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Exploring the Therapeutic Efficacy of Xiaoyaosan in Alleviating Depression: A Mechanistic Insight into the Modulation of Microglial Oxidative Stress Response by Quercetin

Zikuan Bai

Shanghai YK Pao School, Room 2502, No.6, Lane 1288, Xi Kang Road, Shanghai, China

Abstract: Depression is a mental disorder with complicated causes and pathologies. Xiaoyaosan (XYS), a traditional Chinese medicine, has shown promise in depression treatment due to its multi-targeted approach and low side effects. Currently, there are relatively few research that provides a scientific understanding of XYS's treatment mechanism. Here, we explored the potential mechanism to provide proof of XYS's antidepressant effects using network pharmacology and in vitro experiments. Firstly, network pharmacology was conducted to screen key chemicals and potential targets within the major ingredients of XYS's herbal formula. Predicted targets' protein-protein interactions and pathway analysis revealed as the central component effecting multiple core targets shared by depression and XYS's ingredients. Based on that, Quercetin was selected for further in vitro study. A microglial cell model was constructed to evaluate the antioxidant and anti-inflammatory effect of Quercetin under oxidative and bacterial stress induced by H_2O_2 and lipopolysaccharide, respectively. Results showed that Quercetin at appropriate concentrations (20 μM and 50 μM) could maintain microglial cell activation balance and mitochondrial function. Under stressful conditions, exposure to higher concentrations (100 μM) led to cellular morphological damage or apoptosis, but not in healthy or resting microglial cells. Our findings confirmed Quercetin's beneficial impacts on microglial cell health, a critical aspect of depression pathology. This study integrated network pharmacology with in vitro experiments to provide evidence that XYS could mitigate inflammatory and oxidative responses of microglial cells implicated in depression, primarily through the modulating mitochondrial function and cellular morphology.

Keywords: Depression; Xiaoyaosan; Quercetin; Network Pharmacology; Oxidative Stress; Microglia.

1. Introduction

Depression is a prevalent mental disorder globally, with limited pharmaceutical treatment approaches and understood pathophysiology. Major Depression Disorder (MDD) has an overall 6% prevalence in twelve months, rising to 15-18% over a lifetime. This means that nearly one out of every five individuals will experience MDD at some point in their lives [1]. On average, the morbidity age is 25, and females are about twice as likely as males to experience depression.

Depression symptoms encompass a range of emotional and physical manifestations, including anhedonia, worthless and guilty feelings, suicidal thoughts (the second leading cause of death among people aged 10-34), altered sleeping and appetite, fatigue, indecisiveness, and psychomotor retardation or agitation. Various pathologies contribute to depression, including the monoamine hypothesis, which is also the foundation for current antidepressants, hypothalamic-pituitary-adrenal axis (HPA) changes [2], and increased production of pro-inflammatory cytokine that suppress neuroplasticity and neurogenesis [3]. Structural brain changes in depression are also affected, with a smaller hippocampus, increased amygdala activity, hyperactive subgenus anterior cingulate, and hypoactive insulation and dorsal lateral prefrontal cortex [4].

In this essay, investigations of the ingredients of XYS would be conducted and would be instrumental in identifying the active ingredients and elucidating the mechanisms underlying their pharmacological actions. This investigation used network pharmacology to find the key chemical in the XYS herbal formula's ingredients and its molecular mechanism upon effect on the human body, specifically, microglial cells. Microglia cell model in depressive states was constructed to conduct experiment upon different concentrations of Quercetin's effect on mitochondria function and response towards oxidative stress. The results provide a theoretical explanation of XYS's mechanism for depression treatment and provide additional support for molecular mechanism investigation in traditional Chinese medicine. There is also clinical value in the potential targets for depression treatment.

2. Method

2.1. Cell culture and Cell Model Construction

BV2 cell line (Wuhan Pricella Biotechnology Co., Ltd.) derived from C57/BL6 murine was used as the microglial cell model here. Firstly, thawed the cells by taking them out from liquid nitrogen tank and melted in water bath at 37℃. Before the ice is completely melted, add pre-warmed growth medium, which is RPMI 1640 (Biological Industries) containing 10% FBS (Wuhan Pricella Biotechnology Co., Ltd.). Centrifugation is conducted at 800 rpm for 5 minutes, decant the supernatant and resuspend the cells in complete growth medium. Cells were cultured in humidified 5% CO2 incubator at 37℃.

When experiment was finished, we collected cells in the same way and froze them with cell freezing medium (NCM Biotech) Cryotube with cells was placed in an isopropanol freezer container and stored at –80°C overnight. After that, transfer cryopreserved cells to liquid nitrogen.

We used 200 mM H2O2 solution to model for the oxidative stress and 100 ng/mL lipopolysaccharides (LPS) (Selleck chem, s7850) for modeling bacterial stress. The cells were seeded into 96-well plates at 5 x 104 cells/mL and given different combinations of chemicals every 4 wells (n = 4). Also, 10, 20, 50, and 100 μM Quercetin (Selleck chem, s2391) was added to test the antioxidant or anti-inflammatory effect. The specific layout is shown in Figure 1 (Fig.1).

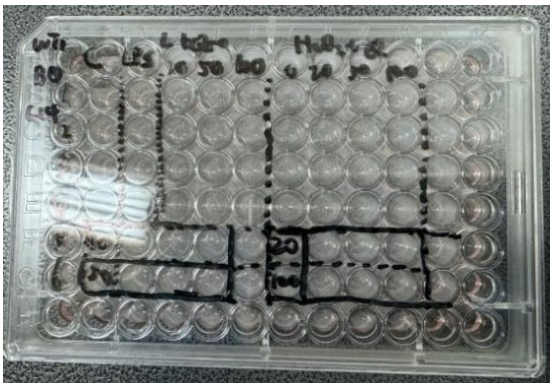


Fig 1. Treatment for CCK8 assay.

Groups: negative control, LPS, H2O2, LPS or H2O2 combined with Quercetin at different concentrations, only Quercetin at different concentrations

2.2. Cell Apoptosis-Related Caspase-3 Activity and Mitochondrial Membrane Potential Detection

The Caspase-3 activity and mitochondrial membrane potential in cells were detected using Caspase-3 Activity and Mitochondrial Membrane Potential Detection Kit for Live Cell (Beyotime, C1073S), which consists of GreenNuc Caspase-3 Substrate and Mito-Tracker Deep Red 633. The working medium consisted of 200 nM GreenNuc Caspase-3 Substrate, 5 μM of Mito-Tracker Deep Red 633(C1073S-2), detection buffer and was put into the 25 wells in the 96 well plate after washed with PBS (Fig.2). The plate was then put in room temperature for 25 minutes and then observed under spectrophotometer (BioTek, Synergy H1). Green fluorescence produced by GreenNuc-DNA complex is observed under Ex=500nm and Em=530nm, while Mitro-Tracker Deep Red 633 may produce a dark red fluorescence observed with Ex=622nm and Em=648nm.

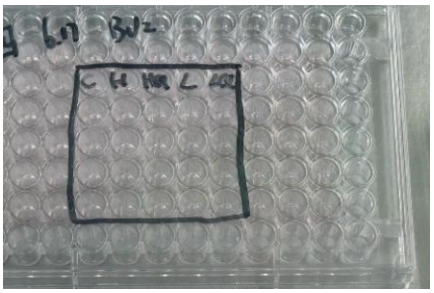


Fig 2. Treatment for Caspase-3 Activity and Mitochondrial Membrane Potential Detection.

Groups: negative control, LPS, H2O2, LPS or H2O2 combined with Quercetin

Then, we removed the working medium and detected it under a confocal microscope (Olympus Fluoview FV3000) to observe mitochondrial color. In another group, different concentrations of Quercetin, specifically, 0 μM, 10 μM, 20 μM, 50 μM, and 100 μM, were added to H2O2 and LPS and sampled in a 96-well plate (n=5). The same steps were performed, and spectrophotometer was used for mitochondrial color observation. The fluorescence enzyme-linked immunosorbent assay setting was Ex/Em = 510/580 nm.

2.3. RNA Extraction, Reverse Transcription and Real-time PCR

RNA extraction was performed using RNA Easy Fast Tissue/Cell Kit (TIANGEN BIOTECH, DP451). To make cell lysis and release DNA and RNA, we added 350 μL of RLA and 10 μL of Proteinase K. After centrifuged at 13400 g for 3 minutes, the supernatant was then added to spin column, centrifuged for 30 seconds to remove DNA and precipitate RNA. [5-11] The same volume of 70% ethanol was then added, and the mixture was put into RNase-Free absorption column CR4 and centrifuged for 30 seconds. RW3 and RW washing buffer were added to the column to remove the protein or polysaccharide impurities. Then, the absorption column was centrifuged for 2 minutes and stewed for two minutes under room temperature to dry the buffer. Afterwards, the columns were moved into new RNase-Free centrifuge tubes, 40 μL of RNase-Free ddH2O was dropped to diffuse the RNA. The concentration was measured under spectrophotometer (Thermo Scientific, NANODROP 2000C). Then, FastKing gDNA Dispelling RT SuperMix (TIANGEN BIOTECH, KR118) was used to conduct reverse transcription. SuperMix was mixed with RNase-Free ddH2O and added to the RNA template, reacted at 42℃ for 15 minutes and 95℃ for 3 minutes to remove gDNA and conduct reverse transcription reaction. After obtaining the cDNA template, SuperReal PreMix Plus (SYBR Green) (TIANGEN BIOTECH, FP205) was used to build Real-Time PCR reaction system. The 20 μL system was used when adding the SuperReal PreMix Plus, primers and RNase-free ddH2O to the template in the PCR tube (Fig. 3). Then Real-Time PCR was conducted using the Bio-Rad C1000 Touch Thermal Cycler (CFX96), setting at 95℃ for 15 minutes, then 40 cycles of 95℃ for 30 s followed by 60℃ for 30 s.

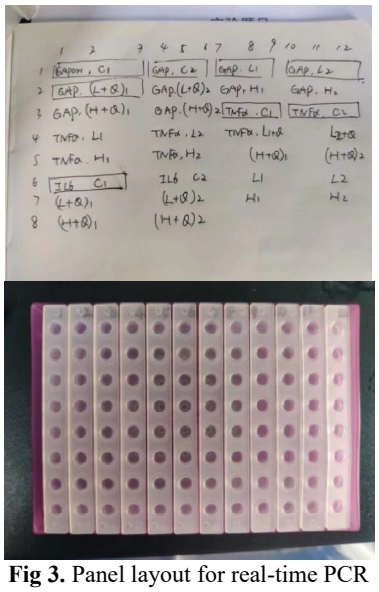


Fig 3. Panel layout for real-time PCR

Groups: negative control, LPS, H2O2, LPS or H2O2 combined with Quercetin

3. Results

3.1. Quercetin is Selected to Conduct Cellular Experiments on Oxidative Stress Based on Network Pharmacology

Through STRING (https://cn.string-db.org/), we converted the 181 target genes of the active compounds in XYS into uniprot ID form, and compared them with the 15649 targets of depression obtained from GeneCards database using Venn diagram. We identified 174 overlapping targets that were likely to play a significant role in the treatment of depression with XYS. Subsequently, we used STRING to construct a PPI network from the 174 obtained shared targets (Fig. 4). We then used Cytoscape software to analyze and visualize the output degree value of the targets' PPI network, which is the number of interactions the targets have with other targets. The resulting PPI network consisted of 170 nodes and 6782 edges. Their degree value ranges from 1 to 125. By analyzing the network, we identified the 149 hub genes with a degree value greater than or equal to 10.

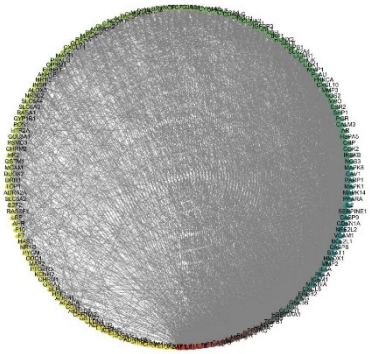


Fig 4. The PPI network of 174 overlapping genes under STRING database and Cytoscape construction.

By using GO analysis module on SRplot website, we identified the major biological process, cellular components, and molecular functions enrichment related to the 149 hub genes. The ten biological processes that have the highest number of involved genes are all involved in response to outside stimuli, both chemical and biological. Chemical stimuli come from drugs and reactive oxygen species, and biological stimuli come from molecules of bacterial origin(Fig. 5).

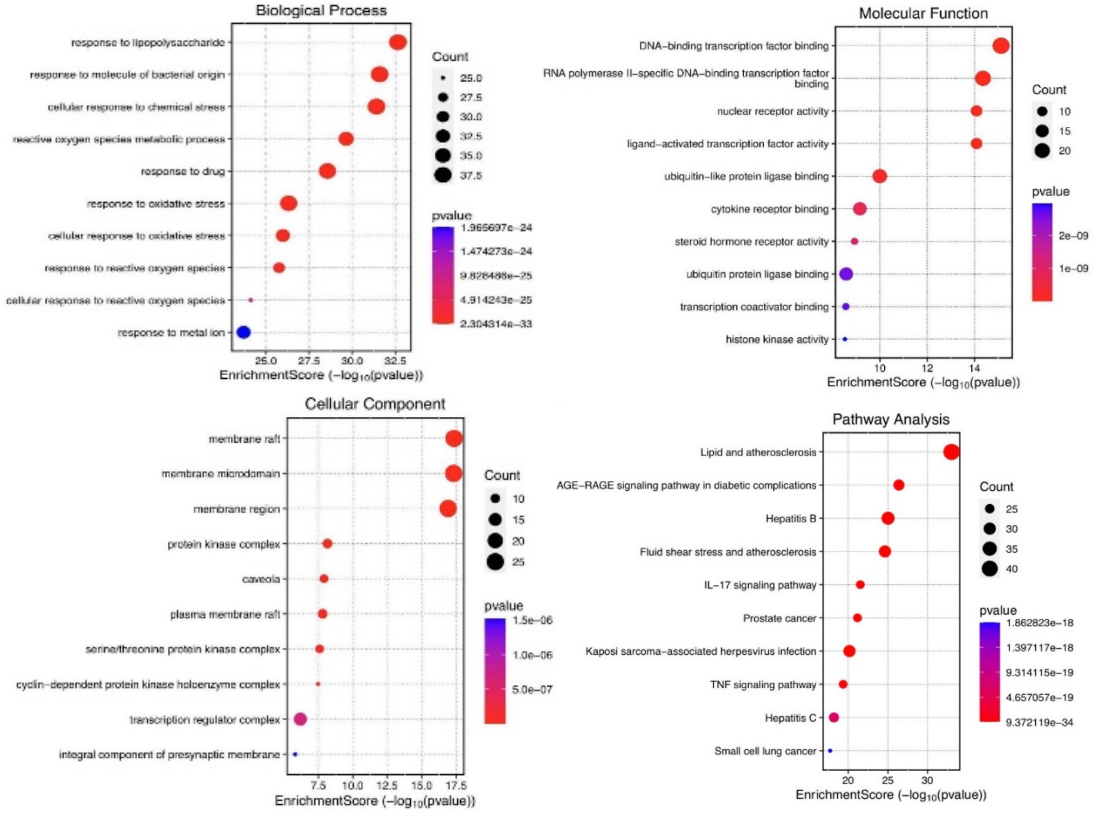


Fig 5. Dot plot of cellular component and enrichment scores

Microglia are the primary immune cells found in the central nervous system (CNS), playing a crucial role in cellular inflammatory response to oxidative stress. In a state of depression, microglia can become chronically activated due to various stressors, leading to a cascade of events that contribute to the development and maintenance of depressive symptoms. The network pharmacology analysis revealed that the main components of XYS predominantly target pathways associated with oxidative stress in the treatment of depression, indicating a significant convergence of their therapeutic effects in this pathway. Thus, we decided to use microglia as a cell model for future in vitro experiments.

3.2. Data of Cell Experiment

Microglia cells (cultured BV2 cell line) were stimulated separately by LPS and H2O2 to mimic the bacterial stress and oxidative stress state on microglia in the brains suffering from depression. CCK-8 testing was then performed to test cell viability. There were three sets of data obtained from the test (Fig. 6). The first set of data is obtained as we added different

concentrations, specifically, 0μM, 10 μM, 20 μM, 50 μM and 100 μM of Quercetin to microglial cells. The cell viability in the control group was normalized as 1.0 when there was no Quercetin added. The ANOVA test revealed that Quercetin at various concentrations did not significantly affect the viability of resting BV2 cells (Fig. 10A, p = 0.8515).

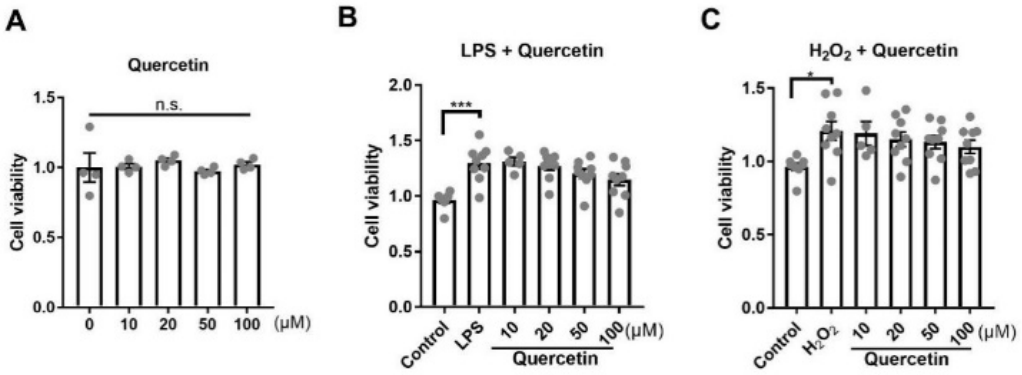


Fig 6. CCK8 assay to evaluate the cell viability in BV2 cells with Quercetin treatment at various doses.

Upon activation, BV2 cells undergo a series of significant morphological transformations. They would typically exhibit a ramified morphology characterized by long, branched processes that continuously survey the surrounding environment. To further ensure the activation status of cells, we observed the cell morphology under a microscope (Fig. 7). We then used DAPI fluorescence to observe the cell nucleus and phalloidin staining of F-actin to observe cytoskeleton (Fig. 8).

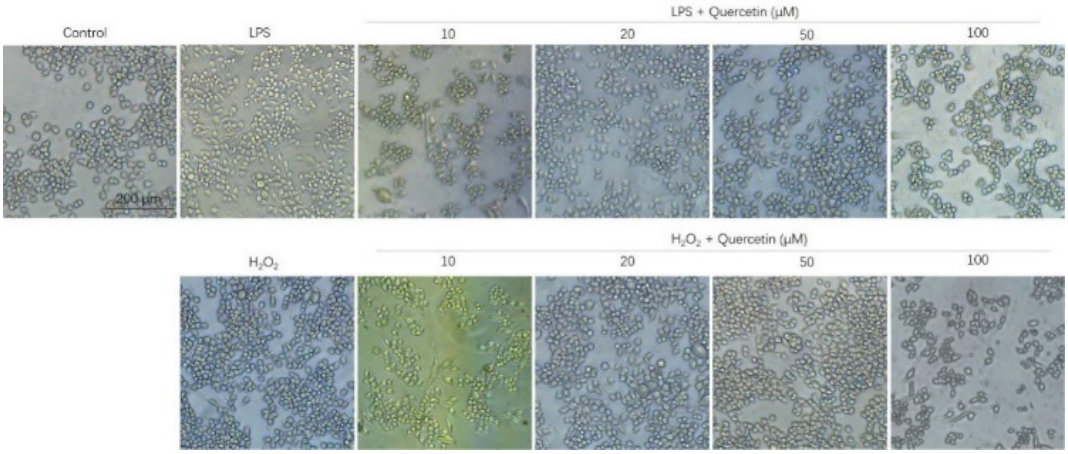


Fig 7. Cell morphology under optical microscope

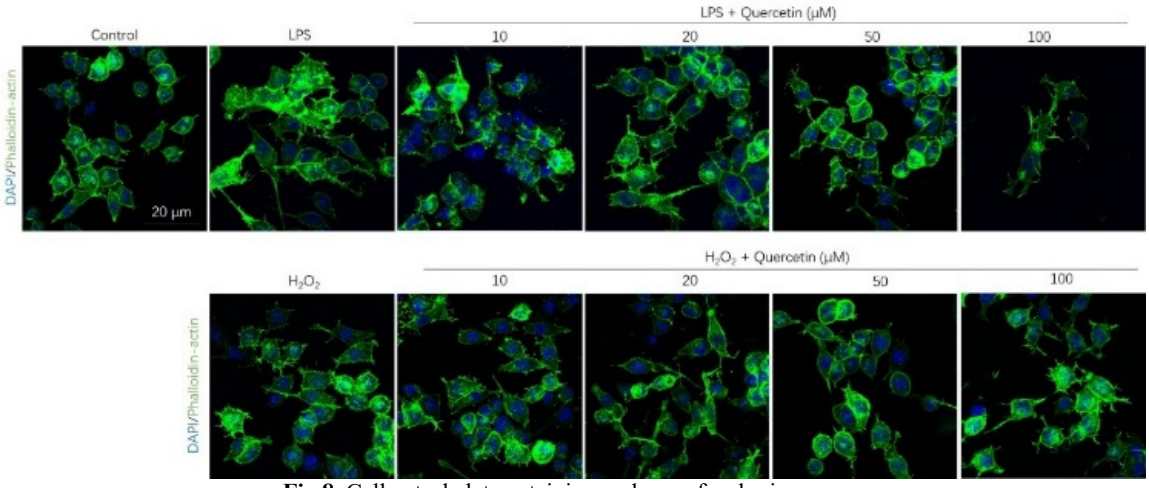


Fig 8. Cell cytoskeleton staining under confocal microscope

In the group of different concentrations of Quercetin being added to LPS, caspase -3 activity which is reflected by the OD value (Fig. 9A) The group of Quercetin at 100 μM concentration showed significantly greater caspase -3 activity relative to all other groups, indicating cell apoptosis occurring and mitochondrion's over-stimulated reaction towards high concentration of Quercetin and bacterial stress. It suggested that Quercetin at 100 μM concentration might lead to cell apoptosis through caspase-3 activation under LPS stimulation. This also corresponds to the results of mitochondrial membrane potential in the same cell modeling group (Fig. 9B).

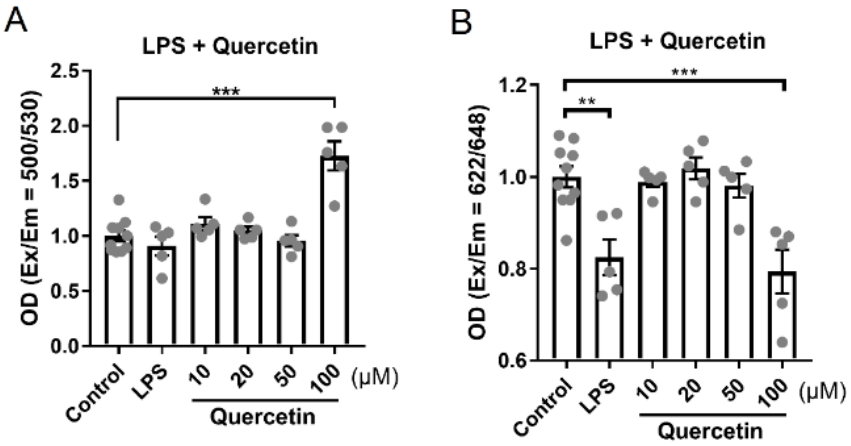


Fig 9. Caspase-3 activity (A) and mitochondrial membrane potential assay (B) to investigate LPS and different concentrations of Quercetin's effect on BV2 cells.

In the group with different concentrations of Quercetin and H2O2 added, the caspase 3 activity showed no significant changes compared to the control group (Fig. 10A). This suggests that cells may have died in a different pathway instead of apoptosis through caspase -3 release and activation, and cell viability significantly decreased before being able to respond to oxidative stress and pressure of high Quercetin concentration. The results of mitochondrial membrane potential were generally similar to the caspase-3 activity (Fig. 10B).

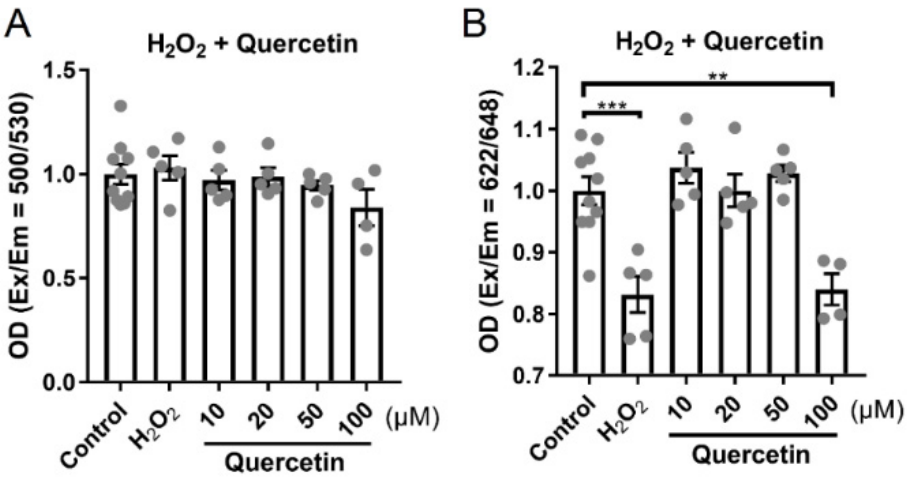


Fig 10. Caspase-3 activity (A) and mitochondrial membrane potential assay (B) to investigate H_2O_2 and different concentrations of Quercetin's effect on BV2 cells.

Cells were observed under a confocal microscope (Fig. 11) and Fluorescence microplate reader (Fig. 12). Observation under a confocal microscope demonstrated a decrease in SOD level as Quercetin concentration increased as visible by the fluorescence intensity of red bubbles.

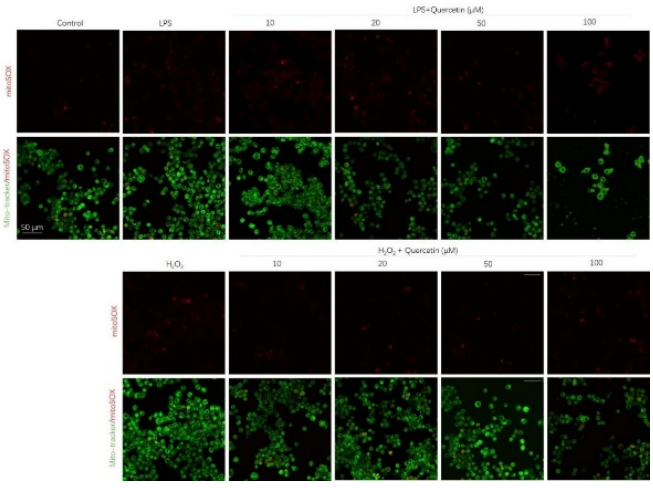


Fig 11. MitoSox Red and Mito-Tracker Green make mitochondria number and superoxide quantity visible for morphology observation

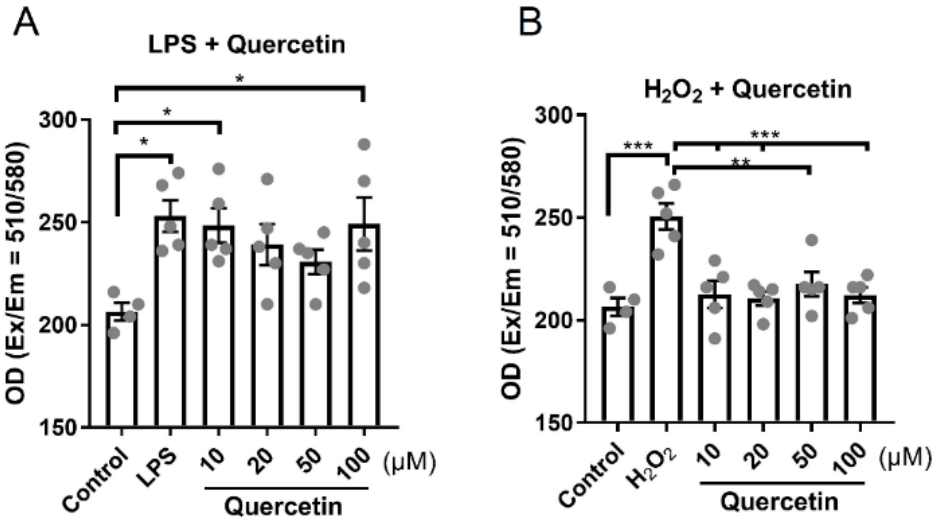


Fig 12. MitoSox Red assay to evaluate fluorescence intensity of SOD level when modeled cells are treated with different concentrations of Quercetin

The inflammatory cytokines, tumor necrosis factor-alpha (*Tnf-a*), and interleukin-6 (*Il6*) expression would increase when microglial cells are M1 activated, their level of expression was tested through real-time PCR to further investigate different concentrations of Quercetin's effect on balancing microglial cell activation and inflammatory responses (Fig. 13).

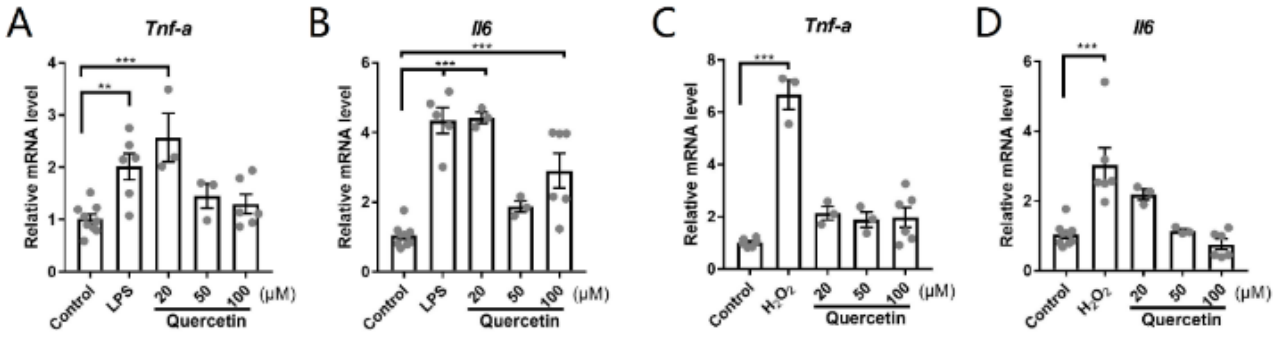


Fig 13. Real-time PCR measuring effects of different concentrations of Quercetin on cellular expression of Tnf-a and Il6 under different stress stimuli

4. Conclusion

The investigation provided clues to potential XYS's depression treatment mechanisms through microglial cells' mitochondrial response and morphology to oxidative and bacterial stress and the different Quercetin concentration's effect on them. The results may provide evidence for future investigations of TCM's disease-treating mechanisms and trustworthiness, and exploration of XYS's depression-treating mechanisms and drug innovation.

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