SAS Journal of Medicine
Abbreviated Key Title: SAS J Med
ISSN 2454-5112
Journal homepage: https://saspublishers.com

OPEN ACCESS

Life Science and Technology

To Explore the Anti-Inflammatory Mechanism of Purslane Based on Network Pharmacology and Molecular Docking

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DOI: 10.36347/sasjm.2023.v09i05.034 | **Received:** 07.04.2023 | **Accepted:** 10.05.2023 | **Published:** 31.05.2023

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Abstract **Original Research Article**

This study was based on network pharmacology and molecular docking combined with experiments in vitro to explore the potential anti-inflammatory mechanism of *Portulaca oleracea* L. flavone (POL-F). First, the core targets of inflammation in purslane were screened through various databases and software, GO and KEGG analysis and molecular docking were performed. Then, mouse macrophage RAW264.7 was selected as the study object, and the safe experimental concentration of POL-F was confirmed by CCK-8 assay. The cells were divided into a control group, an LPS inflammatory cell model group, and an LPS inflammatory cell model group treated with different concentrations of POL-F. The secretion of NO in each group was detected by the Griess method. The expressions of IL-1β, IL-7, and TNF-α in the supernatant were detected by ELISA. The regulatory effects of POL-F on p-PI3K and PAKT signaling pathway proteins in cells of each group were detected by Western blot. The results showed that Pol-F treated with 100, 200, and 400 μg/ mL had no toxic effect on cells. The three concentrations of POL-F showed inhibitory effect on NO secretion of inflammatory cells, and the inhibitory activity was the best at 400 μg/ml. The expression of p-PI3K and PAKT protein was also inhibited after POL-F treatment. In conclusion, POL-F has a significant inhibitory effect on inflammatory response in vitro.

Keywords: POL-F; inflammatory; network pharmacology; molecular; docking in vitro experiment.

INTRODUCTION

Inflammation is the body's defensive response to stimuli, manifesting as redness, swelling, heat, pain, and dysfunction. Normally, inflammation is a beneficial, automatic defense response, but sometimes it can be harmful, such as attacks on the body's own tissues, inflammation in clear tissue, and so on. Excessive inflammation can cause cytokine storms leading to acute respiratory distress syndrome, multiple organ dysfunction syndrome, and even death. The occurrence of inflammation is common in the body, such as arthritis, myocarditis, inflammatory bowel syndrome, body injury, and infection of other diseases. Although the early inflammatory lesions mainly occur in the local area, long-term inflammation will make the local lesions gradually develop into whole-body disease, even cancer. At present, Western medicine generally treats inflammation with non-steroidal drugs and some adrenal corticosteroid drugs for anti-inflammatory. While long-term use of Western medicine anti-inflammatory will produce a variety of side effects, and poor prognostic effect, leading to more inflammatory diseases lack of effective treatment[1]. Therefore, it is of great significance to find out effective drugs from traditional Chinese medicine.

Portulaca oleracea L. is a family of portulaca, Portulacaceae, widely distributed in all parts of the country, is recognized by the Ministry of Health of Medicine and food homologous wild medicinal materials, and has high development and utilization value. *Portulaca oleracea* L. mainly contains alkaloids, flavonoids, terpenoids, coumarins, organic acids, polysaccharides, volatile oils, and other chemical components[2-4]. It has the biological functions of anti-inflammatory, antibacterial, antioxidant, anti-tumor, regulating blood sugar and blood lipid, etc. [5-8]Lingchao Miao[9] et al studied the anti-inflammatory effect of POL-F on lipopolysaccharide (LPS) -induced RAW264.7 cells, indicating that POL-F significantly reduced LPS induced NO synthesis, iNOS, and COX-expression levels in a dose-dependent manner. The

production of TNF-α and IL-6 was also significantly reduced at higher doses of 400 μg/mL. Meanwhile, the expression levels of P65, p-P65, p-MEK, and p-IκB-α were inhibited in a dose-dependent manner. Although Portulaca oleracea is known to have anti-inflammatory and bacteriostatic effects, the mechanism of POL-F's action on inflammation remains unclear. In this study, we explored the potential anti-inflammatory mechanism of POL-F based on network pharmacology and molecular docking binding in vitro experiments.

MATERIALS

Software Platform

Pharmacological analysis platform of Chinese Herbal Medicine System (TCMSP, https://old.tcmsp-e.com/tcmsp.php), UniProt database (https://www.uniprot.org/), Genecards database (www.genecards.org), OMIM database (https://www.omim.org/), providing database (http://db.idrblab.net/ttd/), String11.5 database (https://string-db.org/), Cytoscape3.7.2 software, Venny2.1 (http://www.liuxiaoyuyuan.cn/), Annotated database for Biological Information Metascape (http://Metascape.org), PDB (https://www.rcsb.org/) Protein database, AutoDockVina1.1.2 software

Instruments

Ultrasonic cleaning machine PS-40 (Harbin Ween Trading Co., LTD.), rotary evaporation instrument (Shanghai Yarong Biochemical Instrument Factory), UV-visible spectrophotometer (UK BIBBY Jenway), analytical balance FA2014B (Shanghai Aoping Scientific Instrument Co., LTD.), Electrophoresis Instrument (Beijing Liuyi Biotechnology Co., LTD.), Decolorization Shaker (Shandong Qiansi Scientific Instrument Co., LTD.)

Samples and reagents

Purslane (Anhui Daoyuantang TCM Decoction Piece Co., LTD.); Rutin Standard Product (Jiangxi Baicaoyuan Biotechnology Co., LTD.); Petroleum ether, anhydrous ethanol, ethyl acetate, n-butanol (Liaoning Quanrui Reagent Co., LTD.); AB-8 macroporous resin (Guangzhou Weiber Technology Co., LTD.); CCK8 kit, ELISA kit (Shanghai Xinyu Biotechnology Co., LTD.).

Methods

Network Pharmacology and molecular docking technology

Active ingredients and target screening of *Portulaca oleracea* L.

Chinese Herbal Medicine System Pharmacological Analysis Platform (TCMSP) database was used to obtain active ingredients and target information with "Purslane" as keywords and "OB≥30%" and "DL≥0.18" as screening conditions. Flavonoid components[10] were screened, and protein names and gene names were standardized with the UniProt database.

Acquisition of targets related to inflammation

With the help of the Genecards database, OMIM database, and TTD database, and with the keyword[11] "Inflammation", the corresponding targets for the genes selected in each database will be sorted out and collected, and repeated target genes will be eliminated as the targets for inflammatory diseases. The intersection targets of purslane flavone and inflammation were mapped using the Venny analysis tool.

Construct the protein interaction network

The intersection targets were imported into the String11.5 database, the species was set as "homo sapiens", the minimum interaction fraction value was ≥0.4, the unconnected nodes in the network were not shown, and the network map and the interaction results between active constituents of Purslane and inflammatory target proteins were obtained. Cytoscape3.7.2 software was used for visualization analysis[11], and the CyoHubba plug-in in the software was used for network topology analysis to obtain the core targets for inflammation.

Enrichment function analysis

Mapping targets were imported into the Metascape database, and items with $P < 0.01$, minimum count 3, and enrichment factor > 1.5 were screened for genomic Encyclopedia pathway enrichment analysis (KEGG) and Kyoto gene (GO) analysis. Sequencing was conducted according to the number of targets enriched on the pathway (Count). Subtrees with similarity > 0.3 in the screening process are considered to be a cluster, and the most statistically significant item within each cluster is selected to represent that cluster.

Molecular docking

Based on the PPI protein interaction network analysis, download the core compound Mol2 format file from the TCMSP database and the 3D structure.

PDB format file containing the original ligand from the PDB protein database. PyMol2.5 was used to treat all the receptor proteins, including the removal of water molecules, salt ions, and small molecules. The PyMol plug-in center_of_mass.py was used to define the center of the butt box, the size of which wrapped the whole protein. Using ADFRsuite1. Convert all the processed small molecules and receptor proteins into PDBQT format. Use AutoDockVina1.1.2 software for molecular docking verification, and use the default docking parameters for docking. The docking conformation with the highest output score is considered to be the binding conformation, and finally, the results are visualized with PyMol.

Extraction and purification of POL-F

The dried whole purslane grass was sifted to remove the lime dust, and crushed, and 6x mass volume of petroleum ether was added for ultrasonic degreasing.

The degreasing solution was drained and filtered to recover petroleum ether. Weigh 100 g of defatted purslane, add 30 times the volume of 75% ethanol, and soak in sealed overnight. On the second day, ultrasound was performed twice, after filtration, rotary steaming at 60 ℃, extraction solution was concentrated at 10 times the volume, and ethanol was recovered. Petroleum ether, ethyl acetate, and cold water phase n-butanol extraction were carried out at 3 times the volume successively. N-butanol phase was collected, and organic reagents were concentrated to obtain the total flavone-concentrated solution of Purslane, which was sealed and preserved.

AB-8 macroporous resin was soaked in anhydrous ethanol for 24 h, so that the resin was fully swollen, the damaged resin was removed, washed with anhydrous ethanol until there was no white turbidity in the wash solution with water, and then washed with distilled water until there was no alcohol flavor, and reserved. Ensure that there is no organic solvent in the sample, feed the sample slowly, control the spiral cutting at the slowest speed, wait until the liquid level is level with the top liquid level of the macroporous resin, and then sample again. The purified purslane flavone was obtained by rinsing with gradient ethanol and concentrating the fluxes for 4 hours after complete sampling. The purified purslane flavone was sealed and stored

In Vitro experiment

Cell culture: RAW264.7 cells were cultured using DMEM medium (10% FBS, 0.1% double antibody) and placed in an incubator at 37 ℃ and 5% CO_2. When cell fullness reached 80%, passage culture was carried out. RAW264.7 cells at logarithmic growth stage were inoculated into 96-well plates at a density of 7×104 cells per ml, 100 μL per well, and cultured in CO_2 incubator at 37 ℃, 5% CO_2, and saturated humidity.

Effects of POL-F on cell morphology

RAW264.7 cells were divided into 6 groups, namely blank group, model group, POL-F low-dose, medium-dose and high-dose groups. Cells in the blank group were cultured in serum-free medium for 24 h, and cultured in new medium for 1 h. The model group was cultured in serum-free medium for 24 h and then added into the medium containing 100 μg/L LPS for 1 h. POL-F low-dose, medium-dose and high-dose groups were cultured in medium containing 25, 50 and 100 mg/L POL-F for 24 h, respectively, and then added to medium containing 100 μg/L LPS for another 1 h. The cells were cultured on 6-well plates in groups, and the morphology of the cells was observed by microscope.

The effect of POL-F on cell viability was measured by CCK-8

RAW264.7 cells at logarithmic growth stage were inoculated into 96-well plates and cultured in CO_2 incubator at 37 ℃, 5% CO_2, and saturated humidity. After the cells adhered to the wall, the old medium was discarded and cell culture medium containing 0, 12.5, 25, 50, 100, and 200 mg/L POL-F was added, respectively, and continued to be cultured for 24 h. The survival rate of RAW264.7 cells was detected by the CCK-8 kit. Each group was set with 6 multiple Wells and the experiment was repeated 3 times.

Effects of POL-F on NO secretion in RAW264.7 inflammatory cells

RAW264.7 cells were divided into 6 groups, namely blank group, model group, POL-F low-dose, medium-dose and high-dose groups. Cells in the blank group were cultured in serum-free medium for 24 h, and cultured in new medium for 1 h. The model group was cultured in serum-free medium for 24 h and then added into the medium containing 100 μg/L LPS for 1 h. POL-F low-dose, medium-dose and high-dose groups were cultured with medium containing 25, 50 and 100 mg/L POL-F for 24 h, respectively, and then supplemented with medium containing 100 μg/L LPS for another 1 h. Cells were cultured on 96-well plates in groups, and cell supernatants were collected by groups (six parallel in each group). 100 μL supernatant was taken from each well, and the supernatant was added to Griess Reagent I and Griess ReagentII (50 μL/ well) at room temperature, and mixed gently for 10 min at 37 ℃. The light absorption value of the reagent was determined at 540 nm wavelength. The content of NO in the supernatant of cell culture medium was determined by the Griess method: Cells were inoculated in 96-well plates with 8×104 cells/well for 24 h. After culturing for 24 h, the blank group was treated with culture medium containing 0.1% DMSO, LPS (final concentration 1 μg/mL) was added to the stimulation group, and the drug concentration gradient was adjusted according to the results of the CCK-8 experiment. After intervention for 24 h, 50 μL of supernatant was taken to read OD 540 value. Substituted into the standard curve to calculate NO content.

The content of inflammatory factors in cell supernatant was determined by ELISA

The cell supernatant and cells from the culture plates were collected, and the inflammatory cytokines IL-1β, IL-6, and TNF-α in the culture medium of each group were detected at room temperature according to the instructions of the mouse interleukin 6 (IL-6) ELISA kit.

Western blot was used to detect p-PI3K and PAKT protein expression

The inflammatory cells were scraped off the cell scraper and collected into the EP tube. Add 1 mL PBS, centrifuge 600 g for 5 min, and discard the supernatant. 80 μL cell lysis solution (1 mL cell lysis solution in 10 mL PMSF) was added to the EP tube and ice lysis solution was added for 20 min. After lysis, the EP tube was centrifuged (12000 g,10 min,4 ℃). After

centrifugation, the precipitation was abandoned and the supernatant was retained. Add 20 μL 5buffer and blow well. Let the EP tube sit at 100℃ for 15 minutes, then store it at -20℃. The extracted total protein was separated, electrophoretically transferred to a PVDF membrane by SDS-PAGE, and incubated with the corresponding primary antibody. Overnight at 4 ℃, the film was washed at room temperature, and the second antibody was incubated for 1 h. Color development was performed with a hypersensitive ECL chemiluminescence kit. Protein expression was observed with a gel imager, and the gray values of the bands were measured.

RESULTS

Network Pharmacology and molecular docking analysis

Network pharmacological analysis revealed 143 potential targets of flavonoids in Portulaca oleracea (Figure 1). A total of 1434 potential targets of inflammation were collected through GeneCards, OMIM, and TTD databases, and 114 common targets of drug targets, and disease targets were pooled for analysis (Figure 2). Through the construction of the PPI network (Figure 3) and the calculation and integration of the MCC algorithm, MNC algorithm, and Degree algorithm, seven core genes of Purslane flavone in the treatment of inflammation were screened out as MMP9, PTGS2, TNF, VEGFA, CASP3, IL1B and IL6, respectively. Compounds occupying more than 3 core gene targets were luteolin, quercetin, β-carotene, and kaempferol.

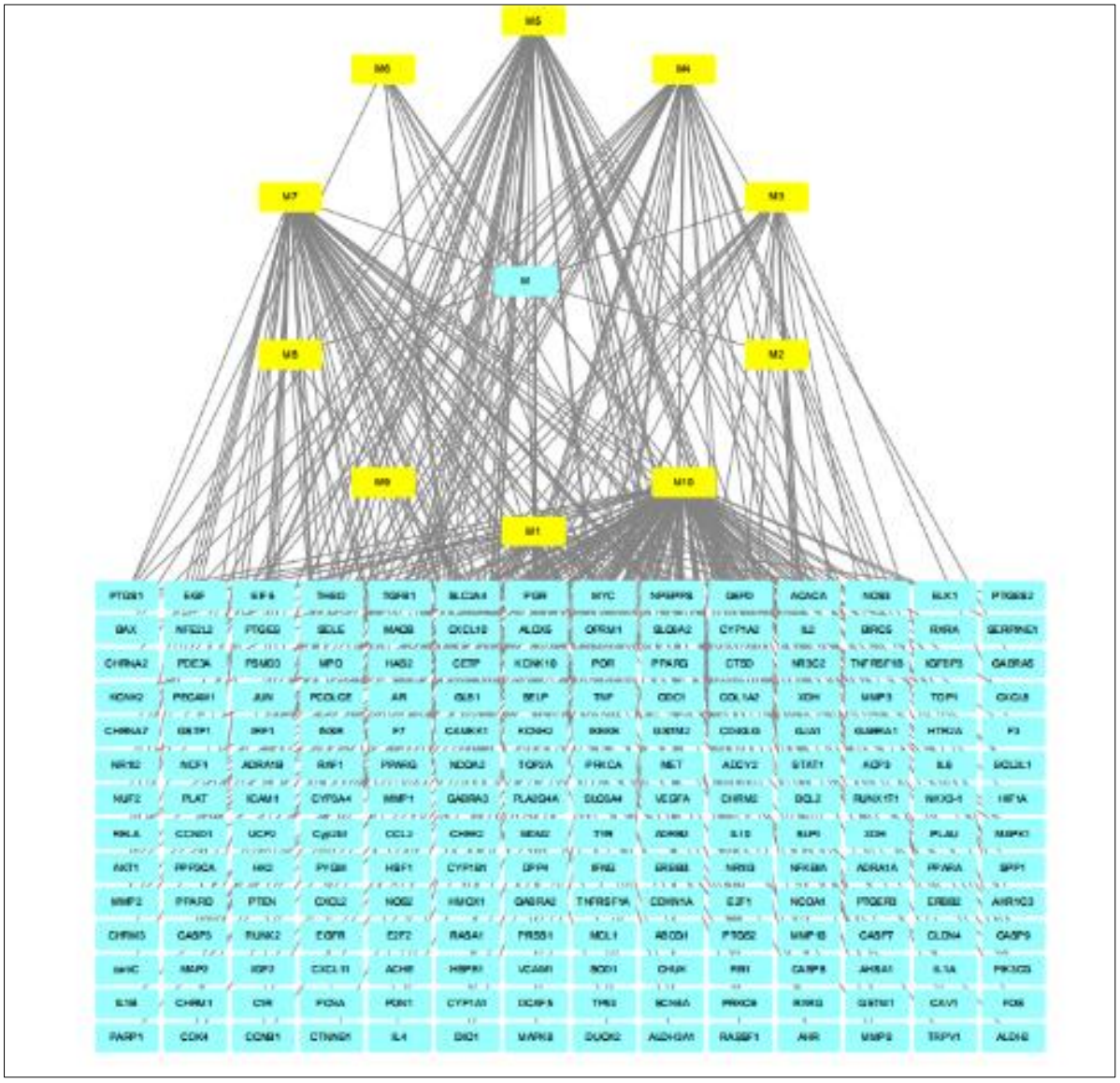


Figure 1: Potential targets of POL-F

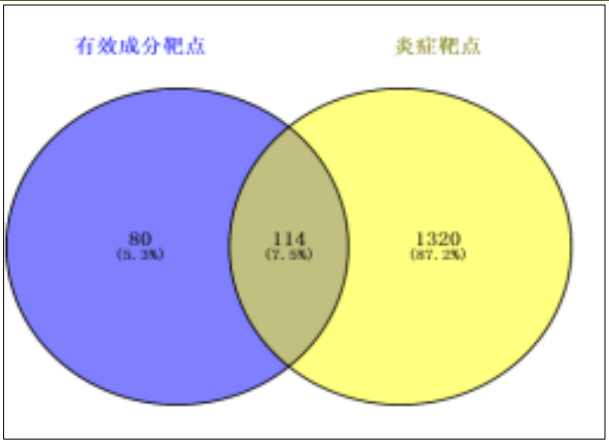


Figure 2: Venny diagram of inflammation and POL-F targets

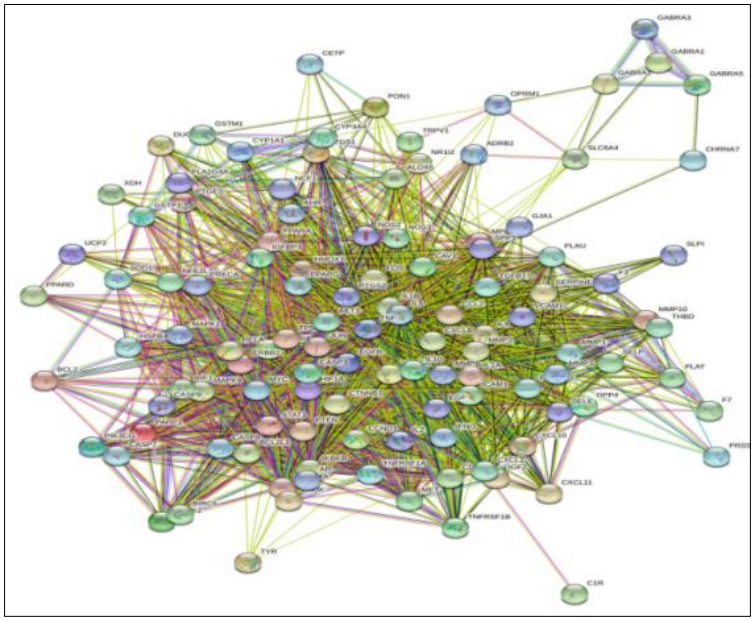


Figure 3: PPI network diagram

GO enrichment analysis (FIG. 4) and KEGG enrichment analysis (FIG. 5) using Metascape showed that biological processes were mainly related to inflammatory response, oxidative stress, signal transduction, and immune response. Cell composition was mainly concentrated in the cell membrane, nucleus, and mitochondria. Molecular functions are mainly involved in protein binding, cytokine activity, and protein kinase activity.

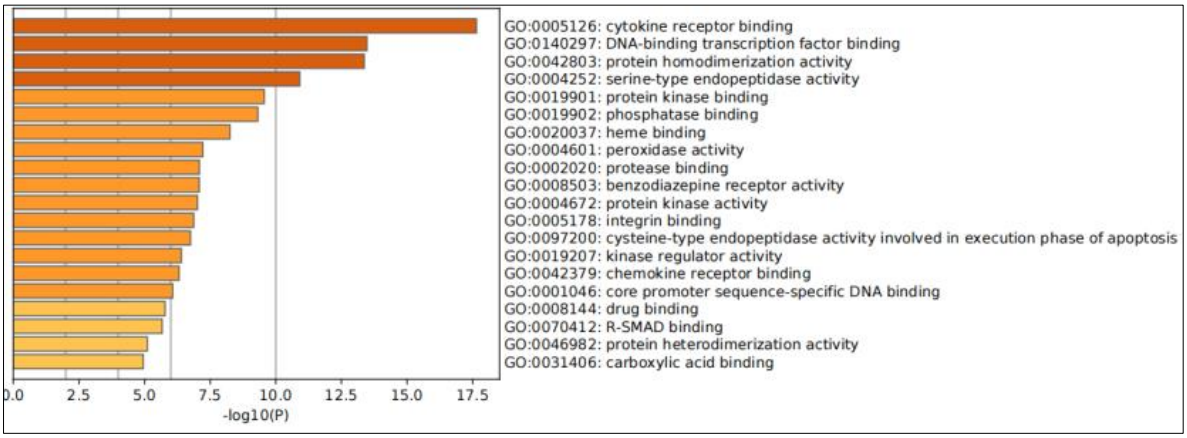


Figure 4: GO functional enrichment

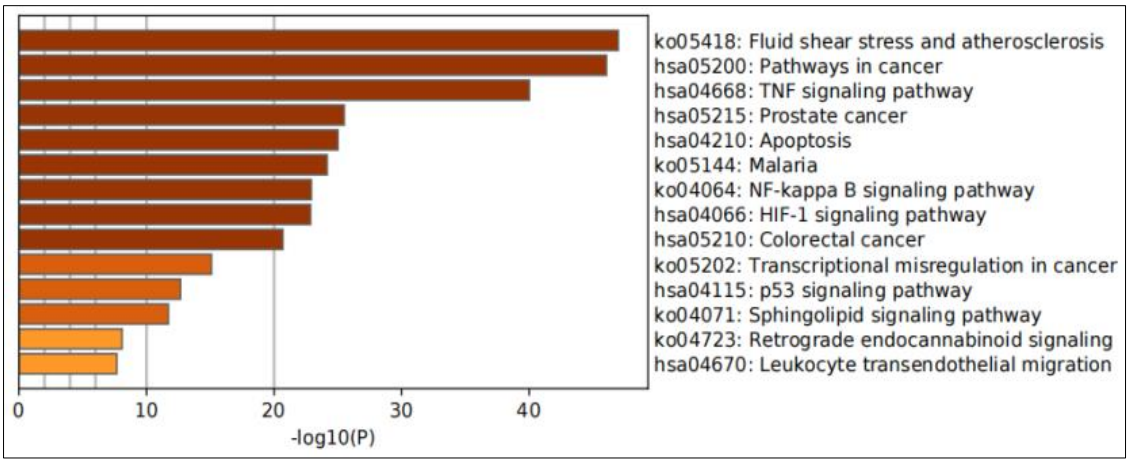


Figure 5: KEGG functional enrichment

The group with the highest binding energy was visualized by molecular docking (Figure 6). Luteolin formed hydrogen bonds with 3 amino acid residues in PTGS2 and 3 amino acid residues in IL-6. Kaempferol formed hydrogen bonds with two amino acid residues in PTGS2; Hesperidin formed hydrogen bonds with one amino acid residue in PTGS2; Arachidonic acid formed hydrogen bonds with 3 amino acid residues in CASP3; Quercetin formed hydrogen bonds with 4 amino acid residues in TNF and 3 amino acid residues in IL1B. It indicated that the flavonoid compounds of purslane had a good affinity with inflammatory protein molecules.

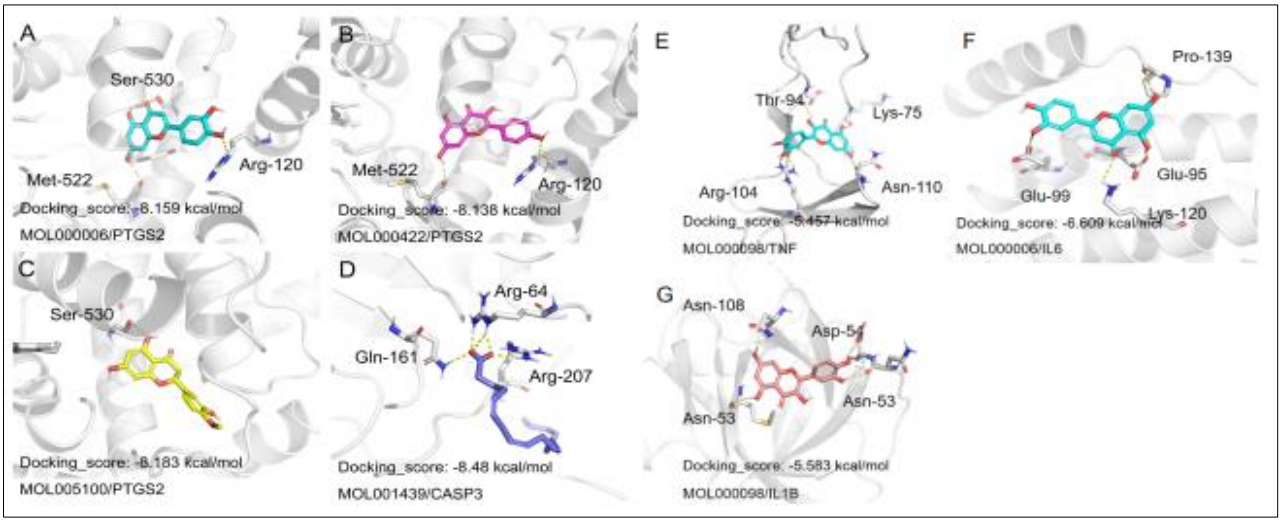


Figure 6: Stereoscopic molecular docking conformation

Analysis of cell experiments in vitro

Morphological observation of POL-F on infected inflammatory cells

The cell morphology in the blank group, model group, and under the influence of low, medium, and high doses of POL-F was shown in Figure 7: RAW246.7 cells in the blank group were round and had weak adherence to walls. Compared with the control group, LPS-induced cell volume increased, and pseudopodia was obvious. The morphology of macrophages was significantly improved after POL-F treatment, and the higher the dose, the more obvious the morphological changes.

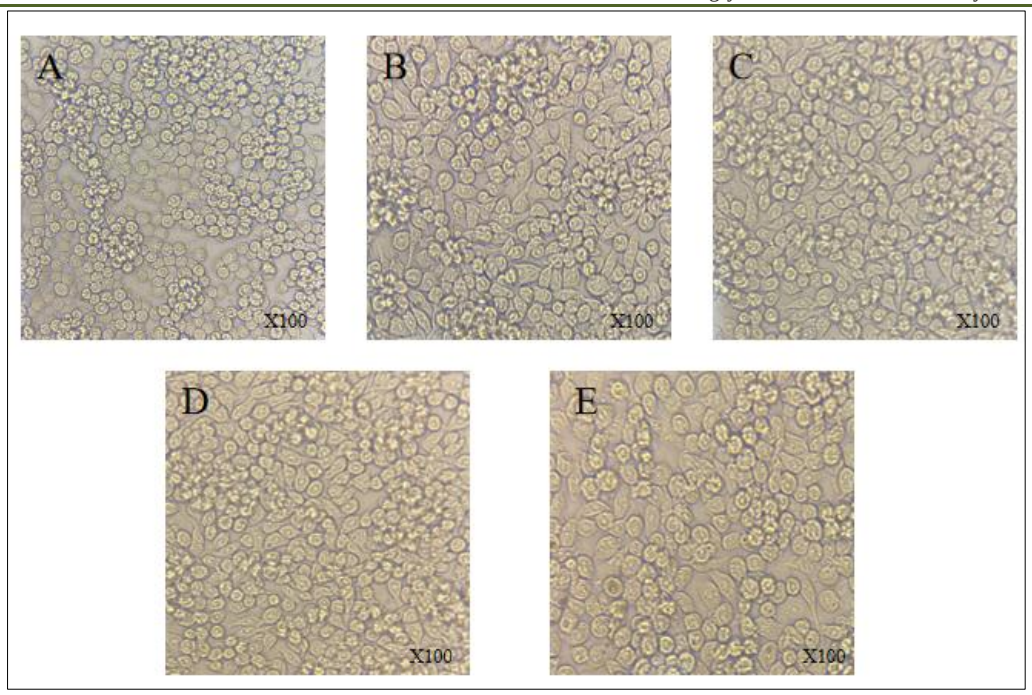


Figure 7: Effect of POL-F on the Morphology of inflammatory cells

Note: A: blank group; B: LPS 1μg/mL; CC:LPS 1μg/mLand POL-F 100 μg/mL D:LPS 1μg/mLand POL-F 200 μg/mL; C:LPS 1μg/mL and POL-F 400 μg/mL

Cytotoxicity and drug dose screening

To study POL cytotoxicity in vitro, CCK-8 was used to detect relative cell viability, and the results are shown in Figure 8. Compared with the control group, the cell activity after treatment with different concentrations of POL was higher than 90%, and the cell survival rate was the highest when the concentration was 400 μg/mL. Therefore, Pol-P with the concentration of 400 μg/mL was used in this study as the condition for further in-depth study.

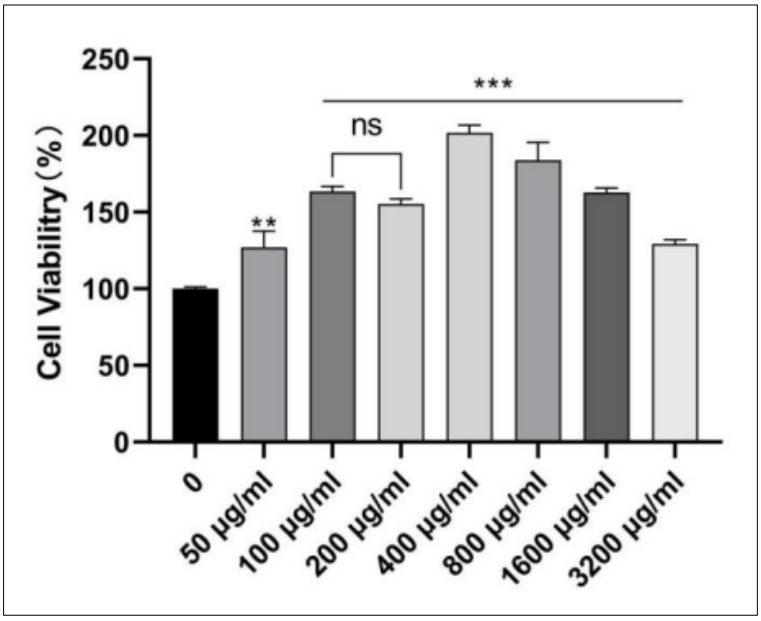


Figure 8: Effects of POL at different concentrations on cell viability

Effect of POL-F on NO secretion in RAW264.7 inflammatory cells

In order to study the effect of POL-F on NO secretion of inflammatory cells, absorbance values of the blank group, model group, POL-F low-dose, medium-dose and high-dose groups were measured at 540 nm wavelength. The results are shown in Figure 9. It can be seen that with the increase of POL-F concentration, NO secretion content decreased continuously, in a concentration-dependent manner.

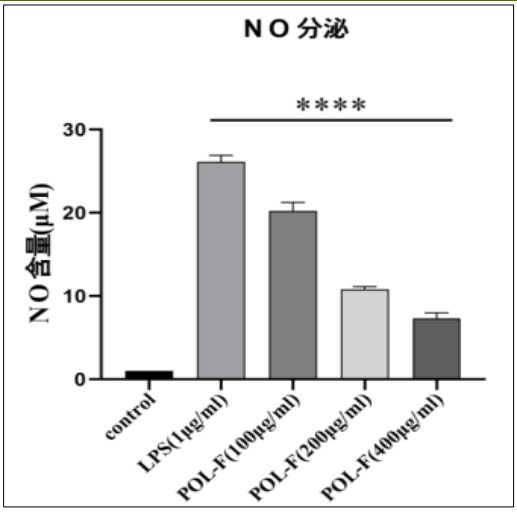


Figure 9: Effect of POL extract on NO secretion of inflammatory cytokines

Cytokine detection

With the concentration of the standard substance as the horizontal coordinate and the corresponding OD value as the vertical coordinate, the linear regression curve of the standard substance was drawn. The concentration values of each sample were calculated according to the curve equation, and the contents of IL-17, TNF-α, and IL-1β were detected. The specific results were shown in Figure 10. With the increase of POL-F content, the contents of IL-17, TNF-α, and IL-1β decreased gradually, indicating that POL-F could inhibit inflammatory factors in vivo in a concentration-dependent manner.

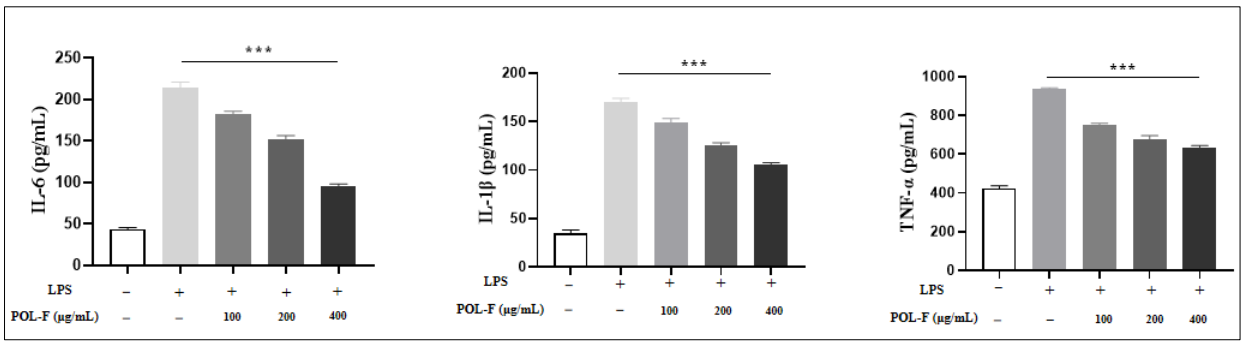
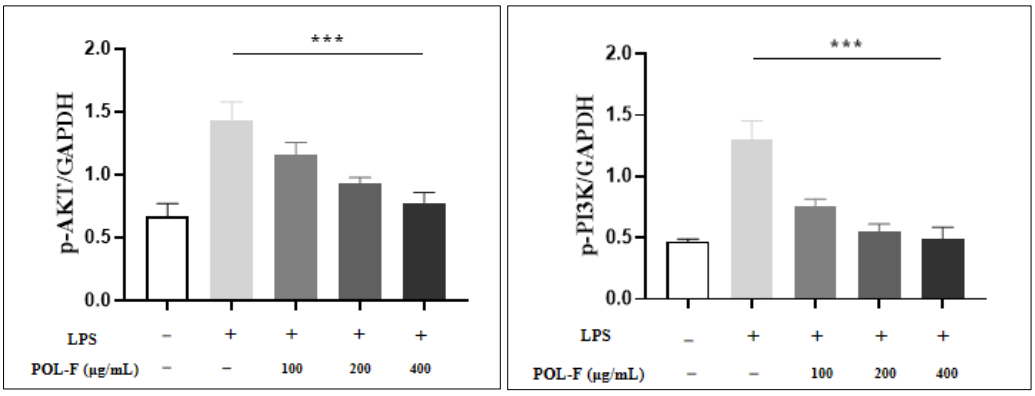


Figure 10: Effects of POL-F on various inflammatory factors

Effects of POL-F on p-PI3K and PAKT proteins

The effects of POL-F on the content and properties of p-PI3K and PAKT protein were detected by Western blot. The inflammatory cells were treated with different concentrations of POL-F (100, 200, 400 μg/mL). Compared with the LPS group and control group, different concentrations of POL-F inhibited inflammatory cytokines, and the inhibitory effect became better and better as the concentration increased (FIG. 11).



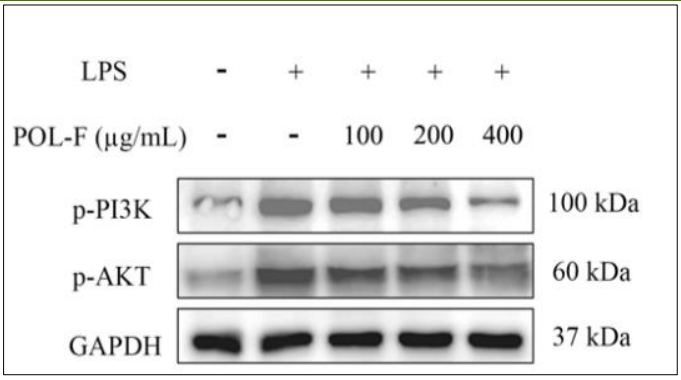


Figure 11: Effects of different concentrations of POL-F on p-PI3K and PAKT proteins

DISCUSSION

In the immune inflammatory response of the body, inflammatory factors such as NO, TNF-α, IL-6, and IL-1β are highly expressed to varying degrees. As a membrane permeability signaling molecule, NO is a central inflammatory mediator. Tnf-alpha, IL-6, and IL-1β were primarily involved in endogenous inflammatory responses and subsequent inflammatory processes[12]. The significant pharmacological effects of traditional Chinese medicine in anti-inflammatory, especially in regulating and weakening the production [13]of inflammatory transmitters NO and iNOS as well as inflammatory factors. POL-F is a class of traditional Chinese medicine with broad-spectrum pharmacological action, which has the effect of anti-tumor cell proliferation and promoting tumor cell apoptosis and has anti-inflammatory function [14-15]in cellular inflammation.

The results of this study showed that POL-F had an effective inhibitory effect on the expression of inflammatory transmitter NO and inflammatory cytokines (TNF-α, IL-7, and IL-1β), and could reduce the degree of the inflammatory response of macrophage RAW264.7. POL-F can regulate the production of downstream inflammatory factors by regulating p-PI3K and PAKT signaling pathways, and then inhibit the inflammatory response. POL-F is concentration-dependent on p-PI3K and PAKT proteins and viral titers. When the concentration is 400 μg/mL, POL-F has the greatest inhibitory effect on the inflammatory response. This study provided a preliminary theoretical basis for the development and utilization of Portulaca oleracea in the treatment of inflammation.

Acknowledgments

This work was supported by Heilongjiang BaYi agricultural university's innovative training project for college students and the "Three Longitudinal" basic cultivation project (XC2022037, ZRCPY202217，202210223093).

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