Th17 cell differentiation: T-helper 17 cell differentiation.

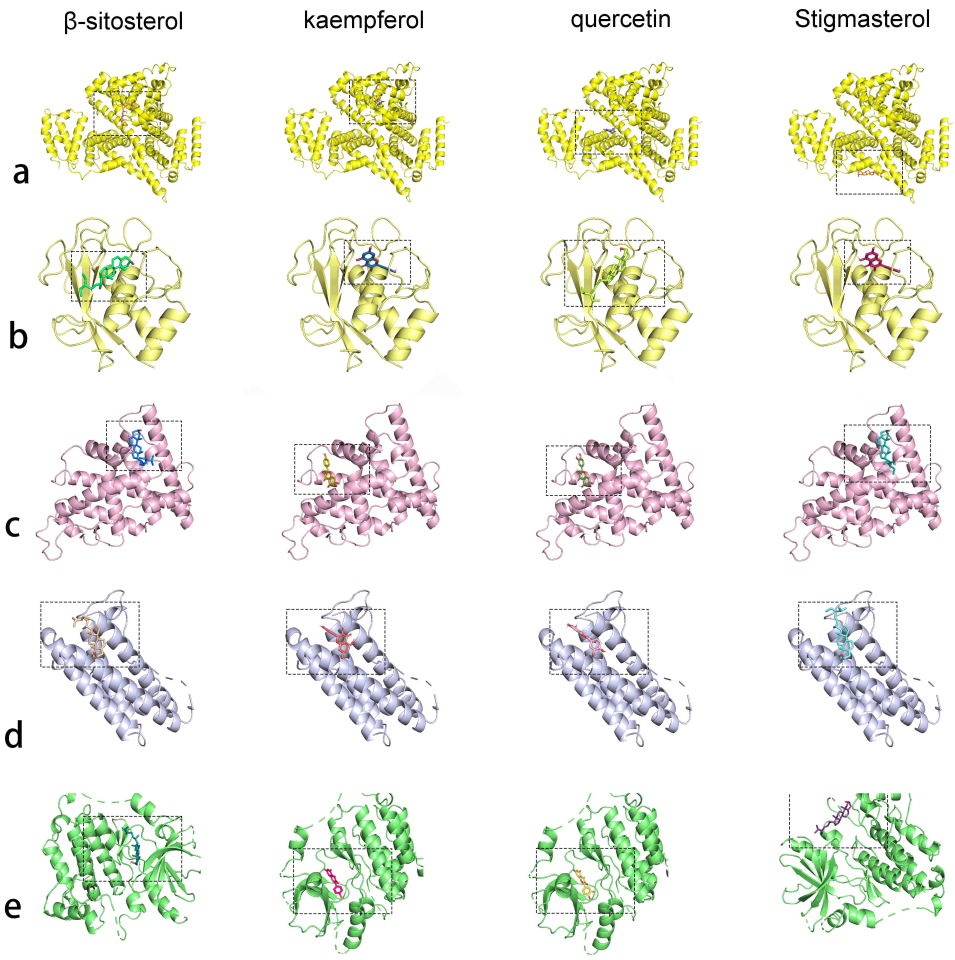

FIGURE 5. Docking Results of Target Proteins and Macromolecular Compounds. a: ALB; b: MMP9; c: ESR1; d: IL-6; e: EGFR. The black dotted box in Fig. 5 represents the binding site where the small molecule interacts with the protein.

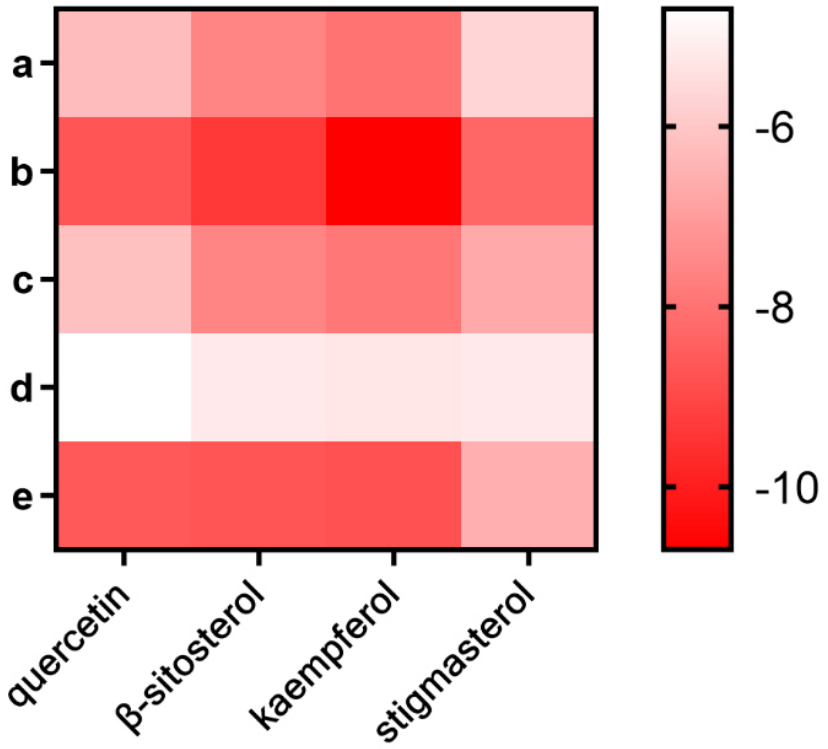

FIGURE 6. Heatmap of Molecular Docking Binding Energy Results. a: ALB; b: MMP9; c: ESR1; d: IL-6; e: EGFR.

increased in cells stimulated with LPS ($p < 0.05$), whereas kaempferol treatment significantly inhibited the increase in MMP9 in a dose-dependent manner ($p < 0.05$) (Fig. 7B). Immunofluorescence analysis further confirmed the changes in MMP9 levels (Fig. 7C). IL-6, a crucial cytokine linked to inflammation, plays a pivotal role in the body's defense mechanism by controlling immune and inflammatory reactions. As illustrated in Fig. 7D kaempferol decreased the expression levels of both IL-6 mRNA and protein during the inflammatory process.

4.7 Inhibitory effect of kaempferol on the ErbB signaling pathway

To verify that the inhibitory effect of kaempferol on MMP9 is mediated through the ErbB signaling pathway, we measured the levels of EGFR in the cell supernatant using ELISA. The findings suggested that the presence of kaempferol led to a notable decrease in the elevated EGFR levels induced by LPS ($p < 0.05$) (Fig. 7E). Moreover, the Western Blot (WB) experiment demonstrated that kaempferol can reduce the increase in protein expression of EGFR, MMP9, IL-6, and TNF-α induced by LPS in cells ($p < 0.05$) (Fig. 7F).

5. Discussion

CP/CPPS is a chronic disease that significantly affects patients' quality of life. BZYQT is a commonly used adjunctive therapy for CP/CPPS and has demonstrated favorable clinical efficacy. Research has demonstrated that the use of BZYQT can hinder the growth of prostate cells by regulating proteins associated with the cell cycle. This, in turn, delays the advancement of abnormal developments, ultimately safeguarding against additional harm and deterioration of prostate tissue [12]. This study investigates the mechanism of BZYQT in chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) and finds that it regulates inflammation and cell proliferation through multiple targets, highlighting its significant therapeutic potential. In the pathology of CP/CPPS, MMP9, EGFR, IL-6 and TNF-α are pivotal players, and the active ingredient kaempferol in BZYQT has the ability to concurrently regulate multiple essential molecules and signaling pathways.

MMP9, a member of the matrix metalloproteinase family, is extensively involved in the breakdown of the extracellular matrix (ECM) and the restructuring of tissues. Overactivation of MMP9 is recognized as a key factor in driving the destruction of inflamed tissues, facilitating the infiltration of inflammatory cells and the deterioration of ECM in the prostate tissue, consequently resulting in tissue impairment and functional irregularities [13]. This study found that BZYQT significantly inhibits MMP9 expression, which is consistent with other findings that MMP9 inhibition helps reduce damage in chronic inflammatory tissues [14]. Extensive research has been conducted on the association between EGFR and MMP9 in a range of disease models, focusing particularly on tumors and inflammation-related conditions. EGFR, as part of the epidermal growth factor receptor family, governs cell proliferation, migration and survival by initiating signaling pathways downstream [15]. Research suggests that EGFR activation induces MMP9 expression, thereby exacerbating tissue degradation and inflammatory responses [16]. Aberrant activation of the EGFR signaling pathway in the pathogenesis of CP/CPPS could exacerbate prostate tissue injury through the enhancement of MMP9 expression [17]. This study shows that BZYQT can downregulate both EGFR and MMP9 expression, suggesting that BZYQT may indirectly inhibit MMP9 activity by suppressing the EGFR signaling pathway, thereby reducing tissue damage and inflammation.

IL-6 and TNF-α are pivotal pro-inflammatory cytokines that are significantly involved in the pathogenesis of CP/CPPS. [18, 19]. IL-6 not only promotes inflammation but also enhances EGFR signaling pathway activity, driving both cell proliferation and inflammation [20]. Research has demonstrated that IL-6 is capable of triggering the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway, leading to the enhancement of the EGFR signaling pathway and intensifying inflammatory reactions [21]. TNF-α, a potent pro-inflammatory factor, also plays a critical role in the pathology of CP/CPPS. The activation of the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway by TNF-α leads to an increase in MMP9 expression, exacerbating tissue degradation and inflammation [22]. Furthermore, TNF-α has the potential to trigger the EGFR signaling pathway, thereby enhancing cell proliferation and causing additional tissue damage [23]. The findings of this research show that the administration of BZYQT leads to a notable decrease in the levels of IL-6 and TNF-α. This action is anticipated to mitigate inflammation and injury in the prostate tissue by concurrently repressing the EGFR signaling pathway. These results are consistent with existing studies indicating that the suppression of TNF-α can ameliorate inflammation within the prostate tissue.

This research uncovers the intimate correlation among MMP9, EGFR, IL-6 and TNF-α in the development of CP/CPPS. Using network pharmacology, it is demonstrated that BZYQT effectively modulates these crucial molecules via various targets and pathways, leading to a notable mitigation of prostate tissue damage and inflammation. The active compound kaempferol in BZYQT may play an important role in alleviating pathological changes in CP/CPPS by simultaneously downregulating the expression of MMP9, EGFR, IL-6 and TNF-α.

Although this study validates the mechanisms of BZYQT in a cellular model, future animal experiments and clinical studies are needed to further explore its long-term regulatory effects on the immune microenvironment. Furthermore, further exploration is needed to investigate the varying effectiveness of BZYQT in different pathological subtypes of CP/CPPS. To sum up, BZYQT, with its intricate network of regulatory mechanisms, presents fresh perspectives and proof for addressing CP/CPPS, laying a molecular groundwork for utilizing traditional Chinese herbal formulas in chronic inflammatory conditions.

6. Conclusions

The treatment of CP/CPPS with BZYQT is characterized by its multi-component nature, targeting multiple aspects and in-

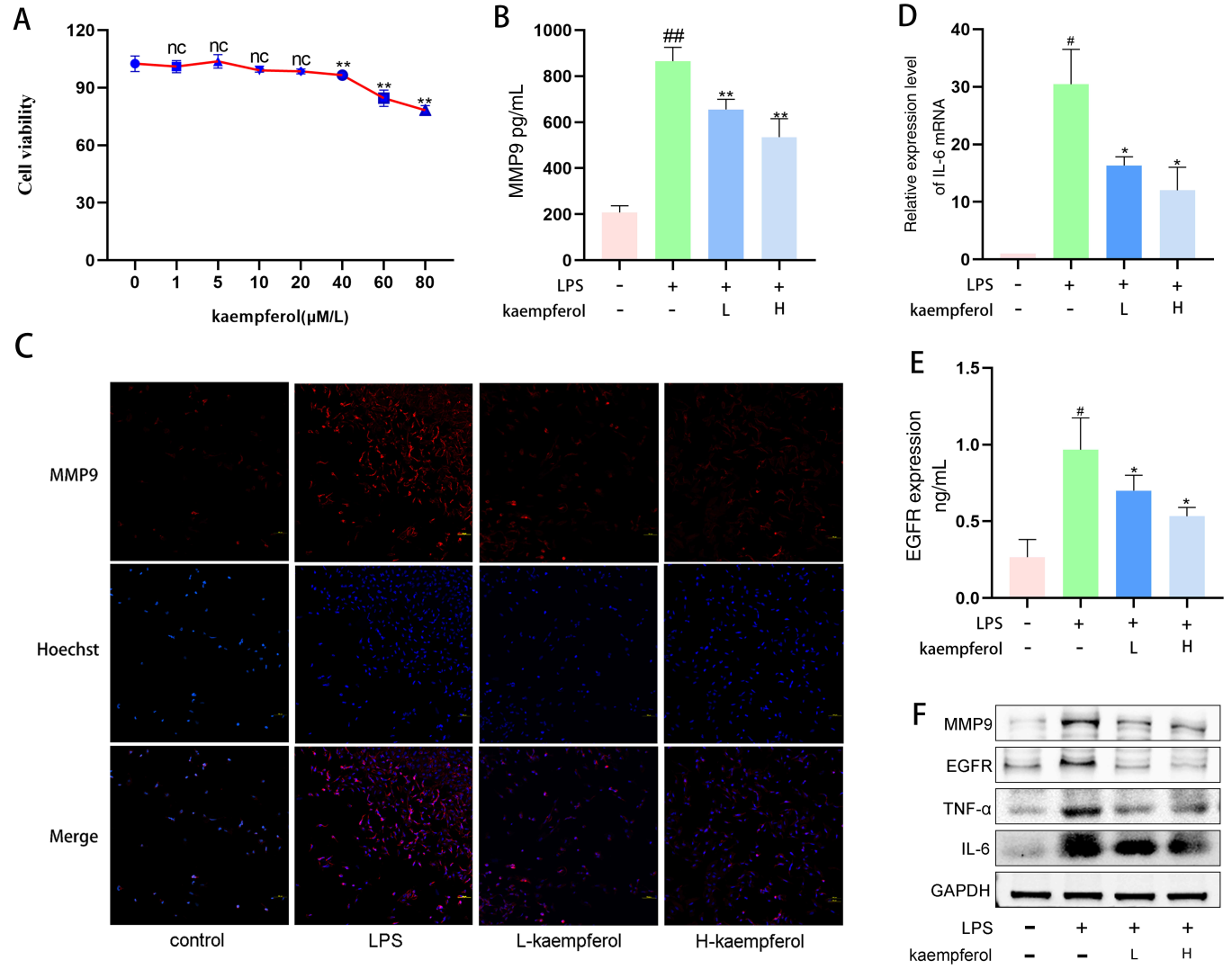

FIGURE 7. Inhibitory effect of kaempferol on MMP9 and the ErbB signaling pathway. (A) The effect of different concentrations of Carnosol on cell viability; (B) ELISA detection of MMP9 expression in various groups; (C) Immunofluorescence detection of the effect of LPS and Carnosol on MMP9 expression; (D) The effects of LPS and kaempferol on EGFR expression were detected by immunofluorescence; (E) RT-qPCR was used to detect the relative expression of IL-6 in each group; (F) The relative expression of MMP9, EGFR, TNF-α, IL-6 protein in each group was detected by WB. nc stands for no statistical difference; * indicates $p < 0.05$ compared to the model group; ** indicates $p < 0.05$ compared to the model group, # indicates $p < 0.05$ compared to the blank group. ## indicates $p < 0.05$ compared to the blank group. + represents addition; − represents no addition; L represents low concentration of the drug; H represents high concentration of the drug. MMP9: Matrix Metallopeptidase 9; IL-6: Interleukin 6; LPS: Lipopolysaccharide; EGFR: Epidermal Growth Factor Receptor; TNF-α: Tumor Necrosis Factor alpha; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase.

volving complex pathways. Through comprehensive analysis in network pharmacology studies, the mechanisms of action of BZYQT in treating CP/CPPS have been systematically elucidated. This not only enhances the comprehension of the pathophysiology of this intricate disease but also furnishes molecular support for the application of traditional Chinese medicine in CP/CPPS treatment. This lays the foundation for further research into the mechanisms of CP/CPPS and may provide crucial insights for developing new therapeutic approaches.

AVAILABILITY OF DATA AND MATERIALS

The data are contained within this article.

AUTHOR CONTRIBUTIONS

PFZ—designed the research study; YX and ZSZ—performed the research; HW and YH—analyzed the data; LYC and QYZ—wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscripts.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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