Balkan Med J 2024;41:286-97

Cannabidiol Alleviates Oral Mucositis by Inhibiting PI3K/Akt/NF-κB-Mediated Pyroptosis

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Background: Cannabidiol (CBD), extracted from *Cannabis sativa*, has anticancer, anti-inflammation, and analgesic effects. Nevertheless, its therapeutic effect and the mechanism by which it alleviates oral mucositis (OM) remain unclear.

Aims: To explore the impact of CBD on OM in mice and on human oral keratinocyte (HOK) cells.

Study Design: Expiremental study.

Methods: The Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform, GeneCard, DisGeNET, and Gene Expression Omnibus databases were used to conduct therapeutic target gene screening for drugs against OM. Cytoscape software was used to build networks linking components, targets, and diseases. The STRING database facilitated analysis of intertarget action relationships, and the target genes were analyzed for Kyoto Encyclopedia of Genes and Genomes pathway enrichment. Occurrence of serum inflammation-related factors, hematoxylin and eosin staining, and immunohistochemistry were used to assess OM injury. Cell proliferation, migration, pyroptosis, and apoptosis of HOK cells under different treatments were assessed. Molecular mechanisms were elucidated through western blot and quantitative real-time polymerase chain reaction analyses.

Results: A total of 49 overlapping genes were pinpointed as potential targets, with NF-κB1, PIK3R1, NF-κBIA, and AKT1 being recognized as hub genes among them. Additionally, the PI3K/Akt/NF-κB and interleukin-17 signaling pathways were identified as relevant. Our in vivo experiments showed that CBD significantly reduced the proportion of lesion area, mitigated oral mucosal tissue lesions, and downregulated the expression levels of genes and levels of proteins, including NLRP3, P65, AKT, and PI3K. In vitro experiments indicated that CBD enhanced HOK cell proliferation and migration and reduced apoptosis through inhibition of the PI3K/Akt/NF-κB signaling pathway and pyroptosis.

Conclusion: Our findings suggest a novel mechanism for controlling OM, in which CBD suppresses the PI3K/Akt/NF-κB signaling pathway and pyroptosis, thereby mitigating OM symptoms.

INTRODUCTION

Oral mucositis (OM) frequently occurs as a complication associated with cancer chemotherapy, causing pain and hindering eating in cancer patients, thereby reducing the quality of life.[1] Around 40% of patients receiving chemotherapy for solid tumors experience mucositis.[2] Patients with severe OM may require a reduction in chemoradiotherapy, potentially delaying the cancer treatment course and worsening the prognosis.[3] Mucosal inflammation has five interrelated stages: initiation, primary damage response, signal amplification, ulceration, and healing.[4,5] Although the pathogenesis of OM is unknown and the condition is challenging to cure, there are currently no effective preventive or therapeutic medications.[6]

The terms "pyro" and "ptosis," which denote inflammatory characteristics and cellular degeneration, combine to form the phrase "pyroptosis."[7] Pyroptosis causes cell swelling and the formation of projections that resemble bubbles, and it exhibits similarities to apoptosis associated with DNA damage and chromatin condensation. Caspase-3 is an essential requirement for apoptosis. The morphological features of cellular pyroptosis are different from those of apoptosis, which can lead to inflammation. Both external

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Received: February 22, 2024 **Accepted:** May 16, 2024 **Available Online Date:** 04.07.2024 • **DOI:** 10.4274/balkanmedj.galenos.2024.2024-2-66

Available at www.balkanmedicaljournal.org

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Cite this article as: Yang J, Lin N, Li S, Wang D, Liu Y, Zhou Y, Yuan H. Cannabidiol Alleviates Oral Mucositis by Inhibiting PI3K/Akt/NF-κB-Mediated Pyroptosis. Balkan Med J.; 2024; 41(4):286-97.

and intracellular stimuli, including bacteria, viruses, toxins, and chemotherapy drugs, can induce cellular pyroptosis. Chemotherapy causes damage and inflammation to the oral mucosa, resulting in symptoms, such as mouth ulcers, discomfort, and infection. The medicines used in chemotherapy destroy normal cells, including the mucosa of the mouth, in addition to killing cancer cells. Periodontitis is associated with pyroptosis.[8] Therefore, finding effective therapy pyroptosis is urgent.

Cannabis contains a range of cannabinoids, with the main components being delta (9)-tetrahydrocannabinol and cannabidiol (CBD). The former is the primary cause of psychoactivity in cannabis, whereas the latter, as the second most abundant cannabinoid, occurs in higher concentrations than the former in some cannabis strains.[9] More importantly, CBD is a non-psychoactive substance with low toxicity and high safety.[10] Recently, CBD has received substantial research attention due to its favorable pharmacological properties, including anti-inflammatory, antioxidant, and neuroprotective effects.[11] It has been used in the treatment of epilepsy, advanced cancer pain, cerebral ischemia, Parkinson's disease, and depression.[12] However, the role of CBD in the treatment of OM and its mechanisms are not yet understood.[13]

Although previous studies have shown that CBD can protect oral ulcers, the specific mechanism has not been elucidated.[14] In this study, bioinformatics methods were used to screen therapeutic target genes for activity against OM, and functional analyses were performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) to identify potential signaling pathways. The therapeutic effects of CBD were examined by constructing an OM mouse model, and a cell model was established to evaluate changes in cell proliferation, migration, and apoptosis. Additionally, the expression levels of genes and proteins associated with the signaling pathway were examined to elucidate the mechanism of CBD in treating OM. Our findings will provide valuable insights and an experimental basis for the clinical treatment of OM with CBD.

METHODS

Experimental animals

The Animal Research Institute of Mudanjiang Medical University provided eight-week-old C57BL/6 mice (20 ± 0.5 g) for the study, which was approved by the Mudanjiang Medical University Medical Science Ethics Committee (approval number: 20230318-11, date: 06.03.2023). The mice were randomly divided into five groups (n = 8): control group (equal volume of 0.9% saline solution), paclitaxel (PTX) group (50 mg/kg/d PTX), low concentration CBD group (7.5 mg/kg/d CBD and 50 mg/kg/d PTX), medium concentration CBD group (15 mg/kg/d CBD and 50 mg/kg/d PTX), and high concentration CBD group (30 mg/kg/d CBD and 50 mg/kg/d PTX). The mice were injected continuously for seven days, and the health conditions were observed daily. Visual assessments were performed to observe alterations in the oral mucosa of the mice, and the diameters of the ulcerated surfaces of the mouth were recorded. Additionally, measurements on changes in the thickness of the mucosa were taken. The dose of PTX used for injection was determined using the standard body surface area conversion methodology.[15] All mice were sacrificed on the 12th day, and relevant experimental indices were determined in accordance with the protocols.

Cell culture

Resuscitated frozen human oral keratinocyte (HOK) cell lines were routinely cultured in a humidified incubator containing 1% penicillin-streptomycin containing DMEM culture and 10% fetal bovine serum, in an environment of 5% CO_2 at 37 °C. Cells were digested and passaged when they reached the logarithmic growth phase (80% to 90% confluence). The cell concentration was readjusted to 4.5 x 10^5 cells/mL. Subsequently, the cells were inoculated into a large dish and cultured until they attached to the surface. After discarding the original medium, HOK cells were rinsed twice with PBS before addition of fresh medium, and treatment with PTX at 10 μmol/L, CBD at 1.5 μmol/L, 3 μmol/L, and 6 μmol/L, and LY294002 at 20 μM, respectively.

Bioinformatic analysis

Network pharmacology is a strategy for analyzing network topology using a systems biology approach. Its application in the study of traditional Chinese medicine and the integration of clinical data has recently contributed to moving from a "one target, one drug" approach to a "network target, multicomponent therapy" approach.[16] In this study, we used the Traditional Chinese Medicine Systems Pharmacology (TCMSP) (https://tcmsp-e.com/tcmspsearch.php) to screen all the chemical components and targets of medicinal marijuana. Subsequently, we searched for the corresponding genes of the targets in various databases. By using "Oral mucositis" as the keyword, we retrieved OM disease-related targets from GeneCards and Disgenet (corresponding URL: https://www.genecards.org/ and https://www.disgenet.org/). Several target genes were obtained from both databases, and duplicates were removed to obtain disease-specific target genes. Relevant target genes for CBD treatment in a mouse model of OM were extracted from the Gene Expression Omnibus (GEO) database (GSE152088).[17] Venn diagrams were generated by intersecting CBD target genes, CBD therapeutic model expression genes, and disease-specific target genes to identify superimposed gene. The genes that overlap between drugs and diseases were uploaded into the STRING database to construct protein-protein interaction (PPI) networks. To ensure reliability of the study, the minimum composite score for the study was set at greater than or equal to 0.9. The related data were analyzed using cytoscape, and the degree of network nodes was calculated. Subnetworks of the first fifteen nodes with the maximum degree of inclusion were extracted using cytoHubba, indicating their core position in the network. The DAVID Functional Annotation Chart tool (version 6.8) was used to perform KEGG enrichment analysis.[18,19]

Hematoxylin & eosin staining

Tongue tissue samples of mice were obtained and fixed in 4% formaldehyde. The tongue tissue was embedded in paraffin before it was sectioned into 4 μm thickness. The specific procedures included hematoxylin staining for 2 min, rinsing in running water for 2 min, treating with 1% hydrochloric acid alcohol for 2 s, rinsing

under running water for 10 s, ammonia solution rebluing for 15 s, running water rinsing for 5 min, 80% alcohol treatment for 20 s, staining with 1% eosin solution for 15 s, 95% alcohol treatment for 20 s, 95% alcohol treatment for 20 s twice, xylene treatment for 3 min twice. The observations were made under an ordinary light microscope to examine the pathological changes in each group.

ELISA

Blood samples of mice were centrifuged in a centrifuge at 3,000 rpm for ten min. ELISA kits were used appropriately to determine the content of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) in mouse serum after extracting the supernatant.

Immunofluorescence staining

Appropriate sections were selected, and routine immunofluorescence procedures were followed. The sections were incubated overnight with the NLRP3 primary antibody (1:100). The primary antibody was washed off, and a fluorescent secondary antibody (1:500) was applied, incubated for 90 min at room temperature, before it was rinsed with PBS. Nuclei were stained by adding an antifluorescence quenching sealing solution containing DAPI dropwise. Slices were sealed simultaneously with staining the nucleus and were finally observed under a fluorescence microscope (Axioscope 5, Zeiss).

Immunohistochemistry

After dewaxing and hydration of paraffin sections, we washed them with PBS for 5 min three times. The sections were incubated with 3% H_2O_2 for 10 min, before they were rinsed with PBS for 5 min. This procedure was repeated three times. We applied 0.04% sodium citrate for antigen retrieval, followed by sealing with BSA for 1 h, followed by PBS washing for 5 min. This procedure was repeated three times. We added primary antibody dropwise and incubated the sections for a full night at 4 °C. The sections were washed in PBS for 5 min three times. After applying the secondary antibody dropwise, the samples were incubated for 90 min at 37 °C. They were washed again PBS for 5 min, three times. A freshly prepared DAB solution was added dropwise to develop the color, followed by microscopic observation. The sections were restained with hematoxylin, before they were rinsed with aseptic water to return to blue, and then dehydrated with gradient alcohol. Transparency and neutral gum sealing were the next steps. To serve as a negative control, the primary antibody was replaced with PBS. We captued five images of the area of interest. We used ImageJ 1.46 software to quantify the area of brown staining in each image.

Cell migration analysis

HOK cells were inoculated into small dishes at a concentration of 200,000 cells per 3 ml of medium overnight. Scratches were created using a 200 µl tip. The cells were gently washed with fresh medium to remove scattered cell fragments. The corresponding reagents were added according to the grouping. Lesion area size was monitored using phase-contrast microscopy.

To evolute the capacity of cells to migrate, a transwell assay was performed using a transwell chamber with an 8 µm pore size (Biosharp, Hefei). In summary, 100 µl of serum-free media was used to seed 1 x 10^5 cells into the transwell insert's upper chamber.

The upper chamber was cultured with serum-free medium, and the lower chamber was cultured with 20% FBS. After 24 h of incubation, cell migration on the transwell membrane was observed. Formaldehyde was used to fix the cells. The fixed cells were stained with 0.1% gentian violet for 20 min at room temperature, rinsed with PBS, and photographed. The cells that had migrated were counted in five randomly selected fields of view using an inverted microscope.

Cell proliferation analysis

Cell proliferation was observed using a 5-ethynyl-2'-deoxyuridine (EdU) kit (Beyotime, Shanghai). After successful modeling, PTX (10 µM), LY294002 (20 µM), and CBD (1.5, 3, and 6 µM) were added over 24 h, Subsequently, cells were treated with EdU (10 µM, 37 °C, 2 h), before they were washed with PBS. The cells were fixed in 4% paraformaldehyde for ten min. The cells were stained with 100 µl of Apollo staining solution for 30 min, followed by staining with Hoechst 33342 at room temperature for 10 min. We obtained images using a fluorescence microscope (Axioscope 5, Zeiss) and analyzed the images using ImageJ 1.46 software.

Flow cytometry detection

To detect apoptosis, a fluorescein-labeled annexin-FITC was added to the cell suspension of the aforementioned groups. The mixture was incubated at room temperature for 30 min in the dark. Following this step, an additional 30 min were allowed to pass after adding propidium iodide and allowing it to bind at room temperature. Subsequently, 2 ml of PBS containing 0.09% sodium azide were included, and the mixture was shaken and agitated well. The cells were then centrifuged and resuspended in PBS. Apoptosis was detected using flow cytometry.

Western blot analysis

Total protein extracted from mouse tongue tissue and HOK cells underwent western blot procedures. Primary antibodies, including PI3K, P65, Cleaved-Caspase-1, GSDMD-N, AKT, NLRP3, Pro-caspase-1, IL-17A, Bax, and Bcl-2 (ABclonal Technology, Wuhan, China), as well as β-actin (Santa Cruz Biotechnology), were incubated overnight at 4 °C at a dilution of 1:1,000. The secondary antibodies, including antirabbit/antimouse immunoglobulin G antibodies (from ABclonal Technology, Wuhan, China), were diluted to 1:10,000 and incubated for 1 h at room temperature. Specific protein bands were visualized using an enhanced chemiluminescent kit from Biosharp in Hefei, and detection was performed using a multiplex fluorescent imaging system from ProteinSimple in CA.

Quantitative real-time polymerase chain reaction analysis

All RNAs were extracted from tissues and HOK cells using the RNeasy Mini Kit (Qiagen) to determine the mRNA expression of TNF-α, IL-6, PI3K, AKT, P65, GSDMD-N, NLRP3, and IL-17A. PCR was conducted using 2 ul of cDNA, equivalent to 0.4 µg of total cDNA, and appropriate primers (Table 1). cDNA was synthesized from 1 µg

TABLE 1. Nucleotide Sequences of the Primers Used for RT-PCR

Genes	Forward (5'-3')	Reverse (5'-3')
GAPDH	GAAGGAATGGGTCGGAGTC	GAAGATTGGGATGGGATTTC
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
IL-6	CCTGAACCTTCCAAAGATGGC	TTCACCAGGCAAGTCTCCTCA
PI3K	TGCCCCTCCTGATGTTGC	TCGGCGAGATAGCGTTTG
AKT	GCTACAAGGAACGGCCTCAG	GTTTCCACATGGAAGGTGCG
NLRP3	GTAGGTGTGGAAGCAGGACT	CTTGCTGACTGAGGACCTGA
P65	CCCACGAGCTTGTAGGAAAGG	GGATTCCCAGGTTCTGGAAAC
GSDMD-N	CCATTGGGAGCATGGCCTC	GCGGTCTCCAGAATCGTGAA
IL-17A	TCCCACGAAATCCAGGATGC	GGATGTTCAGGTTGACCATCAC

RT-PCR, Real-Time Polymerase Chain Reaction.

of RNA. The mRNA levels of target genes were quantified using the Qiagen miScript SYBR Green PCR Kit on the CFX96 Touch Real-Time Polymerase Chain Reaction (RT-PCR) System (Bio-Rad, USA). The reaction conditions were as follows: predenaturation at 94 °C for 4 min, followed by denaturation at 94 °C for 20 s, annealing for 30 s, and extension at 72 °C for 30 s. The reaction was cycled 35 times, and a final extension was performed at 72 °C for 10 min. Relative mRNA levels were determined using the ΔΔCT method, with GAPDH as a reference and internal standard for quantification.

Statistical analysis

All experiments were replicated at least three. All quantitative data were expressed as mean value ± standard error of mean. ANOVA was used to test for significant difference among treatment group means. When ANOVA detected significant differences the Bonferroni multiple comparison test was performed to locate difference. All statistical analyses were performed in GraphPad Prism (version 9.0). $P < 0.05$ was considered statistically significant.

RESULTS

Cannabidiol reduces oral mucosal damage in oral mucositis animal model

After administering the treatments according to the experimental procedure, mice in the PTX group developed ulcers on the upper and lower lips, buccal mucosa, floor of the mouth, and particularly the tongue, but those in the vehicle group did not (Figure 1b, c). Hematoxylin and eosin staining revealed that the oral mucosal surface of mice in the PTX group was covered with a gray-yellow pseudomembrane, displaying visible ulcers and areas of detachment. Additionally, the epithelial thickness of the mucosal surface was significantly reduced. Relative to the PTX group, CBD at varying concentrations significantly reduced the aforementioned changes in a dose-dependent manner (Figure 1d, e).

Bioinformatics results identify key drug target genes

A total of 133 chemical components and targets of medicinal marijuana were screened using TCMSP. Using bioinformatics methods with GeneCard and DisGeNET, a comprehensive analysis identified a total of 2,033 genes associated with the OM disease. Additionally, 2,653 genes expressed in the CBD treatment model for OM were obtained from GEO. The intersection of these three datasets was determined using Wayne's method, resulting in identification of 49 intersected genes (Figure 2a). KEGG pathway enrichment analyses were conducted, revealing significant enrichment in the PI3K/AKT, NF-κB, and IL-17 signaling pathways (Figure 2b). The STRING online database was used to construct PPI networks, which were then imported into cytoscape for node degree calculations. Hub target genes, namely, NF-κB1, PIK3R1, NF-κBIA, and AKT1, were detected based on their rankings and scores surpassing the average score (Figure 2c).

Cannabidiol restrains inflammation and pyroptosis in the oral mucositis mice

TNF-α and IL-6 are cytokines that play a role in immune processes and inflammatory responses. ELISA showed that the mice in the PTX group had significantly higher concentration of serum than those in the vehicle group. Treatment with varying concentrations of CBD significantly decreased the expression of TNF-α and IL-6 (Figure 3a, b). These findings indicate that CBD mitigated the inflammation induced by the PTX treatment. Furthermore, the quantitative real-time polymerase chain reaction (qRT-PCR) results were consistent with those of ELISA in showing the same degree of expression of mRNA of TNF-α and IL-6 in tongue tissues (Figure 3c, d). Immunofluorescence staining was used to assess NLRP3 and GSDMD-N protein expression in tongue sections. These findings reveal a notable increase in NLRP3 and GSDMD-N protein expression in the PTX groups in comparison with the vehicle groups following PTX modeling injury. Moreover, NLRP3 and GSDMD-N protein expression showed significant dose-dependent down-regulation in the various concentrations of CBD groups relative to the PTX groups. (Figure 3e, f, h). The immunohistochemical results indicated that the levels of proteins PI3K, AKT, and P65 in the mouse tongue tissues were higher in the PTX groups than in the vehicle groups. But the protein expression was significantly downregulated in the various concentrations of CBD group (Figure 3g, i-k).

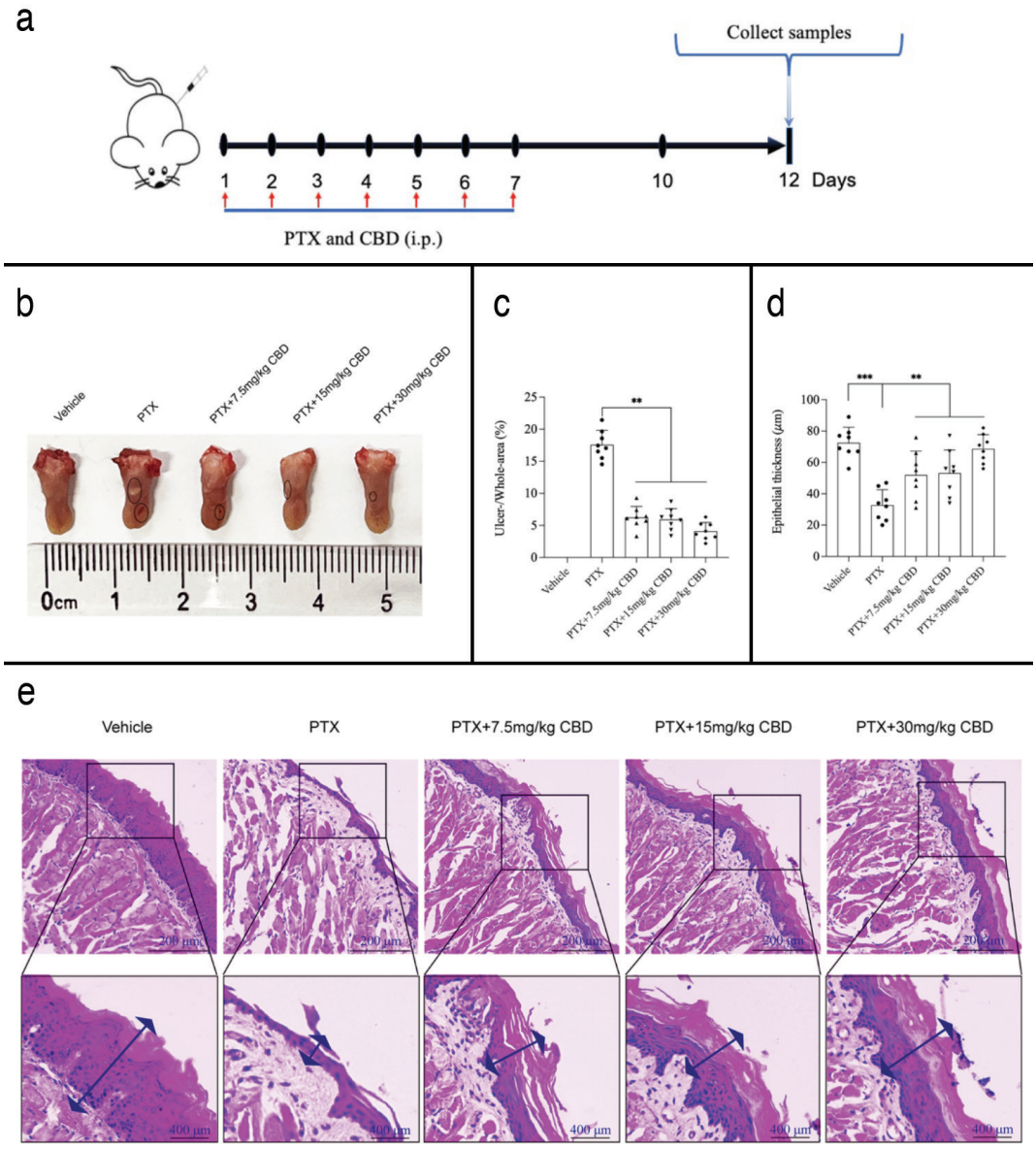

FIG. 1. Cannabidiol reduces oral mucosal damage in OM mice. The C57BL/6 mouse was selected as the research object of the OM model, and the related indexes were determined at 12 days. (a) Scheme of the in vivo experiment, (b) ulcers on the tongue of mice, (c) changes in ulcer size, (d) changes in epithelial thickness of the tongue, (e) H&E staining of a tongue tissue section. $^{**}p < 0.01$ vs. PTX group (n = 8); $^{***}p < 0.001$ vs. vehicle group.

OM, oral mucositis; H&E, hematoxylin and eosin; PTX, paclitaxel.

Cannabidiol enhances human oral keratinocyte cell migration and proliferation while inhibiting apoptosis

To study the regulatory effect of CBD in the progression of OM, we performed cell proliferation and migration assays. Scratch assay indicated that, the cells' lateral migration ability in the PTX group was lower than in the control group, whereas the lateral migration ability of the cell in the various concentrations of CBD group was significantly increased (Figure 4a). EdU staining showed that the proliferation ability of cells in PTX group was lower than in the control group, whereas the proliferation ability of cells in various concentrations of CBD treatment was markedly increased in a dose related way (Figure 4b). Flow cytometry was used to assess the apoptosis levels, revealing a significant increase in apoptosis within the PTX group relative to the control group. Conversely, apoptosis level notably declined after different concentrations of CBD processing (Figure 4c). Following the aforementioned experiments, it was observed that the most optimal effects were achieved with a dose of 3 μM of CBD treatment. Consequently, this particular dose was chosen for subsequent experiments aimed at verifying the underlying mechanisms.

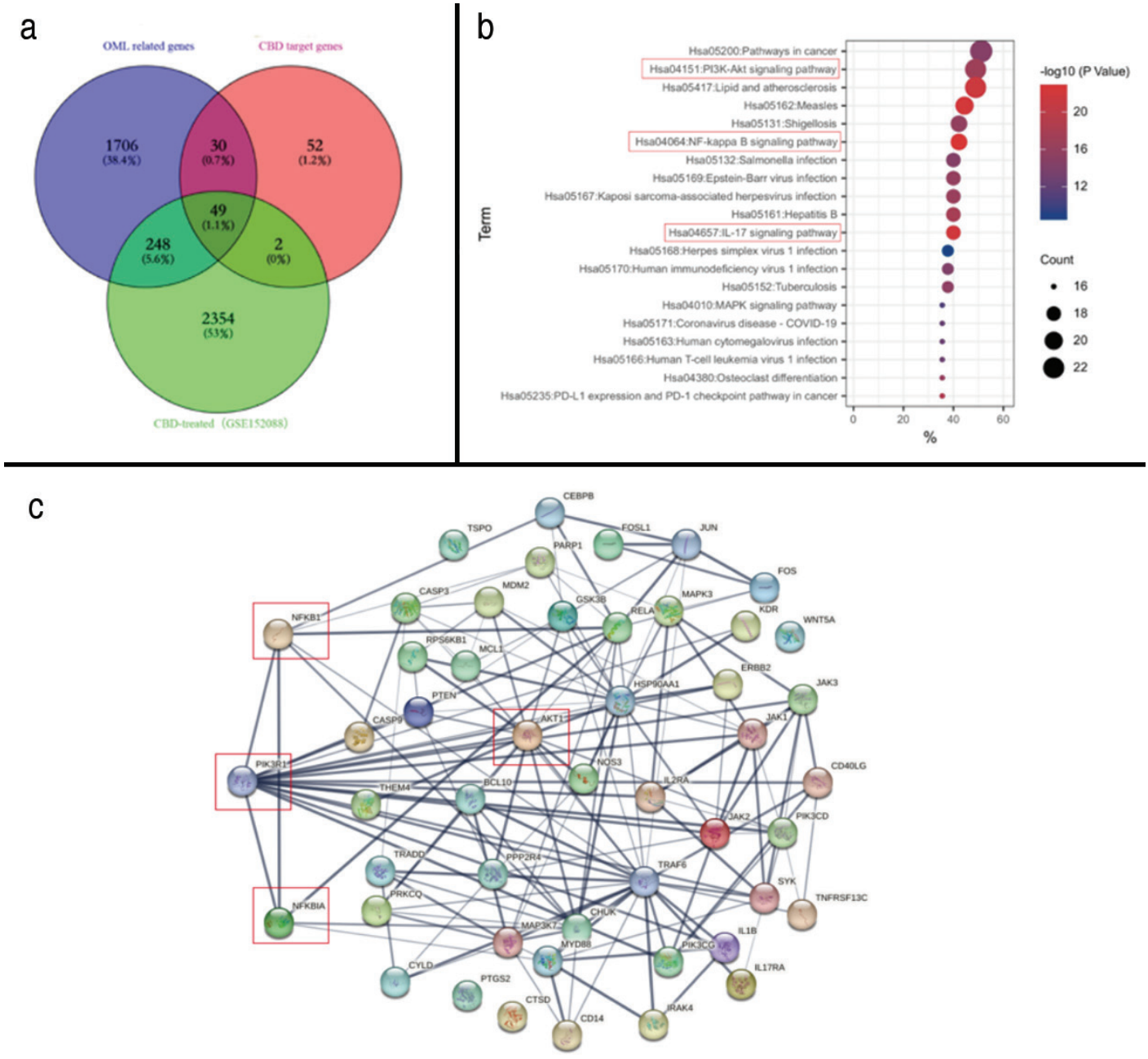

FIG. 2. Network pharmacology procedures. (a) Identifying the crossover target genes between CBD and OM through the use of a Venn diagram, (b) KEGG enrichment analysis of the crossover target genes, (c) constructing the PPI network and screening for hub targets.

CBD, cannabidiol; OM, oral mucositis; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction.

Cannabidiol inhibits pyroptosis by suppressing the PI3K/AKT/NF-κB pathway

Bioinformatic analysis indicated that the PI3K/AKT/NF-κB signaling pathways were crucial players in the development of OM. Consequently, western blot analysis was used to evaluate the expression of PI3K, AKT, and P65 proteins. The expression levels of these proteins were higher in the PTX group than in control group. Notably, CBD treatment exhibited a significant downregulation effect on the expression of each protein (Figure 5a). We measured the expression levels of the proteins GSDMD-N, NLRP3, Cleaved-Caspase-1, and IL-17A by WB analysis. The above expression levels of the four proteins were visibly higher in the PTX group than in the control group, and the administration of CBD treatment markedly reversed the above trends (Figure 5b). Apoptosis-related protein detection reveals that the expression level of Bax was significantly

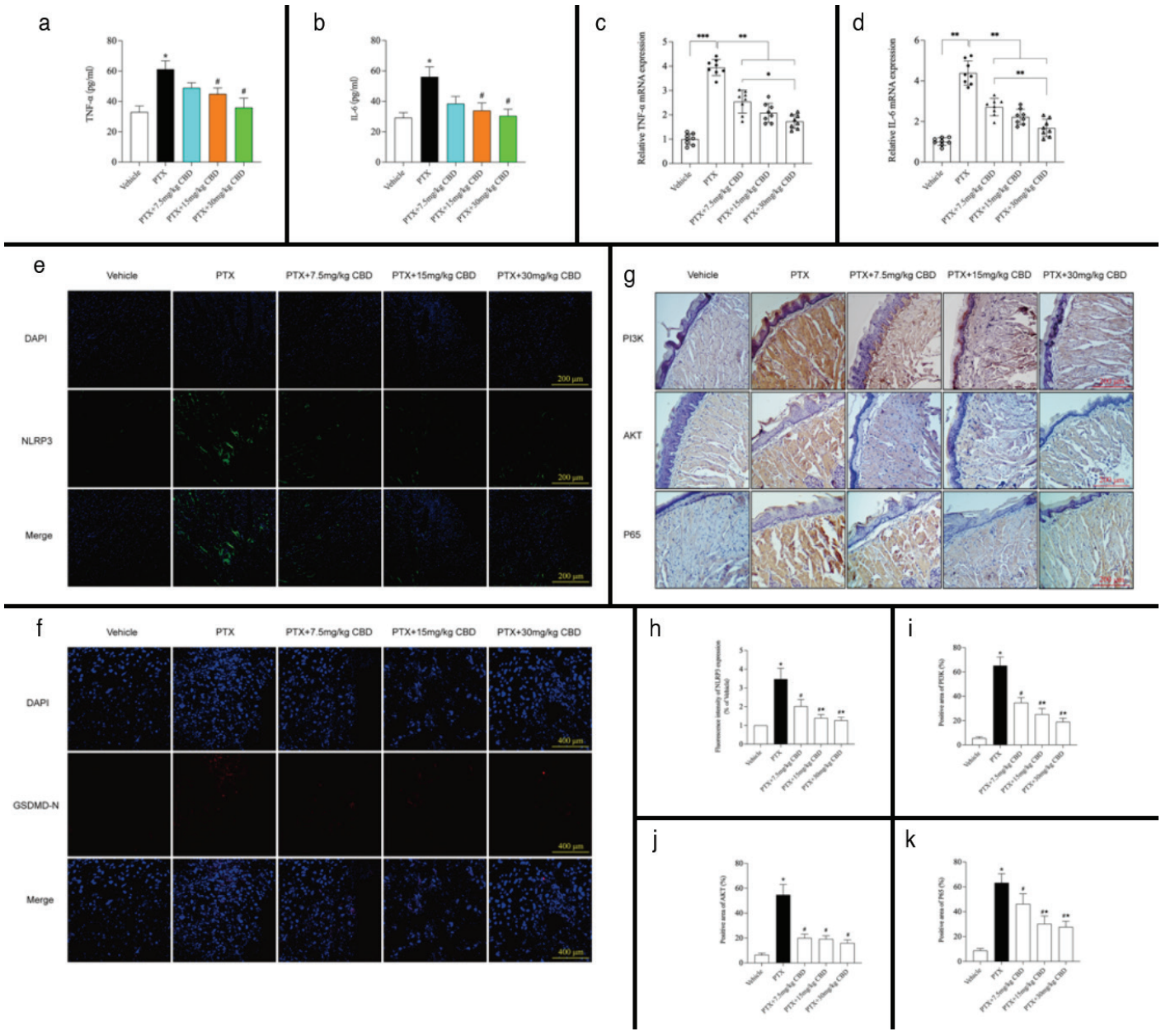

FIG. 3. Cannabidiol suppresses inflammation and pyroptosis. The network pharmacology analysis indicates that OM is involved in the PI3K/AKT and NF-κB pathway. (a) expression level of TNF-α, (b) IL-6 expression level, (c) mRNA expression of TNF-α, (d) mRNA expression of IL-6, (e) detection of NLRP3 expression by immunofluorescence, (f) detection of GSDMD-N expression by immunofluorescence, (g) evaluation of the expression of PI3K, AKT and P65 by immunohistochemistry analysis, (h) fluorescence intensity of NLRP3 expression, (i) positive area of PI3K, (j) positive area of AKT, (k) positive area of P65. $^{*}p < 0.05$ vs. vehicle group; $^{\#}p < 0.05$ vs. PTX group; $^{\star}p < 0.05$ vs. PTX + 7.5 mg/kg CBD group; $^{**}p < 0.01$, $^{***}p < 0.001$.

OM, oral mucositis; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; PTX, paclitaxel; CBD, cannabidiol.

higher in the PTX group than in the control group, whereas it was significantly downregulated in the CBD-treated groups. Conversely, Bcl-2 exhibited opposite expression trends (Figure 5c).

After establishing the beneficial effects of CBD on the typical pathological features of OM, we further explored to understand the underlying pathway mechanisms. Preliminary experiments highlighted the PI3K/Akt/NF-κB pathway as a key focus, corroborating findings of network pharmacology studies and KEGG pathway enrichment analyses. Therefore, a specific PI3K/Akt pathway inhibitor (LY294002) was added to the subsequent cell experiments to explore the underlying mechanism. The qRT-PCR analysis showed that PI3K, GSDMD-N, AKT, NLRP3, P65, and IL-17A mRNA expression levels were significantly lower in the PTX + LY294002 group than in the PTX group. A consistent trend and no

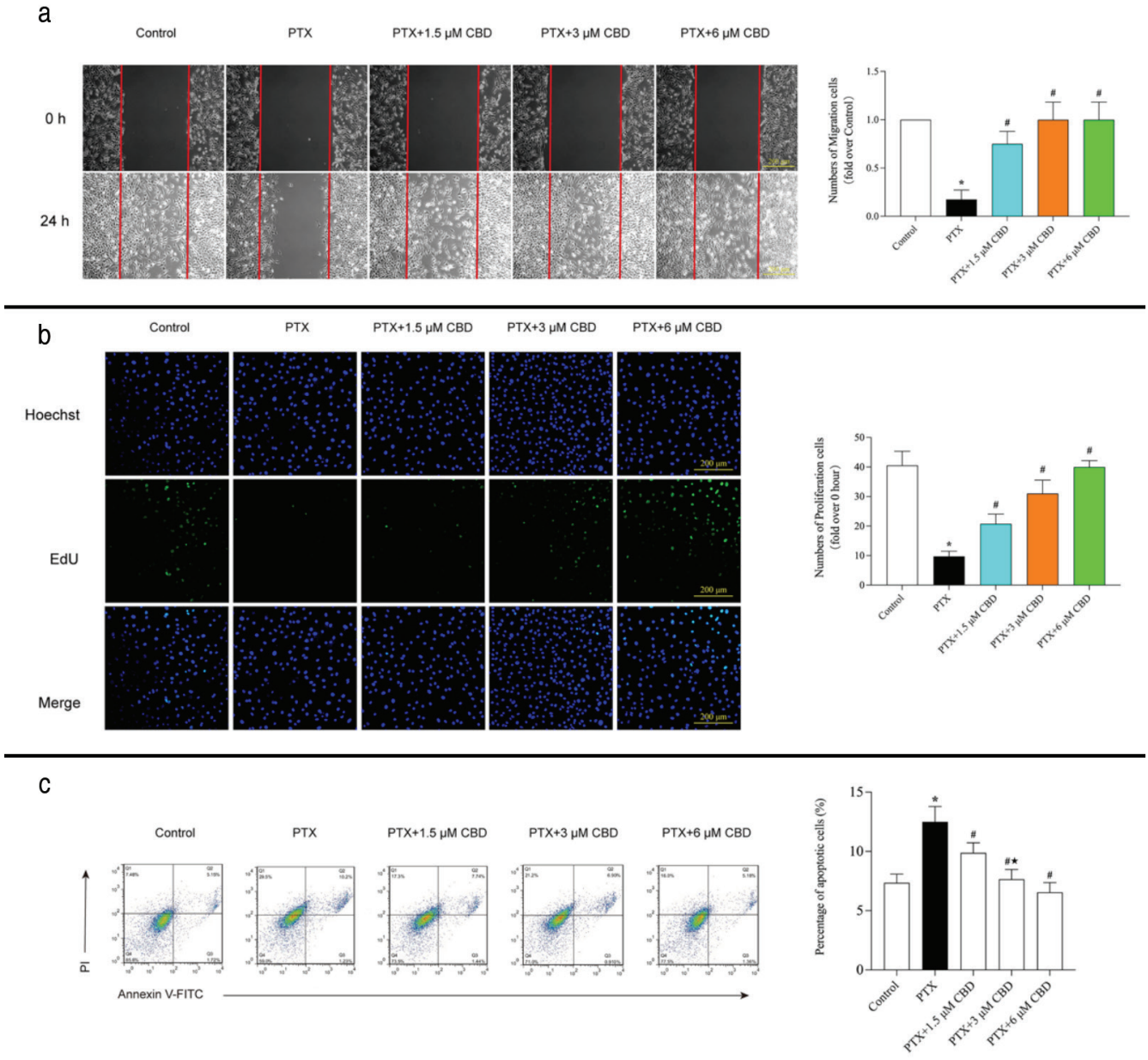

FIG. 4. Effects of cannabidiol on proliferation, migration, and apoptosis of the HOK cells. (a) HOK cell migration was assessed by scratch wound repair, (b) detection of the proliferation of HOK by EdU assay, (c) detection of apoptosis levels of HOK cells, $^{*}p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. PTX group; $^{*}p < 0.05$ vs. PTX + 1.5 μM CBD group.

HOK, human oral keratinocyte; EdU, 5-ethynyl-2'-deoxyuridine; PTX, paclitaxel; CBD, cannabidiol.

obvious variation from each other in the two groups, which was also observed in the PTX + 3 μM CBD treatment group (Figure 6a).

Cell proliferation was assessed using EdU staining. The PTX + LY294002 group and PTX + 3 μM CBD group exhibited higher cell proliferation ability than in the PTX group (Figure 6b, e). Scratch healing experiments revealed a significantly higher lateral cell migration rate in the PTX + 3 μM CBD group and PTX + LY294002 group than in the PTX group (Figure 6c, f), with no marked change between the two groups. Additionally, the transwell assay showed that PTX reduced the amount of migrating cell significantly, whereas CBD and LY294002 increased the longitudinal migration of cells (Figure 6d, g). These findings suggest that CBD may mitigate pyroptosis through suppressing the PI3K/AKT/NF-κB signaling pathway, thereby ameliorating OM (Figure 6h).

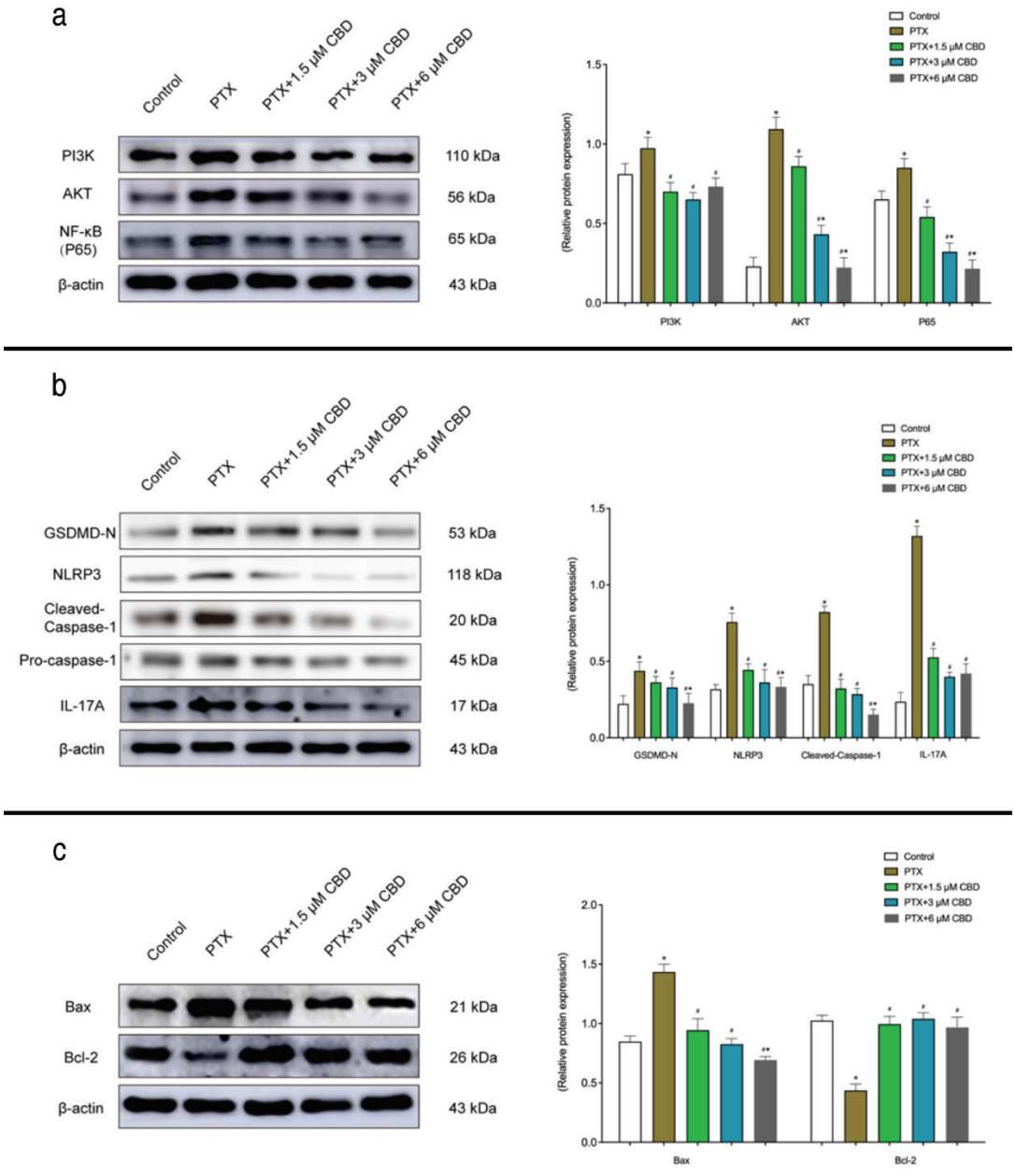

FIG. 5. Cannabidiol impedes the activation of the PI3K/AKT/NF-κB pathway and pyroptosis. The gray scale values were determined and the relative expression of the proteins was calculated. (a) The protein expression levels of PI3K, AKT, and P65 associated with the PI3K/AKT/NF-κB pathway, (b) the protein expression levels of GSDMD-N, NLRP3, Cleaved-Caspase-1, and IL-17A associated with pyroptosis, (c) the protein expression levels of Bax and Bcl-2 associated with apoptosis. *$P < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. PTX group; *$p < 0.05$ vs. PTX + 1.5 μM CBD group (n ≥ 3).

IL-17A, interleukin-17A; PTX, paclitaxel; CBD, cannabidiol.

DISCUSSION

CBD is widely available, but lacks consistent quality and dosage accuracy. It exhibits a range of therapeutic benefits, such as pain relief, neuroprotection, and anticancer properties. Additionally, ongoing research aims to explore its potential in the treatment or prevention of COVID-19 and its complications.[20] Although there are claims that CBD has various uses, there is limited evidence supporting its efficacy, except for its use in refractory seizures. There are promising benefits of CBD in controlling anxiety and schizophrenia, but there are potential adverse effects and drug interactions. Further research is needed, especially on the impact of CBD on liver function and its potential for inducing suicidal

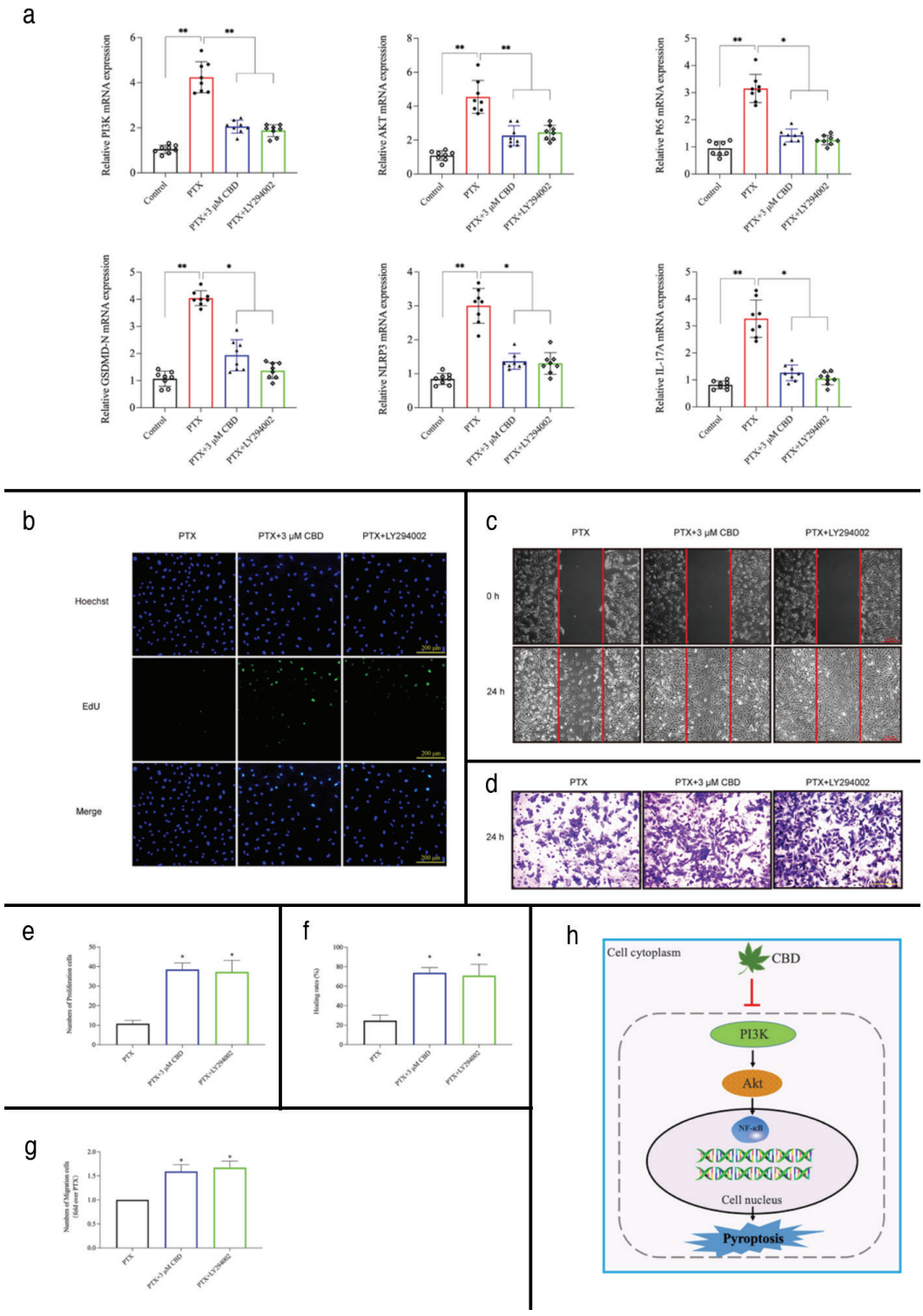

FIG. 6. Cannabidiol (CBD) treatment results similar to pathway inhibitor treatment. The 3 μM CBD treatment dose was the ideal choice for mechanism verification experiments, yielding the optimal effects. (a) mRNA expression levels of PI3K, AKT, P65, GSDMD-N, NLRP3, and IL-17A, (b) detection of proliferation of HOK cells by EdU assay, (c) detection of HOK migration using scratch wound repair, (d) detection of HOK cell migration using transwell assay, (e) statistical analysis of data on proliferation of HOK cells, (f) statistical analysis of data on HOK cell migration rate, (g) statistical analysis of data on HOK cell migration, (h) schematic diagram of the mechanism of CBD alleviating OM. *$P < 0.05$ vs. PTX group; **$p < 0.01$.

CBD, cannabidiol; IL-17A, interleukin-17A; HOK, human oral keratinocyte; EdU, 5-ethynyl-2'-deoxyuridine; OM, oral mucositis; PTX, paclitaxel.

ideation.[21] PTX, a microtubule stabilizer, has shown considerable efficacy in treating several cancers, especially breast and ovarian cancer.[22] However, its utility is constrained by issues such as drug resistance and side effects. Recent investigations have focused on synergistic approaches, such as combining PTX with other drugs or treatment modalities, to augment its effectiveness, particularly through targeted drug combinations.[23,24]

Pyroptosis is a vital mode of programmed cell death in vertebrates, playing a crucial role in defending against infections. It initiates a pro-inflammatory response, which attracts innate immune cells to the site of injury or infection. This process is indispensable for maintaining a robust immune system and overall well-being. Understanding the mechanisms of pyroptosis is essential for developing novel strategies to control diverse diseases and conditions. Inflammatory cysteoaspartic enzymes, which are key effector substrates, play a crucial role in pyroptosis.[25] Gasdermin D (GSDMD) plays a crucial role as an executive factor that triggers pyroptosis,[26,27] and its cleavage produces GSDMD-N, which forms transmembrane pores that ultimately lead to intense inflammation and cell death.[28] NLRP3 detects danger signals and subsequently activates caspase-1, leading to the maturation of IL-17A and the processing of GSDMD, which in turn mediates cytokine release and cell pyroptosis.[29,30] The PI3K/Akt pathway is fundamental for physiological protein synthesis and can induce various intracellular pathways, including the NF-κB signaling pathway.[31] IL-17A is an activator of the NLRP3 inflammasome, triggering the production of IL-1β and IL-18, culminating in pyroptosis.[32] In our preliminary experiments, we observed a significant upregulation of the expression of PI3K, AKT, P65, NLRP3, caspase-1, GSDMD-N, and IL-17A in PTX-treated mice. Treatment with CBD effectively inhibited their expression. Additionally, CBD reduced the mRNA expression levels of PI3K, AKT, P65, GSDMD-N, NLRP3, and IL-17A in OM mice, demonstrating its anti-inflammation and antipyroptosis properties.[33] OM is closely associated with signaling, and stimulation of the NF-κB pathway, which leads to the induction of proinflammatory cytokines production (for example, IL-6, IL-1β, and TNF-α).[34] Multiple cytokines interact synergistically,[35] leading to high rate of cell death and the activation of additional cellular signaling pathways. The PI3K/Akt pathway is important in inflammatory and cancerous environments, as it is involved in processes such as angiogenesis and recruitment of inflammatory factors.[36] CBD has anti-inflammatory and antioxidant properties, which potentially mitigate the adverse effects induced by chemotherapeutic agents.[37]

Based on our findings and those of previous studies, we postulate that the continued administration of cancer drugs in mice with OM induces oral mucosal damage and triggers inflammatory reactions, ultimately resulting in pyroptosis. Through a network pharmacology approach, we identified 49 potential therapeutic genes associated with CBD intervention in OM-induced pyroptosis. Among these genes, NF-κB1, PIK3R1, NF-κBIA, and AKT1 have been identified as potential core genes involved in therapy. Preliminary experiments suggest that CBD exhibits therapeutic effects against OM, as indicated by morphological results, the percentage of OM area, and pathological changes. To further test our hypothesis, we constructed an OM model by administering PTX.[38] Consistent with previous findings, our results indicate that CBD inhibits pyroptosis and decreases the generation of inflammatory factors. In vivo experiments showed that CBD effectively reduces production of inflammatory factors and suppresses expression of pyroptosis-related proteins. In vitro studies revealed that CBD enhances cell migration and proliferation, inhibits apoptosis, and decreases expression levels of pyroptosis-related genes and proteins. Furthermore, findings suggest a dose-dependent effect of CBD on cell viability. The outcomes of both animal studies and cellular experiments suggest that CBD alleviates OM via suppressing the PI3K/AKT/NF-κB signaling pathway and decreasing pyroptosis through integrating pathway inhibitors.

We acknowledge that this study had limitations. First, caution should be exercised in generalizing the model's findings to clinical practice, as the effects of various factors within the human body need to be considered. Second, although our results indicate that CBD mitigated PTX-induced pyroptosis and positively influenced cell viability, proliferation, migration, and displayed some anti-inflammatory effects in animal models, the detailed analysis focused on only one cancer drug. Other factor need to be investigated. Lastly, further research is needed on the interaction between CBD and other cell types. CBD impacts various cell types in the body, including human gingival MSCs and lymphocytes.[39] Therefore, we can hypothesize that CBD may exert therapeutic effects on OM by acting on various cell types in the body. This potential therapeutic impact of CBD on OM is an exciting area of research that merits further exploration.

Our study used bioinformatics data analysis alongside experimental validation, providing novel insights into the therapeutic role of CBD in OM. We discovered that CBD can alleviate pyroptosis in OM through the PI3K/AKT/NF-κB pathway. Although these findings hint at potential therapeutic applications in this domain, further research is crucial to explore this potential.

Acknowledgements: We would like to thank the members in our research group who are not listed in the authors.

Ethics Committee Approval: The study was approved by the Mudanjiang Medical University Ethics Committee (approval number: 20230318-11, date: 06.03.2023).

Data Sharing Statement: The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Authorship Contributions: Concept- H.Y.; Design- H.Y.; Supervision- H.Y.; Fundings- N.L., Y.Z., H.Y.; Materials- J.Y., N.L., S.L., Y.L., Y.Z.; Data Collection or Processing- J.Y., N.L.; Analysis or Interpretation- J.Y., N.L., D.W., H.Y.; Literature Search- S.L., D.W.; Writing- J.Y., H.Y.

Conflict of Interest: The authors declare that they have no conflict of interest.

Funding: This work was supported by the Basic Scientific Research Business Research Project of Heilongjiang (2020-KYYWFMY-0766); Scientific research project of Heilongjiang Provincial Health Commission (No.20230808030122); Doctoral Research Start-up Fund Project of Mudanjiang Medical University (no: 2021-MYBSKY-040); Heilongjiang Natural Science Foundation Project (no: LH2022H097; Science and technology plan project of Mudanjiang City (no: Z2017s0078).

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