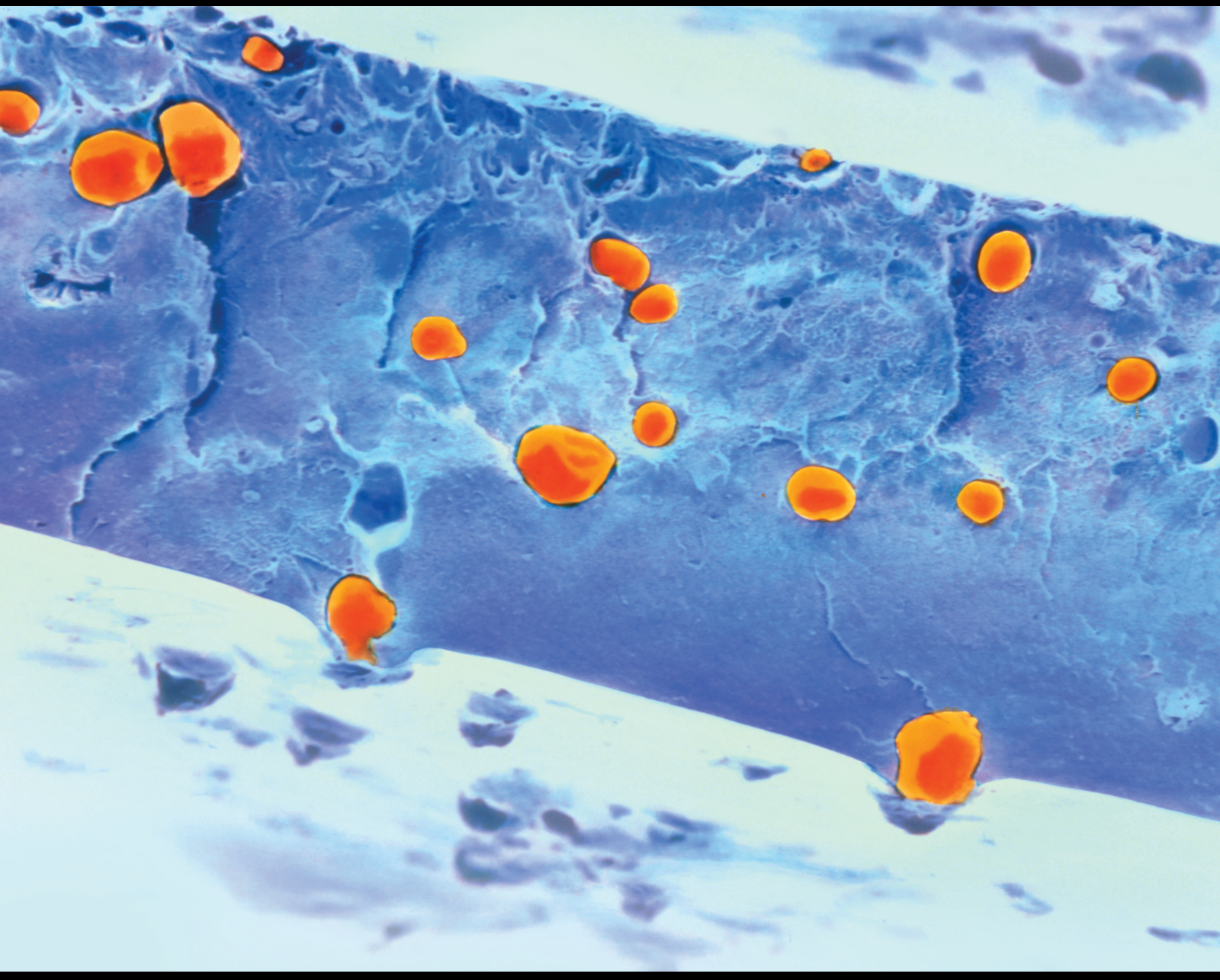
International Journal of Polymer Science

Synthesis and Biomedical Applications of Functional Polymers 2021

Lead Guest Editor: Jianxun Ding
Guest Editors: Di Li, Jingxiao Chen, Chao Zhao, and Parisa P. Abadi



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Research Article

The Effect of Angelica sinensis Polysaccharide on Neuronal Apoptosis in Cerebral Ischemia-Reperfusion Injury via PI3K/AKT Pathway

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In the present study, the protective effects and mechanism of Angelica sinensis polysaccharide (ASP) were investigated in rats with cerebral ischemia-reperfusion injury (CIRI). Rats were randomly divided into sham group, CIRI group, ASP treatment group, and ASP and LY294002 treatment group. H&E results confirmed the successful induction of CIRI in Sprague-Dawley rats. Compared with the sham group, the neurological function score, percentage of myocardial infarction area, neuronal apoptosis, oxidative stress, and inflammation in the CIRI group were significantly increased. Compared with the CIRI group, the ASP group's neurological function score, percentage of myocardial infarction area, neuronal apoptosis, oxidative stress, and inflammation were significantly reduced. However, compared with the ASP group, LY294002 inhibited the effect of ASP in CIRI rats. CIRI downregulated the PI3K/AKT pathway and upregulated the apoptosis level. And ASP activated the PI3K/AKT pathway and Bcl-2 protein expression, while it inhibited caspase-3 and Bax expression. LY294002 could significantly inhibit the protective effect of ASP on nerve injury and the expression and phosphorylation of PI3K and Akt protein in CIRI rats. ASP could effectively improve nerve function and nerve cell apoptosis of CIRI rats by activating the PI3K/AKT signaling pathway.

1. Introduction

Cerebral ischemia-reperfusion injury (CIRI) is a brain injury after ischemic stroke [1]. The most typical feature of CIRI is transient ischemia following reperfusion [2]. Neuronal apoptosis by CIRI causes damage to the hippocampus and cortical neurons [3]. Currently, thrombolytic therapy is the most common clinical treatment for CIRI [4]. However, perfusion increases the production of oxides from reactive proteins, causing intracellular DNA damage, oxidative stress-related injuries, protein oxidation, lipid peroxidation, and thus further worsening the blood-brain barrier and edema [5]. Hence, it is necessary to look for new treatment strategies for post-CIRI.

Traditional Chinese medicine is a rich source of biologically active substances and can be used to prevent or treat various human diseases. In the past few years, it has been proved that the extraction of polysaccharides from traditional Chinese medicine is beneficial to pharmacological activity with low toxicity [6]. *Angelica* has been used as Chinese herbal medicine and functional food in many Asian countries [7]. A water-soluble polysaccharide was isolated from the dried roots of *Angelica sinensis* and named *Angelica sinensis* polysaccharide (ASP). ASP is one of the active ingredients of *Angelica sinensis*. It is composed of xylose, galactose, glucose, arabinose, fructose, and glucuronic acid [8, 9]. ASP has been proven to have various pharmacological effects, with gastrointestinal protective effects, immunomodulatory effects, hematopoietic antitumor activities, antiaging, antidamage, antioxidation, and anti-inflammatory activities [10, 11]. Studies have shown that ASP has a protective effect on cerebral ischemic brain injury. However, there is still a lack of corresponding research on the impact and specific mechanisms of CIRI [12].

The phosphoinositide 3-kinase/AKT (PI3K/AKT) signaling pathway is one of the critical members involved in the

process of brain tissue damage [13]. The PI3K/AKT signaling pathway plays an important regulatory role in a variety of diseases, and it is involved in nerve cell apoptosis, oxidative stress, and inflammation [14]. AKT inhibits apoptosis by regulating apoptosis-related protein cleaved-caspase-3 and apoptosis regulator Bcl-2/Bax after activation. LY294002 is a specific inhibitor of PI3K. The purpose of this paper is to study the effect of ASP on neuronal apoptosis induced by CIRI in rats and to explore the mechanism of the PI3K/AKT pathway in the protective effect of ASP.

2. Methods

2.1. Reagents. Angelica sinensis was purchased from Yutiancheng Pharmacy and collected from Min County, Gansu Province, and the collection time was mid-October 2019. LY294002 was purchased from Thermo Fisher Scientific (California, USA). SOD, MDA, IL-6, and TNF-α detection kits were purchased from Nanjing Jiancheng Biotechnology Company (Nanjing, China). The primary antibodies caspase-3, Bcl-2, and Bax were purchased from Cell Signaling Technology (Massachusetts, USA), and the primary antibodies PI3K, AKT, p-AKT, and GAPDH antibodies were purchased from Abcam (Cambridge, UK).

2.2. Experimental Protocols

2.2.1. Separation and Purification of ASP. The materials of *Angelica sinensis* were pulverized and sieved; then, 100 g powder was added into 800 mL distilled water. After heating and refluxing for boiling for 30 minutes, the obtained decoction was filtered, and the filtrate was lyophilized. The average yield of lyophilized powder was about 19%. The crude ASP was extracted using repeated freezing and thawing to remove protein. The purified ASP was obtained by ultrafiltration, dialysis (MWCO = 3.5 kD), gel filtration chromatography (Sephadex G-50), and lyophilization. Then, the purified ASP was analyzed by high-performance liquid chromatography (HPLC). 20 μL ASP was injected into a Waters Ultrahydrogel™ Linear column (7.8 mm × 300 mm) (Massachusetts, USA). The detector was Waters 2410 refractive index detector. The elution was performed with 0.1 M $NaNO_3$ at a flow rate of 0.9 mL/min.

2.2.2. Preparation of Rat Model of Cerebral Ischemia Reperfusion (CIRI). Sprague-Dawley (SD) male healthy rats (280 ± 30 g body weight) were obtained from Zhejiang University of Traditional Chinese Medicine. Rats are given standard feeding conditions, and an adaptive feeding for one week. This experiment was approved by the Ethics Committee of Zhejiang Rehabilitation Medical Center. The rat CIRI model was established referring to the method of Li et al. [15]: (1) anesthesia: 3% sodium pentobarbital (40 mg/kg) for intraperitoneal injection; (2) preoperative preparation: fixed the rat in the supine position, and prepared skin for disinfection; (3) separate blood vessels and thread: exposed the right common carotid artery, neck external artery, and internal carotid artery; (4) ligation; (5) insert the slit; (6) sewing leather; and (7) postoperative: raised the head slightly in prone position in the suitable temperature and humidity. In the sham group, except that the nylon fishing line was not inserted to occlude the middle cerebral artery, the rest of the steps were the same as the model group.

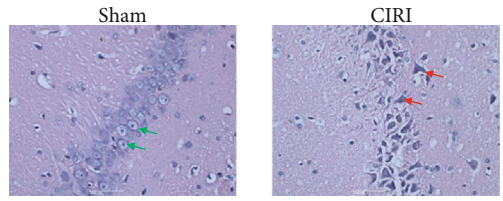

Figure 1: Histopathological changes in hippocampus tissues of rats in the sham and CIRI group were observed by H&E staining. Green arrows point to living cells; red arrows point to dead cells. Magnification ×200.

Hematoxylin-eosin (H&E) staining was used to observe histopathological changes. The neurons in the sham group were regular in shape and neatly arranged, with specific gaps and integrity; the pyramidal neurons in CIRI rats were scattered, the cell bodies were swollen and vacuolated, the nuclei were contracted, and the overall alignment was disordered, as shown in Figure 1. We further confirmed the success of the CIRI rat model.

2.2.3. Grouping and Administration. The rats were randomly divided into sham operation groups, CIRI group, ASP group (intraperitoneal injection of 10 mg/kg ASP solution in CIRI rats), and ASP+LY group (intraperitoneal injection of 10 mg/kg ASP and 0.3 mg/kg LY294002 in CIRI rats), with 10 rats in each group. It is administered once a day for two consecutive weeks.

2.2.4. Neurological Deficit Score. This neurological deficit assessment is a 5-point scale. 0 point: no nerve function damage; 1 point: left forelimb extension disorder; 2 points: circling leftward when walking; 3 points: left leaning when walking; 4 points: coma; and 5 points: death.

2.2.5. Detection of Percentage of Cerebral Infarction Area. The rats in each group were quickly sacrificed, and their brains were removed and sectioned into 2 mm coronal slices. The brain slices were suspended in 1% 2,3,5-triphenyltetrazolium chloride (TTC) and incubated at 37°C in the dark for 30 minutes. Mitochondrial dehydrogenases oxidize TTC, making living tissues appear dark red and necrotic tissues appear pale. Use ImageJ software to calculate the percentage of cerebral infarction area as the following formula: total pale area/total brain area × 100%.

2.2.6. Apoptosis Detection. Annexin V-PI staining was used to detect neuronal apoptosis. The brain tissue of each group was ground into a cell suspension, centrifuged at 800 rpm at 4°C for 5 min, and the supernatant was discarded. Wash the cells twice with precooled PBS, and centrifuge at 800 rpm at 4°C for 5 minutes each time. Discard the supernatant, add 500 μL of Annexin V binding buffer, and mix by pipetting. Add 5 μL of Annexin V-FITC and 10 μL of propidium iodide (PI), mix gently, and incubate for 15-20 min in the dark at room temperature. Flow cytometry was used to detect and analyze the percentage of apoptosis of the cells.

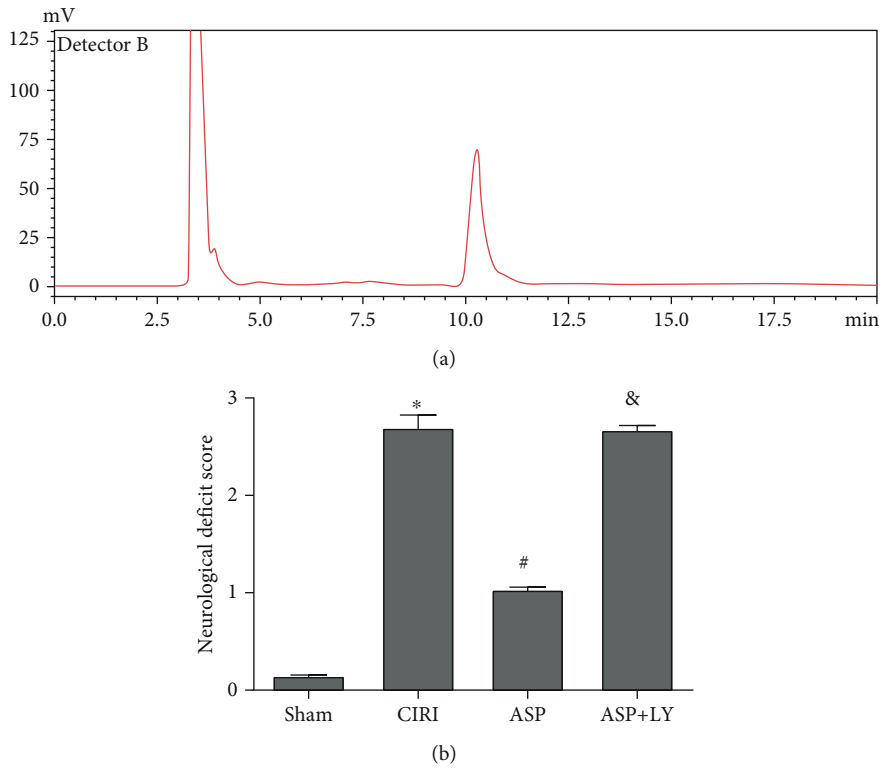

Figure 2: Effect of ASP treatment on rat neurological deficit after CIRI. (a) HPLC profile of ASP. (b) ASP significantly reduces the neurological deficit score of CIRI rats. Note: compared with the sham group, $^{*}P < 0.05$; compared with the CIRI group, $^{\#}P < 0.05$; compared with the ASP group, $^{\&}P < 0.05$. $N = 10$ per group.

2.2.7. ELISA Detection of SOD, MDA, IL-8, and TNF-α Level. The rat brain homogenate was centrifuged at 3500 rpm for 10 min to make a supernatant. The SOD, MDA, IL-8, and TNF-α level was determined by ELISA according to the manufacturer's instructions. The absorbance value of the sample was converted to the concentration according to the standard curve.

2.2.8. Western Blot Analysis. The rat brain was made into homogenate and added with cell lysate to extract total protein. The protein concentration was determined using a BCA kit (Beyotime, Shanghai, China). The quantified protein was loaded, and the protein was separated by SDS-PAGE, and the membrane was electrotransferred to the PVDF membrane. The protein was sealed by 5% skim milk at room temperature for two hours. The membrane was washed and incubated with the primary antibody overnight at 4°C. The primary antibodies used in this study were caspase-3 (1 : 1500), Bcl-2 (1 : 1000), Bax (1 : 1000), PI3K (1 : 1500), Akt (1 : 2000), p-Akt (1 : 2000), and GAPDH (1 : 800). On the next day, the membrane was washed and incubated with the secondary antibodies at room temperature for 2 hours. After washing the membrane, the membrane was developed, imaged, and quantitatively analyzed by Image Lab 3.0 software. GAPDH was used as an internal reference.

2.2.9. Statistical Analysis. SPSS 17.0 software was used to analyze data, and the results were expressed as mean ± standard deviation. The statistical significance among groups was analyzed using one-way ANOVA. The Student t-test was used to compare the difference between the 2 groups. A significance was considered at $P < 0.05$.

3. Results

3.1. The Effects of ASP on Neuronal Functions in CIRI Rats. After the ASP was purified, it was analyzed by HPLC. And a single peak was detected, suggesting that purified ASP was a homogeneous polysaccharide (Figure 2(a)). The neurological deficit score was detected in each rat. As shown in Figure 2(b), compared to the sham group, the scores of neurological deficit in the CIRI group increased significantly ($P < 0.05$). The scores of neurological deficit in the ASP group were significantly lower than those in the CIRI group ($P < 0.05$). Compared to the ASP group, the neurological deficit scores of rats in the ASP+LY group were upregulated significantly ($P < 0.05$).

3.2. Effect of ASP on Percentage of Cerebral Infarction Area in CIRI Rats. TTC staining was used to analyze the cerebral infarct of the rats in each group. The results are shown in Figure 3. Compared to the sham group, the cerebral infarction area percentage in the CIRI group increased significantly ($P < 0.05$). Compared to the CIRI group, the cerebral infarct decreased significantly in the ASP group ($P < 0.05$). Compared to the ASP group, the cerebral infarct in the ASP+LY group increased ($P < 0.05$).

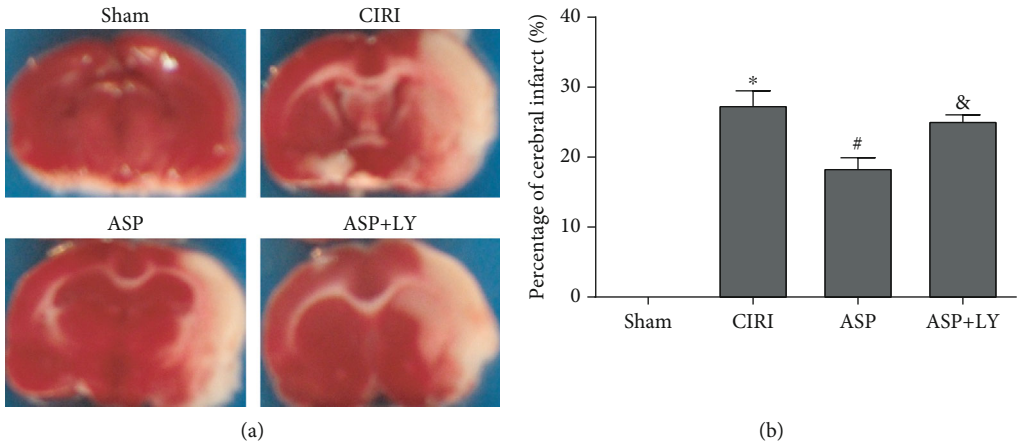

Figure 3: Effect of ASP on cerebral infarct of CIRI rats. (a) Cerebral infarction in SD rats and the represented image of TTC staining. (b) The percentage of cerebral infarction area was quantified and analyzed. Note: compared with the sham group, $^{*}P < 0.05$; compared with the CIRI group, $^{\#}P < 0.05$; compared with the ASP group, $^{\&}P < 0.05$. $N = 10$ per group.

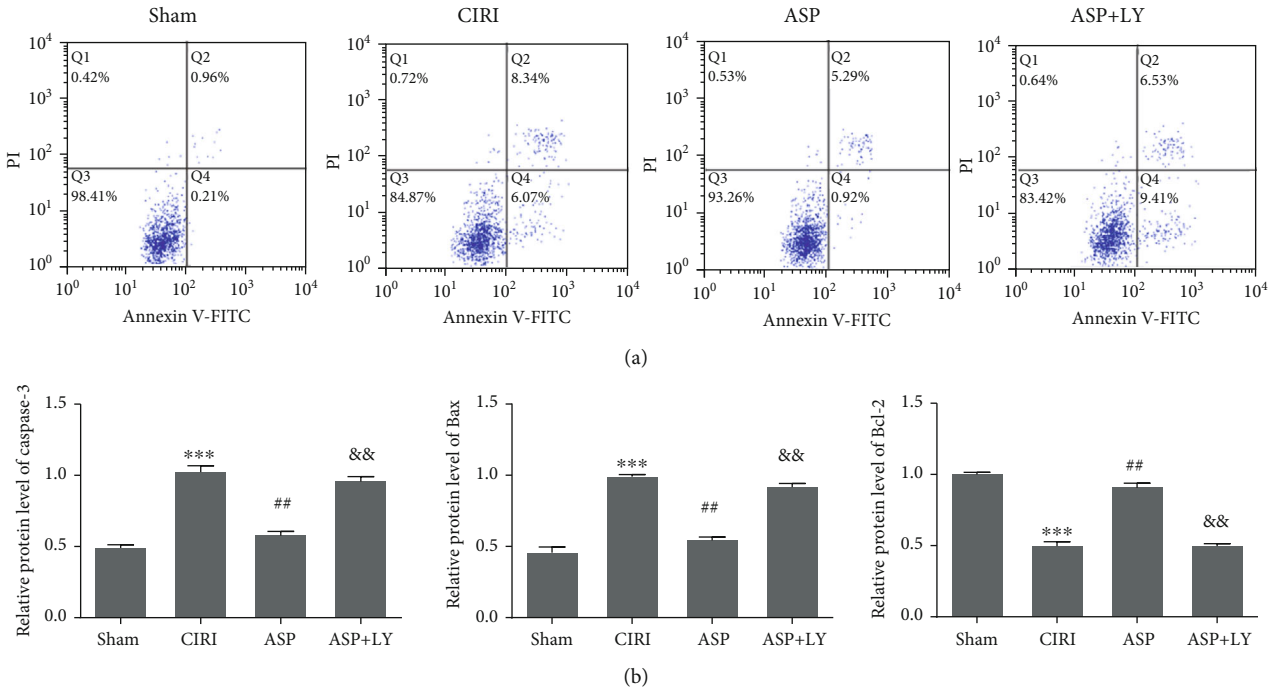

Figure 4: The effect of ASP on cell apoptosis of CIRI rats. (a) Flow cytometry analysis of the apoptosis of nerve cells in the brain tissues of rats in different groups. (b) Western blotting assay for the apoptosis related-proteins expression of caspase-3, Bax, and Bcl-2. Note: compared with the sham group, $^{***}P < 0.001$; compared with the CIRI group, $^{\#\#}P < 0.01$; compared with the ASP group, $^{\&\&}P < 0.01$. $N = 10$ per group.

3.3. Effects of ASP on Neuron Apoptosis Induced by CIRI in Rats. Annexin V-PI flow cytometry was used to detect neuronal apoptosis in the rat brain. As shown in Figure 4(a), compared with the sham group, the apoptosis of neurons in the CIRI group increased significantly ($P < 0.05$). Compared to the CIRI group, neuronal apoptosis decreased significantly in the ASP group ($P < 0.05$). Compared to the ASP group, the apoptosis of nerve cells in the ASP+LY group increased ($P < 0.05$).

Further, apoptosis-related protein expression was detected by western blotting. As shown in Figures 4(b), compared with the sham group, the caspase-3 and Bax expression levels of the rat brain in the CIRI group increased significantly, and Bcl-2 protein expression decreased significantly ($P < 0.001$). Compared to the CIRI group, the caspase-3 and Bax expression levels of brain tissue in the ASP group decreased significantly, while Bcl-2 protein expression significantly increased ($P < 0.01$). Compared to the ASP group, the caspase-3 and Bax expression levels of brain tissue in ASP+LY groups were increased, and Bcl-2 protein expression was decreased ($P < 0.01$). It was suggested that ASP might protect nerve cell from damage and inhibit nerve cell apoptosis.

3.4. Effects of ASP on Oxidative Stress and Inflammatory Factors in CIRI Rats. Cerebral ischemia usually leads to oxidative stress and inflammation. Next, we analyze SOD, MDA, IL-6, and TNF-α levels. As shown in Figure 5,

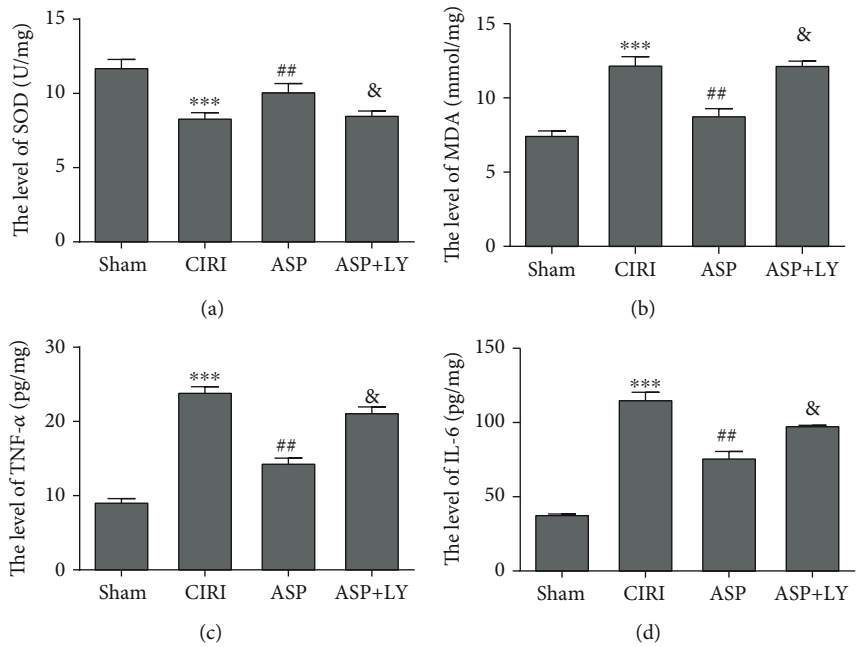

Figure 5: The effect of ASP on the levels of oxidative stress and inflammatory cytokines in rats with cerebral ischemia reperfusion. (a) The levels of SOD. (b) The levels of MDA. (c) The levels of TNF-α. (d) The level of IL-6. Note: compared with the sham group, $^{***}P < 0.001$; compared with the CIRI group, $^{\#\#}P < 0.01$; compared with the ASP group, $^{\&}P < 0.05$. $N = 10$ per group.

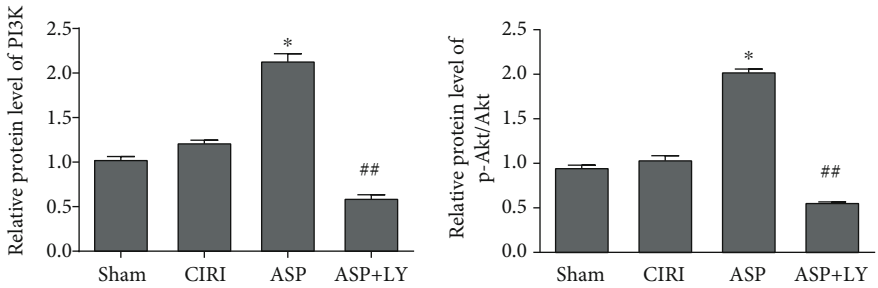

Figure 6: ASP activated PI3K/AKT signaling pathways. PI3K, AKT, and p-AKT protein levels in CIRI rats measured by western blot analysis. Note: compared with the CIRI group, $^{*}P < 0.05$; compared with the ASP group, $^{\#\#}P < 0.01$. $N = 10$ per group.

compared to the sham group, SOD activity decreased significantly in the CIRI group ($P < 0.001$), while MDA, IL-6, and TNF-α levels increased considerably ($P < 0.001$). Compared to the CIRI group, SOD activity had a significant increase in the ASP group ($P < 0.01$), while there was a significant decrease of MDA, IL-6, and TNF-α levels in the ASP group ($P < 0.01$). Compared to the ASP group, SOD activity decreased in the ASP+LY group ($P < 0.05$), whereas MDA, IL-6, and TNF-α levels increased ($P < 0.05$). Therefore, oxidative stress and inflammation occurred after CIRI. ASP had the function of resisting oxidative stress and reducing the expression of inflammatory cytokines.

3.5. ASP Activated the PI3K/AKT Signaling Pathway in CIRI Rats. By western blotting assay, the expression of critical proteins in the PI3K/AKT signaling pathway was detected. As shown in Figure 6, the expression level of PI3K and the ratio of p-AKT/AKT in the ASP group increased significantly in CIRI rats ($P < 0.05$) compared to the CIRI group. Compared to the ASP group, PI3K level and the ratio of p-AKT/AKT expression levels decreased ($P < 0.01$) in the ASP+LY group. The results showed that LY294002 intervention significantly inhibited the effect of ASP on the expression and phosphorylation of PI3K and AKT protein in rats after CIRI. ASP may affect neuronal cells in CIRI rats by activating the PI3K/AKT signaling pathway.

4. Discussion

Stroke is one of the major diseases with the highest mortality and disability rates [16]. It is classified as ischemic stroke and hemorrhagic stroke. Ischemic stroke is caused by a sudden occlusion of cerebral vessels, accounting for about 70% of all strokes [17]. Early and timely reperfusion is the most effective method to limit infarction and improve the clinical prognosis. However, it can also cause harmful effects such as secondary contamination and neuronal loss. Secondary neuronal loss is one of the most critical factors affecting neural function. Neural cells are considered to be the basis of the central nervous system, and the loss of nerve cells is one of

the golden clinical predictors of long-term prognosis. Neuronal loss caused by CIRI is a complex pathological process [18] such as energy failure, neuroinflammation, neuronal apoptosis, oxidative stress, and calcium overload. In this study, it was shown that CIRI rats suffered the pathological symptoms and characteristics, including deteriorated neuronal deficit and cerebral infarction, induced nerve cell apoptosis, inflammation, and oxidative stress. After ASP treatment, it was found that ASP has a protective effect on the nerve damage in CIRI rats and improves the nerve function and cerebral infarct of CIRI rats. The results were partly similar to the previous reports, which confirmed the disease protection of ASP. It has been reported that ASP has a protective effect on acute liver injury in mice induced by concanavalin A or acetaminophen [19]. ASP also has a certain protective effect in colitis [20] and myocardial cells [21]. This study confirmed the protective effect of ASP from CIRI and expanded the pharmacological action of ASP.

Nerve cell apoptosis is the primary manifestation after CIRI. Many studies have shown that the process of apoptosis plays an essential role in CIRI [22]. It was detected that the expression of antiapoptotic proteins Bcl-2 and Bcl-xl was downregulated. In contrast, apoptotic proteins such as cleaved-caspase-3, Bax, and cytochrome c were upregulated after reperfusion [23–25]. The peak period of cell apoptosis is about 24-48 hours after transient ischemia [26]. There is also much evidence that blocking the process of cell apoptosis after cerebral ischemia-reperfusion has a neuroprotective effect. Overexpression of antiapoptotic proteins, such as Bcl-2 and Bcl-xl, or knocking out Bid, caspases, and other proteins with proapoptotic genes can lead to smaller infarctions [27]. Many potential targets or drugs for neuroprotection have been reported through their effects on the process of apoptosis [28]. In this study, it was found that ASP could effectively inhibit neuronal cell apoptosis after CIRI, inhibit the expression levels of caspase-3 and Bax, and increase the expression of Bcl-2 protein to protect neuronal cell damage. The antiapoptosis effect of ASP has also been demonstrated in other diseases. ASP inhibits the oxidative stress-induced chondrocyte apoptosis [29]. In acute liver injury, ASP suppresses the hepatic apoptosis *in vivo* and *in vitro* [30].

Ischemic stroke involves the interaction of many pathophysiological processes. Neuroinflammation and oxidative stress play an essential role in the pathological process [31, 32]. Neuroinflammation is an important marker of the pathology of ischemic stroke, which involves a series of inflammatory responses. Proinflammatory cytokines, TNF-α and IL-6, increased significantly during cerebral ischemia and stroke [33, 34]. In this study, the experimental results showed that after CIRI, the SOD activity decreased significantly. The MDA, IL-6, and TNF-α levels increased significantly, and oxidative stress and neuroinflammation occurred, while in the ASP group, the results showed a significant increase in SOD activity and a significant decrease in MDA, IL-6, and TNF-α levels, indicating that ASP effectively improved oxidative stress and inflammatory response after CIRI. Antioxidant effect is one of the main characteristics of ASP. Several studies have confirmed that ASP play the role of *in vivo* antioxidant effect in liver injury [35], diabetes [36], colitis [37], and urolithiasis [38]. ASP also play the role of antioxidant to chondrocytes*in vitro* [29]. In addition, the previous studies confirmed that ASP has an anti-inflammatory effect, which is similar to the results in this study. In liver injury, ASP pretreatment significantly reduces levels of proinflammatory cytokines (TNF-α, INF-γ, IL-2, and IL-6) [39]. In chronic kidney disease, ASP even inhibits the inflammatory NF-κB signaling pathway [40].

The PI3K/AKT pathway is one of the main signaling pathways affecting apoptosis. The PI3K/Akt pathway plays an important role in regulating cell proliferation, growth, survival, and angiogenesis [41]. Activation of PI3K/Akt pathways has been shown to reduce inflammatory genes and apoptotic protein [42]. In this present study, it was found that ASP activated the PI3K/AKT signaling pathway and exerted the protective effects on rats CIRI against neuronal damage, cerebral infarct, apoptosis oxidative stress, and inflammatory response. Then, the effect of ASP was intervened by LY294002, which was a specific inhibitor of PI3K. The results in this study showed that LY294002 inhibit the signaling pathway induced by ASP, such as PI3K and p-AKT protein expression, and destroyed the protective effect of ASP. This study revealed that ASP may have a protective effect on neuronal cells in CIRI rats by activating the PI3K/Akt signaling pathway.

It was found that ASP could improve the neurobiological function and cerebral infarct of CIRI rats. ASP increased SOD activity; decreased the levels of MDA, IL-6, and TNF-α; and significantly alleviated the apoptosis of nerve cells by activating the PI3K/AKT pathway. However, natural products may have multiple target therapeutic mechanisms and complex signal pathways. The finding of this study is not enough to explain the mechanism on CIRI by ASP in-depth, but the finding suggests that the reasonable combination of ASP and chemical drugs may provide a new thinking for the study of CIRI therapy. In summary, ASP may have a particular therapeutic effect on CIRI rats, providing a theoretical basis for clinical CIRI treatment.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Acknowledgments

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Research Article

Effects of Rhizopus Nigricans Exopolysaccharide on Proliferation, Apoptosis, and Migration of Breast Cancer MCF-7 Cells and Akt Signaling Pathway

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Objective. To study the effect of Rhizopus nigricans exopolysaccharide EPS1-1 on the proliferation, apoptosis, and migration of breast cancer MCF-7 cells. *Methods*. Human breast cancer MCF-7 cells were cultured in vitro and treated with different concentrations of EPS1-1. The effect of EPS1-1 on cell proliferation was tested by the CCK-8 experiment, and the effect of EPS1-1 on cell apoptosis was determined by flow cytometry. And the scratch test was used to detect the impact of EPS1-1 on cell migration. Western blot then was used to measure the expression changes of related proteins in the Akt signaling pathway. *Results*. Compared with the control group, treatment with EPS1-1 significantly reduced the proliferation, migration, and invasion ability of MCF-7 cells and promoted the apoptosis of MCF-7 cells in a dose-dependent manner. In terms of the underlying mechanism, EPS1-1 can significantly inhibit the phosphorylation of Akt at threonine 308 and serine 473 and cause the expression changes of downstream proliferation-related genes CCND1 and p21, apoptosis-related genes Bcl-2 and Bax, and migration-related genes Vimentin and E-cadherin in terms of their protein levels. *Conclusion*. EPS1-1 can inhibit the proliferation, migration, and invasion of breast cancer MCF-7 cells and promote the apoptosis of MCF-7 cells by inhibiting the activation of the Akt signaling pathway. Therefore, EPS1-1 can be used as a potential new drug or adjuvant drug for the treatment of breast cancer.

1. Introduction

Breast cancer (BC) is one of the most common and the leading cause of cancer-related death among women around the world [1]. Every year, a large number of cancer-related deaths are caused by BC [2]. Complex etiology including genetic factors and environmental factors, such as late age at birth, exogenous hormone intake, smoking, drinking and obesity, leads to the heterogeneity of BC [3]. Although advanced strategies including surgery, chemotherapy, radiotherapy, hormone therapy, and molecular targeted therapy have been widely used in BC treatment recent years, the prognosis of BC patients is still very poor [4]. Therefore, the development of new effective therapeutic drugs is a problem that needs to be solved urgently.

Biologically active polysaccharides have been widely studied and applied in the food and pharmaceutical industries due to their therapeutic properties and small side effects. The antitumor activity [5], immunomodulatory activity [6], and antibacterial activity [7] of polysaccharides have been well studied and applied in the fields of biochemistry and medicine. Yu et al. [8] extracted an extracellular polysaccharide, namely, EPS1-1 from the fermentation broth of Rhizopus nigricans. Studies have shown that EPS1-1 could significantly inhibit the tumor growth and increase the immune organs index of CT26 tumor-bearing mice. Also, EPS treatment

increased the productions of interleukin-2 (IL-2) and tumor necrosis factor-α (TNF-α) levels in serum, as well as increased the percentage of CD8(+) cytotoxic T cells among total spleen T lymphocyte [9]. EPS1-1 can also present antitumor activity and prolong the survival period of S180 tumor bearing mice [10]. Moreover, EPS1-1 can significantly enhance the body immunity through cellular immunity and humoral immunity by increasing the activities of phagocytosis and acid phosphatase and the production of NO, IL-2, and TNF-α [11]. These results indicate that EPS1-1 may become a new drug or adjuvant drug for tumor chemotherapy.

In this study, we extracted EPS1-1 from the fermentation broth of Rhizopus nigricans according to Yu [8] and studied its effect on the proliferation, apoptosis, and migration of breast cancer cell MCF-7 and the corresponding mechanism. Therefore, this study provides a theoretical basis for the clinical application of EPS1-1 as a new drug or auxiliary drug for the treatment of breast cancer.

2. Materials and Methods

2.1. Extraction and Identification of EPS1-1. The extraction of EPS1-1 followed the method reported by Yu et al. [8]. According to the results of high-performance liquid chromatography, EPS1-1 is composed of GLC, MAN, GAL, and FRU, with a polarity ratio of 5.89 : 3.64 : 3.20 : 1.00.

2.2. Cell Culture and Processing. The human breast cancer cell MCF-7 cell line and normal human breast epithelial cells (MCF-10A) were purchased from ATCC (United States) and cultured in DMEM medium containing 10% fetal bovine serum at 37°C and with 5% CO_2. The cells were then treated with the different concentration of EPS1-1 control (0 μg/mL) and treatment groups (including 200 μg/mL, 400 μg/mL, 600 μg/mL, 800 μg/mL, and 1000 μg/mL in each treatment group).

2.3. CCK-8 Cell Proliferation Experiment. The CCK8 kit was purchased from Dojindo, Japan. MCF-7 cells were seeded in a 96-well plate with about 8,000 cells per well. After culturing in an incubator for 24 h, the supernatant was removed, and the culture medium containing different concentrations of EPS1-1 was then added. After incubating for 24, 48, and 72 h, 10ul CCK8 reagent was then added to each well, and the cells were then continuously incubated in a 37°C incubator for an appropriate period. After that, a microplate reader was used to measure the absorbance at 450 nm.

2.4. Colony Formation Assay. Cells were collected, counted, and seeded in a 6 cm culture plate. After 2 weeks of conventional culture, the cells were fixed with methanol at room temperature for 15 minutes and then stained with 1% crystal violet solution for 10 minutes to calculate the number of colonies.

2.5. Cell Scratch Test. The MCF-7 cells were seeded in a 6-well plate and cultured in an incubator until the cell fusion rate reaches 95%. A pipette tip was used to draw a straight line with a width of 1 mm on the fused monolayer of cells in each well. The cells were washed 3 times with PBS, and a culture medium containing different concentrations of EPS1-1 was then added. After 0, 48, and 72 h of culture, the scratched area was photographed, and the cell migration rate was calculated.

2.6. Apoptosis Measured by Flow Cytometry. The MCF-7 cells were seeded in a 6-well plate and cultured for 24 h, then culture medium containing different concentrations of EPS1-1 was added, and cells were continuously cultured for 48 and 72 h. A single-cell suspension after digestion with trypsin was then prepared, and according to the instructions, Annexin V-FITC/PI Apoptosis Kit (Sigma, USA) and flow cytometry were then used to detect the apoptosis rate.

2.7. Transwell Assay. The Transwell test was performed on the 8.0 μm Transwell plate with matrix gel (Corning, USA). The cells were resuspended in 200 μL serum-free DMEM medium and added to the upper chamber of Transwell. 600 μL DMEM medium containing 10% FBS was added to the lower chamber. After 24 hours of culture, the submembranous cells were fixed with methanol at room temperature for 10 minutes and stained with 0.1% crystal violet solution for 15 minutes to calculate cell number.

2.8. Western Blot. The MCF-7 cells were seeded in a 6-well plate and cultured for 24 h, and then, a culture medium containing different concentrations of EPS1-1 was added, and cells were continuously cultured for 72 h. The protein was extracted with RIPA lysis buffer, and the protein concentration was determined by the BCA method. 20 μg protein sample was added to each well for electrophoresis, followed by transferring the protein to the membrane, blockage of the membrane, and incubations with primary and secondary antibodies before the development of the image. All the antibodies used in this experiment were purchased from Abcam, USA.

2.9. Statistical Analysis. SPSS 19.0 statistical software was used for data analysis. The results were expressed as mean ± standard deviation ($x \pm s$). The difference between the groups was analyzed with Student's t-test or one-way analysis of variance. Each experiment was repeated at least three times, and $P < 0.05$ indicated that the difference was statistically significant.

3. Results

3.1. The Effect of EPS1-1 on the Proliferation of MCF-7 Cells. As shown in Figure 1(a), compared with the control group, EPS1-1 significantly reduced the proliferation activity of MCF-7 cells in a dose-dependent manner. The inhibitory effect at 48 h and 72 h was statistically different (IC50 = 387.1 and 357.2, respectively), and when the concentration was greater than 600 μg/mL, the proliferation activity of MCF-7 cells did not decrease significantly. Therefore, 48 h and 72 h were used as the treatment time in the follow-up test, and the concentration of EPS1-1 was ranged from 0 to 600 μg/mL. We also found that when the concentration was less than 600 μg/mL, EPS1-1 showed no significant toxicity to MCF-10A cells (Figure 1(b)). As shown in Figure 1(c), compared with the control group, EPS1-1 also significantly

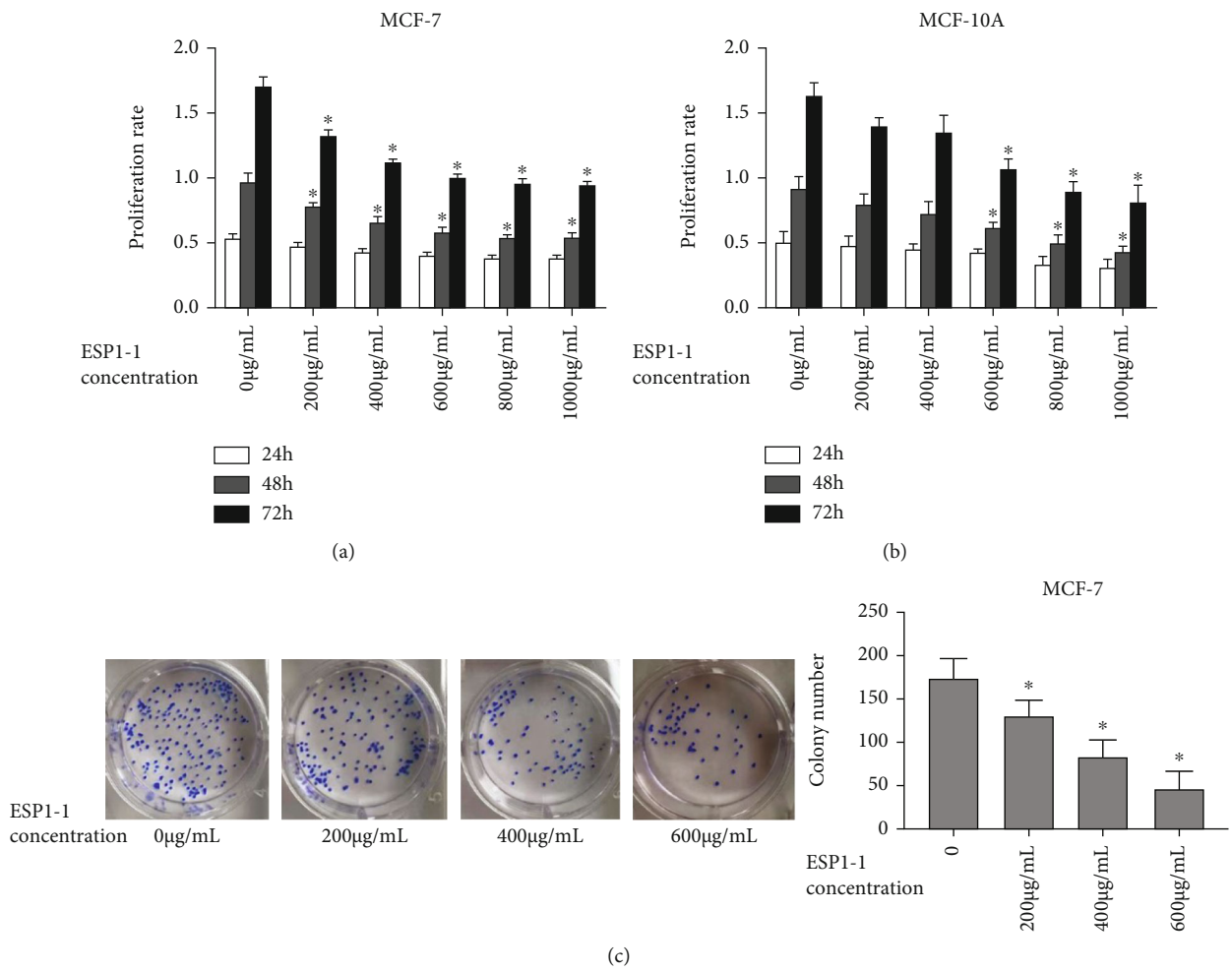

Figure 1: The effect of EPS1-1 on the proliferation activity of MCF-7 cells, $N = 3$, $^{*}P < 0.05$.

reduced the cell colony formation activity of MCF-7 cells in a dose-dependent manner.

3.2. The Effect of EPS1-1 on the Apoptosis of MCF-7 Cells. As shown in Figure 2, compared with the control group, after EPS1-1 treatment for 48 h and 72 h, the apoptotic ratio of MCF-7 cells increased significantly in a dose-dependent manner ($P < 0.05$). The above results indicate that EPS1-1 can promote the apoptosis of MCF-7 cells.

3.3. Effect of EPS1-1 on the Migration of MCF-7 Cells. As shown in Figure 3, compared with the control group, after 48 h and 72 h of EPS1-1 treatment, the closure ratio of MCF-7 cell scratches decreased significantly and showed a dose-dependent manner ($P < 0.05$), therefore indicating that EPS1-1 can inhibit the migration of MCF-7 cells.

3.4. Effect of EPS1-1 on the Invasion of MCF-7 Cells. As shown in Figure 4, compared with the control group, after 48 h of EPS1-1 treatment, the invasion ability of MCF-7 cell decreased significantly and showed a dose-dependent manner ($P < 0.05$), therefore indicating that EPS1-1 can inhibit the invasion of MCF-7 cells.

3.5. The Impact of EPS1-1 on Akt Signal Pathway. After 72 h of treatment of MCF-7 cells with EPS1-1, we found that, compared with the control group, EPS1-1 can significantly inhibit the phosphorylation of Akt at threonine (Thr) 308 and serine (Ser) 473 (see Figure 4). We also detected the protein expression involved in the downstream of Akt signaling pathway including proliferation-related genes CCND1 and p21, apoptosis-related genes Bcl-2 and Bax, and migration-related genes Vimentin and E-cadherin and found that the expression levels of these genes were affected by the treatments of EPS1-1 (see Figure 5).

4. Discussion

Natural polysaccharides have attracted widespread attention due to their medicinal value, especially in the treatment of cancer. Many polysaccharides in plants, fungi, algae, and bacteria can inhibit the occurrence and development of cancer, such as liver cancer, breast cancer, lung cancer, and colon cancer. In this study, we extracted EPS1-1 from the fermentation broth of Rhizopus nigricans and studied its action and mechanism in the inhibition of breast cancer cells.

Functional assays showed that EPS1-1 can inhibit different perspective of the MCF-7 cells, specifically, we found that

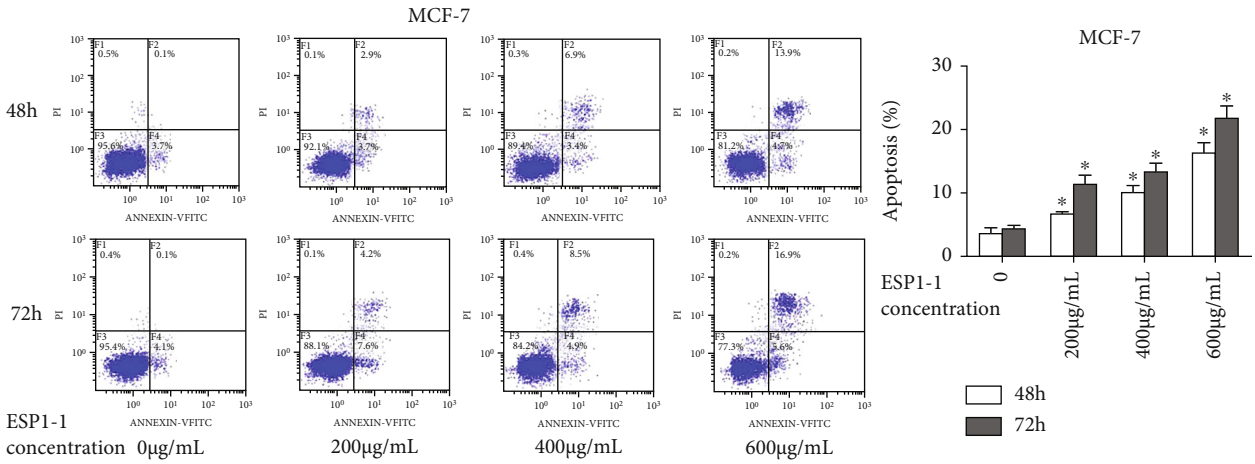

Figure 2: The effect of EPS1-1 on the apoptosis of MCF-7 cell, $N = 3$, $^{*}P < 0.05$.

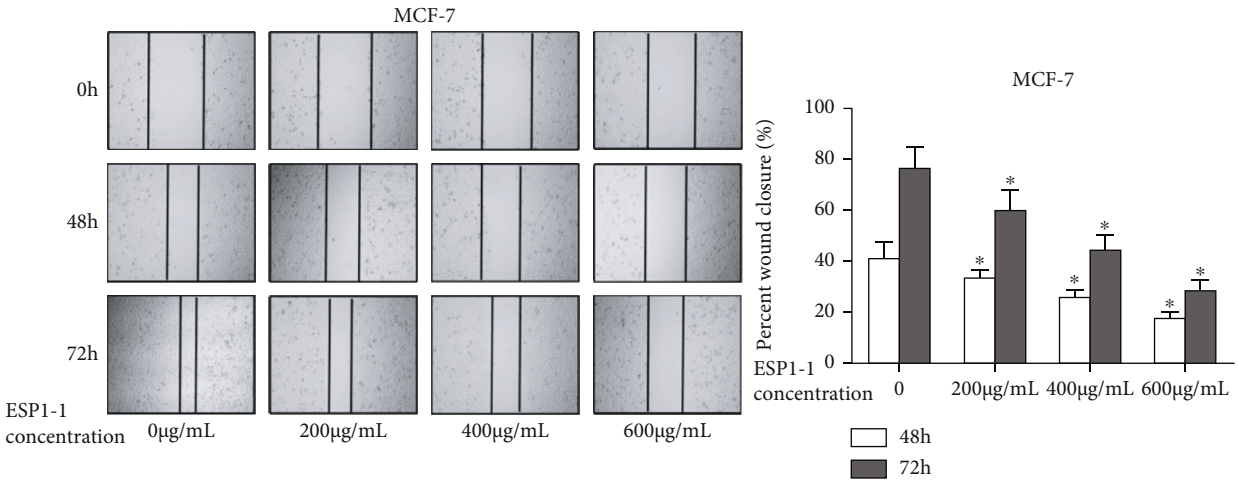

Figure 3: The effect of EPS1-1 on the migration of MCF-7 cells, $N = 3$, $^{*}P < 0.05$.

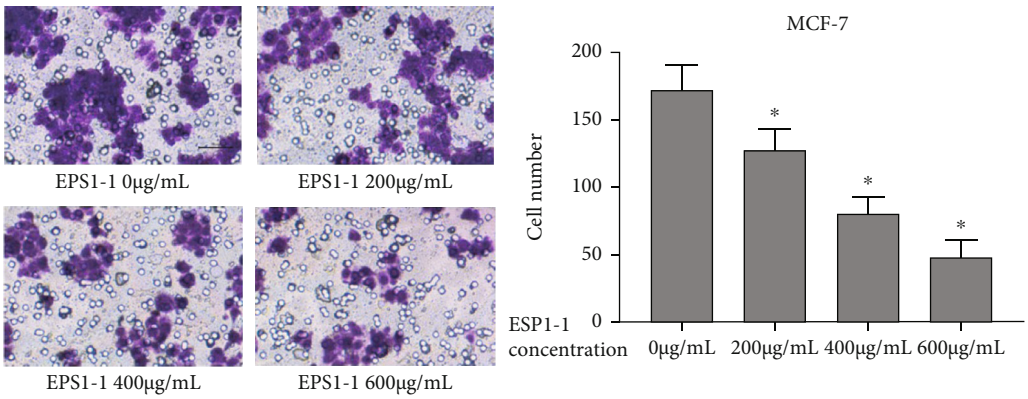

Figure 4: The effect of EPS1-1 on the invasion of MCF-7 cells, $N = 3$, $^{*}P < 0.05$.

EPS1-1 can inhibit the proliferation of MCF-7 cells through the CCK8 experiment and colony formation assay. Moreover, we showed that EPS1-1 can promote the apoptosis of MCF-7 cells via flow cytometry and inhibit MCF-7 cell migration and invasion via the scratch experiment and Transwell assay. Therefore, our results show that in breast cancer cells, EPS1-1 has obvious tumor suppressor activity, and has the potential to be used as a new drug or auxiliary

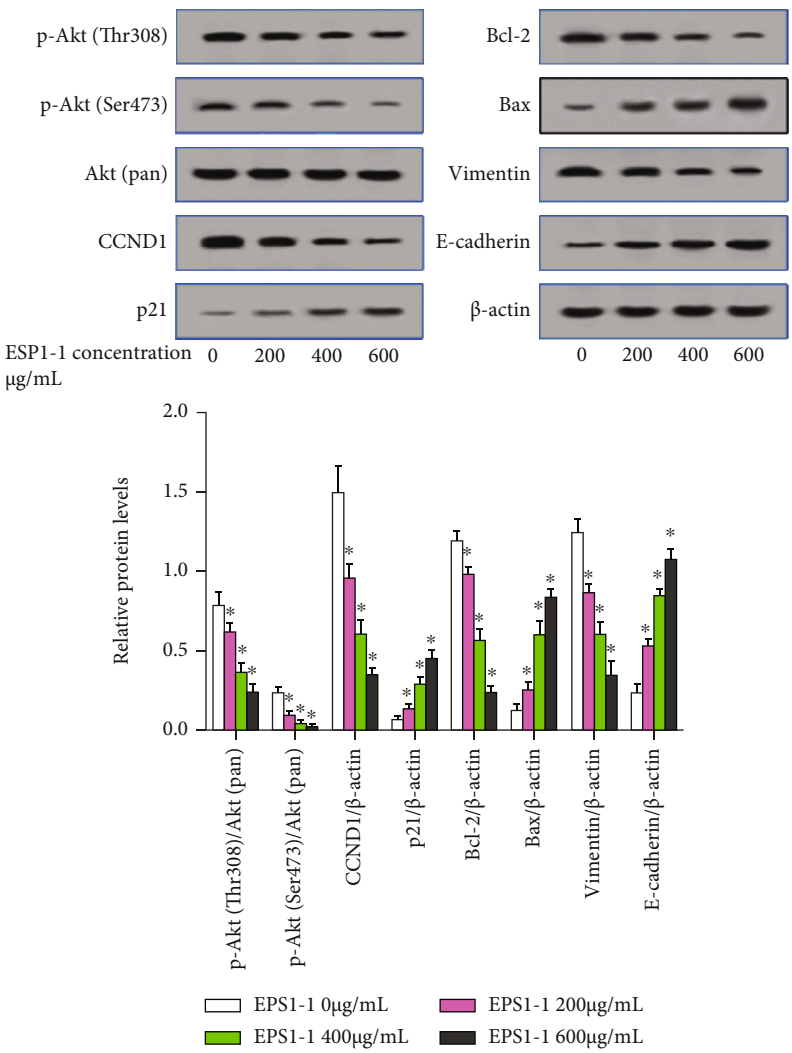

Figure 5: The effect of EPS1-1 on Akt signaling pathway and its downstream genes, $N = 3$, $^{*}P < 0.05$.

drug in the treatment of breast cancers. Therefore, the anticancer role of EPS1-1 in breast cancer is the same as that in other tumors previously reported [8–13].

Previous studies have investigated the mechanisms of EPS1-1 in anticancer application. On the one side, EPS1-1 may increase the productions of interleukin-2 (IL-2) and tumor necrosis factor-α (TNF-α) levels in serum, as well as increased the percentage of CD8(+) cytotoxic T cells among total spleen T lymphocyte [9]. In addition, EPS1-1 can significantly enhance the body immunity through cellular immunity and humoral immunity by increasing the activities of phagocytosis and acid phosphatase and the production of NO, IL-2, and TNF-α [11]. Herein, we found that EPS1-1 can inhibit the activation of the Akt signaling pathway. The serine/threonine kinase Akt (also known as protein kinase B or PKB) is a protooncogene that plays an important role in regulating various cell functions including metabolism, proliferation, apoptosis, migration, and protein synthesis. Phosphorylation of threonine 308 of Akt can cause partial activation of Akt, and serine 473 phosphorylation can stimulate the complete enzymatic activity of Akt [14]. We found that EPS1-1 can inhibit the phosphorylation of Akt at these two sites. CCND1 [15] and p21 [16] are factors related to cell proliferation downstream of Akt. We found that after EPS1-1 treatment, the expression of CCND1 was significantly reduced, while the expression of p21 was significantly increased. CCND1 can promote cell proliferation, while p21 inhibits cell proliferation, the contrasting responses of these two genes upon the EPS1-1 treatment explains the inhibitory effect of EPS1-1 on MCF-7 cell proliferation. Bcl-2 and Bax are factors related to apoptosis downstream of Akt [17]. Our results show that after EPS1-1 treatment, the expression of Bcl-2 was significantly reduced, while the expression of Bax was significantly increased. Bcl-2 can inhibit cell apoptosis, while Bax promotes cell apoptosis, which explains the apoptosis-promoting effect of EPS1-1 on MCF-7 cells. Vimentin and E-cadherin are factors related to cell migration downstream of Akt [18]. We found that after EPS1-1 treatment, the expression of Vimentin was significantly reduced, while the expression of E-cadherin was significantly increased. Vimentin can promote cell migration, while E-cadherin inhibits cell migration, which explains the

inhibitory effect of EPS1-1 on MCF-7 cell migration. In summary, EPS1-1 inhibits the proliferation and migration of MCF-7 cells by inhibiting the activation of the Akt signaling pathway and promotes the apoptosis of MCF-7 cells, which explains the anticancer mechanism of EPS1-1 on the molecular level.

This study lacks animal experiments and clinical experiments and only explored the potential value and theoretical basis of EPS1-1 as a cancer suppressor, so it has certain limitations.

5. Conclusion

In this study, EPS1-1 was extracted from the fermentation broth of Rhizopus nigricans, and in vitro experiments confirmed that EPS1-1 can inhibit the proliferation, migration, and invasion of breast cancer MCF-7 cells by inhibiting the activation of the Akt signaling pathway and can promote the apoptosis of MCF-7 cells. Therefore, this study provides a theoretical basis for the clinical application of EPS1-1 as a new drug or auxiliary drug for the treatment of breast cancer.

Data Availability

All data generated or analyzed during this study are included in this published article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Momordica Charantia Polysaccharides Attenuates MPP$^+$-Induced Injury in Parkinson's Disease Mice and Cell Models by Regulating TLR4/MyD88/NF-κB Pathway

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Objective. To investigate the potential role of Momordica charantia polysaccharides (MCPs) in Parkinson's disease (PD) and reveal the molecular mechanism of its function. *Method*. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1-methyl-4-phenylpyridinium (1-methyl-4-phenylpyridinium, MPP$^+$) were used to establish PD mice and cell models. The mice and cells were divided into 4 groups: Control group, Control+MCPs group, PD group, and PD+MCPs group. Pole climbing experiment and Rotarod experiment were used to observe the coordination ability of mice. High-performance liquid chromatography and enzyme-linked immunosorbent assay (ELISA) were used to determine neurotransmitters and metabolites, inflammatory factors TNF-α and IL-1β, oxidative stress-related markers SOD, MDA, and GSH in striatum tissues. Western blot was used to determine the protein levels of tyrosine hydroxylase (TH), oxidative stress-related protein Cytochrome C (Cytochrome C), and apoptosis-related proteins Bcl-2, Bax, and cleaved Caspase-3 in tissues and cells. Moreover, flow cytometry, PI staining, and fluorescence were used to observe cell apoptosis. Finally, the activation effect of MCPs on TLR4/MyD88/NF-κB signaling pathway was observed and verified. *Results*. Compared with the Control group, MPTP treatment can induce brain damage in mice (all $P < 0.05$), change the metabolic state of neurotransmitters (all $P < 0.05$), induce inflammation (all $P < 0.05$), and induce apoptosis and the occurrence of oxidation reaction (all $P < 0.05$); however, MCPs treatment can significantly reverse the above changes (all $P < 0.05$). In cell models, studies have found that MCPs can play a protective role by regulating the activation state of TLR4/MyD88/NF-κB pathway. *Conclusion*. This study found that the application of MCPs therapy can play anti-inflammatory, antioxidative stress, and antiapoptotic effects in PD by regulating the activation of the TLR4/MyD88/NF-κB pathway.

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disease second only to Alzheimer's disease [1, 2]. Nowadays, the incidence of PD in my country is rising rapidly with age [3], and it has become one of the main threats to the elderly. Although the etiology of PD is not yet clear, it has been confirmed that genetic factors and environmental factors (such as poisons) are involved in the occurrence and development of PD [4]. The common cause of PD is the loss of dopaminergic neurons in the substantia nigra compact area and the accumulation of Lewy bodies in the brain [5]. A large amount of evidence indicates that inflammatory damage and oxidative damage are the main causes of PD neurodegeneration and neuroinflammation [6]. N-methyl-4-phenylpyridinium (MPP$^+$) is the active metabolite of 1-methyl-4-phenyl-

1,2,3,6-tetrahydropyridine (MPTP). MPTP is a neurotoxin that selectively destroys dopaminergic neurons, and the exposure can cause PD [7]. Therefore, elucidating the mechanism of MPTP and MPP^+ inducing PD is of great significance to elucidating the pathogenesis of PD.

In recent years, more and more scholars have begun to study the possible mechanism of traditional Chinese medicine in treating PD [8]. For example, studies have found that Poria can delay the progression of PD through immune regulation and other effects [9, 10]; ursolic acid can reduce oxidative stress in the substantia nigra striatum of mice and improve neurobehavior [11]; South Africa Cigarette can reduce the symptoms of Parkinson's disease by inhibiting the apoptosis of dopaminergic neurons [12]. Chinese medicine mechanisms mainly include participation in the protection of dopaminergic neurons [13], improvement of mitochondrial function [14], reduction of neuritis [15], enhancement of immune response [16], reduction of excitotoxicity [17], antiapoptosis [18], autophagy induction [19], and the inhibition of the accumulation of abnormal proteins [20]. It is widely known that a variety of natural molecules in food, such as plant polysaccharides, can protect the brain and delay aging. Studies have shown that foods rich in antioxidants, such as fruits, vegetables, and nuts, play a beneficial role in improving cognitive impairment by preventing or delaying the occurrence of cognitive decline during aging and neurodegeneration [21, 22]. Momordica charantia belongs to the Cucurbitaceae family. It is an important multifunctional edible and medicinal plant widely distributed throughout Asia. As a natural compound in daily food, Momordica charantia polysaccharides (MCPs) are famous for their antioxidant, anti-inflammatory, antitumor, hypoglycemic, and antidiabetic effects [23]; however, little is known about its role from the perspective of neurogenesis regulation. Studies have found that MCPs can protect nerve damage after stroke by scavenging free radicals [24].The above studies suggest that MCPs can play a protective role in neurological diseases and injuries.

In view of the fact that there is no research report on the relationship between MCPs and PD, therefore, this study is the first to investigate whether MCPs have antineuronal apoptosis and inhibit inflammation effects in mice and cellular PD models. In addition, it is known that TLR4/MyD88/NF-κB is an important signaling pathway involved in apoptosis and inflammatory response [26]. In addition to exploring the protective effects of MCPs on tissues and cells, this study also detected changes in the expression of the TLR4/MyD88/NF-κB signal axis, so as to explore the potential mechanism of MCP. It provides a theoretical basis for in-depth understanding of the role of MCP in the treatment of PD and the transformation of population research.

2. Method

2.1. Preparation and Analysis of MCPs. Bitter melon purchased from the local market in our city in July 2020 was washed, dried, cut into pieces, seeded, then ground into a homogeneous powder (40-60 mesh), and stored in a dry, ventilated place for later use. The bitter melon-dried fruit powder was extracted with 80°C water for 2 h. The extract was filtered with glass wool and centrifuged at 6,000 g for 10 min to separate the supernatant and sample residues. The Sevag method was used to remove related proteins in the solution. After removing the Sevag reagent, the aqueous phase was concentrated and precipitated with ethanol, and the polysaccharide was precipitated overnight in a refrigerator at 4°C. The precipitate was collected, redissolved in water, and then further dialyzed in distilled water (MWCO 8000) for 24 h [27]. After concentration, the precipitate was frozen and made into freeze-dried powder.

The anthrone sulfuric acid method was used as the standard, and d-glucose was used as the internal standard to determine the total sugar content of MCPs. Analyze protein content with the folic acid reagent. According to the method of Blumenkrantz and Asboe-Hansen [28], d-glucuronic acid was used as the internal standard to determine the content of uronic acid. The high-performance liquid chromatography (HPLC) was equipped with an ultra-hydrogel linear column (7.8 mm × 300 mm). 0.5% MCPs (20 μL) was dissolved in distilled water, 0.7% NaCl was used as mobile phase, 0.5 mL/min, 35°C as HPLC conditions, and T series dextran was used as a standard for calibration. On a Shim Pack C18 column (4.6 mm × 250 mm), using an HPLC system, using 82.0% PBS (0.1 mol/L, pH 7.0) and 18.0% acetonitrile at a flow rate of 1 mL/min, dimethyl trifluoroacetic acid was used for hydrolysis After labeling the hydrolysate with 1-phenyl-3-methyl-5-pyrazolone (PMP), the monoacid composition of MCPs was analyzed by HPLC.

2.2. The Establishment and Administration of PD Animal Model. In the study, 40 SPF grade C57B/6 mice (male, 5-8 weeks old) were purchased. Place the mice in a 12-hour light-dark cycle, 22 ± 2°C breeding cage, and drink and eat freely. All experimental protocols involving animals in this study have been reviewed and approved by the ethics committee of our hospital and comply with the requirements of national health institutions.

The mice were randomly divided into 4 groups (10 in each group). PD model group (PD, $n = 10$): mice were intraperitoneally injected with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) 25 mg/kg, once a day, for 7 days, to construct the PD model. Control group (Control, $n = 10$): the same amount of saline was given at the same time as the model group stimulated with MPTP. Control+MCP group (Control+MCPs, $n = 10$): after being treated with saline for 7 days, MCPs (100 mg/kg) were given for 7 days. PD model+MCP group (PD+MCPs, $n = 10$): after 7 days of MPTP treatment, MCPs (100 mg/kg) were given for 7 days [29, 30]. At the same time, mice in the Control group and PD model group were given the same volume of normal saline as the MCP treatment. After the motor function test was completed, all mice were weighed, and the neck was cut to death. The brain tissue (striatum) of each mouse was collected, and part of it was stored in liquid nitrogen for future analysis. Besides, the mouse venous blood was collected in the coagulation tube, and the serum was collected by centrifugation at 5,000 g for 20 min at room temperature and frozen for later use.

2.3. Cell Culture and Processing. The human neuroblastoma cell line (SK-N-SH) was purchased from Shanghai Fuheng Biotechnology Co., Ltd. DMEM (Invitrogen, USA) medium containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA) was used to culture cells in a humidified incubator at 37°C and 5% CO_2. The cells were divided into 4 groups: (1) PD model group (PD): SK-N-SH cells were placed at 37°C and stimulated with 1 mM MPP^+ for 24 hours to establish a PD cell model. (2) Control group: the cells were treated with PBS for 24 h at the same time as the model group stimulated with MPP^+. (3) Control +MCP group (Control+MCPs): after being treated with PBS for 24 hours, given MCPs (80 μg/mL) for 24 hours. (4) PD model+MCP group (PD+MCPs): after MPP^+ treatment for 24 h, MCPs (80 μg/mL) were given for 24 h. At the same time, the Control group and PD model group were given the same volume of PBS as the MCP treatment.

2.4. Pole Climbing Experiment. Pole climbing experiment was used to assess the degree of retardation. On the 7th day of the MCP treatment, the mice were placed on the top of a rod with a length of 50 cm and a radius of 4 mm, and the following activities were recorded three times: the time it takes for the mouse to climb down the upper half, the time it takes for the mouse to climb the lower half, and the time it takes for the mouse to complete the total length of the climbing pole. If the mouse completes the above three steps in 3 s, 6 s, or more than 6 s, the motor coordination score will be 3, 2, or 1 point. The scores of the three steps were accumulated, the test was performed 3 times, and the average value was taken.

2.5. Rotarod Experiment. Rotarod experiment was used to analyze the motor function of mice. It was performed on the 5-7th day of MCPs treatment. Before using ROTA ROD (UgoBasile, Italy) for a 1-day experiment, all mice received a 2-day training (5 times/day). On day 3, the mice were tested 4 times in accelerated mode (4-40 rpm in 5 minutes). The mouse did not fall from the rod after the maximum residence time of 300 s. The average residence time before falling off was measured, and the average of the 3 longest residence times for each animal was analyzed.

2.6. The Measurement of Neurotransmitters and Metabolites by High-Performance Liquid Chromatography. Weigh the striatum tissue and homogenize it in 0.1 μM perchloric acid. After incubating for 1 h on ice, centrifuging at 12,000 g at 4°C for 20 min. Then, the supernatant was collected and filtered through a 0.22 μM filter, and 25 μL of the sample was injected into the column and mixed with the HPLC mobile phase. The HPLC mobile phase includes water, acetonitrile, and 0.01 μM phosphate buffer (adjusted to pH 4 with phosphoric acid), and gradient elution was adopted. At the same time, by diluting the stock solution in the mobile phase, freshly prepared the dopamine (DA), serotonin (5-HT), and its metabolites (including dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindole acetic acid (5-HIAA)) and hydrochloride standard solution (Sigma-Aldrich, USA). The serial concentration of the standard solution was used to determine the linear range of the above substances before testing.

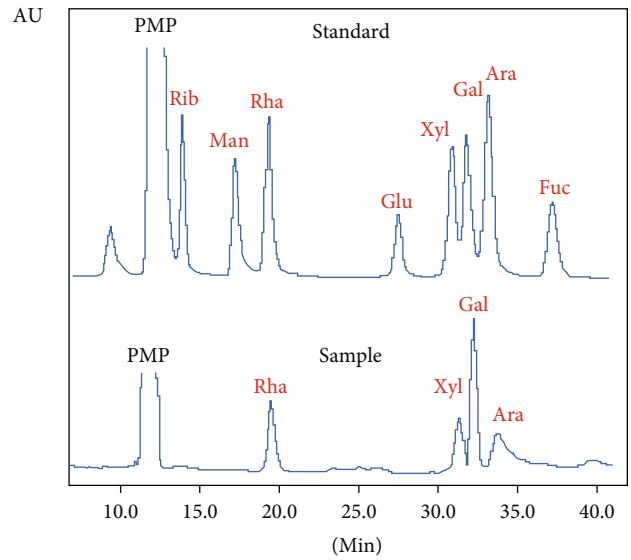


Figure 1: HPLC analysis of MCPs monosaccharide composition. Monosaccharides can be identified and quantified by comparison with standard products. Rib: ribose; Man: mannose; Rha: rhamnose; Glu: glucose; Xyl: xylose; Gal: galactose; Ara: arabinose; Fuc: fucose.

2.7. Enzyme-Linked Immunosorbent Test (ELISA). Strictly follow the instructions, and the ELISA kit (Biotech (Shanghai) Co., Ltd., China) was used to detect the concentration of tumor necrosis factor α (TNF-α) and interleukin-1β (interleukin-1β, IL) in tissues, and the concentration of inflammatory factors is expressed in pg/mg protein.

2.8. The Evaluation of Oxidative Stress Markers. According to the instructions, the kit (Nanjing Jiancheng Institute of Bioengineering, China) was used to measure the levels of GSH, SOD, and MDA in the supernatant of the striatal brain tissue, and the concentration is expressed in μg/mg protein.

2.9. The Detection of Cell Viability. Cell counting kit (CCK-8, Dojin, Japan) was used to determine cell viability. The cells were seeded in a 96-well plate (10^4 cells/well), and after the corresponding treatment the next day, 10 μL of CCK-8 reagent was added to each well and placed in an incubator to continue culturing for 3 hours. The absorbance was recorded with a spectrophotometer at a wavelength of 450 nm.

2.10. The Determination of Apoptosis. Flow cytometry was used to determine the rate of apoptosis. SK-N-SH cells were inoculated in a 24-well culture plate, and after corresponding treatments the next day, the cells were washed twice with precooled PBS. After that, the cells were resuspended in 200 μL binding buffer (1%) by centrifugation, and 5 μL each of Annexin V/FITC and PI staining solution (Invitrogen, USA) was added, which was then incubated at room

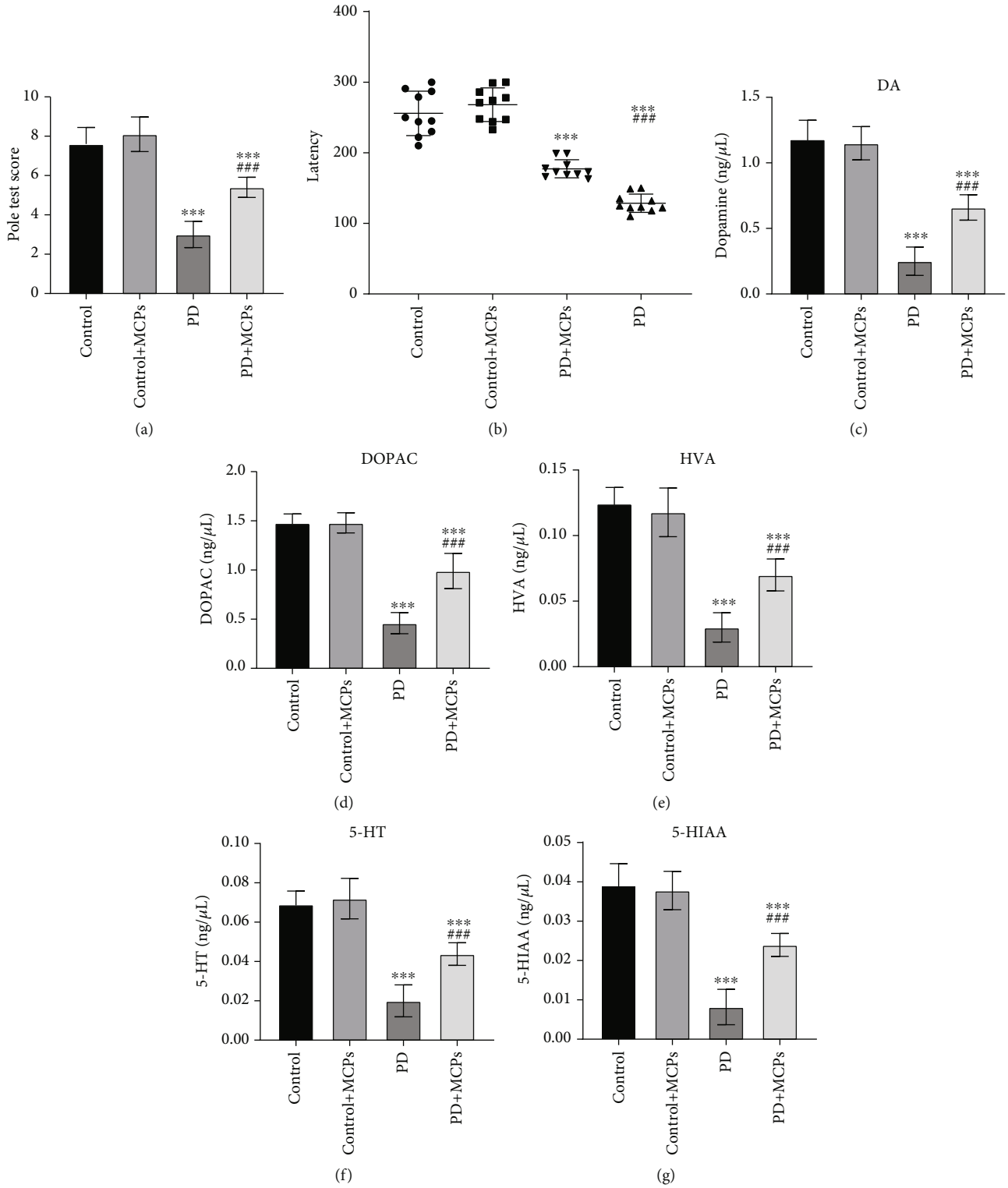
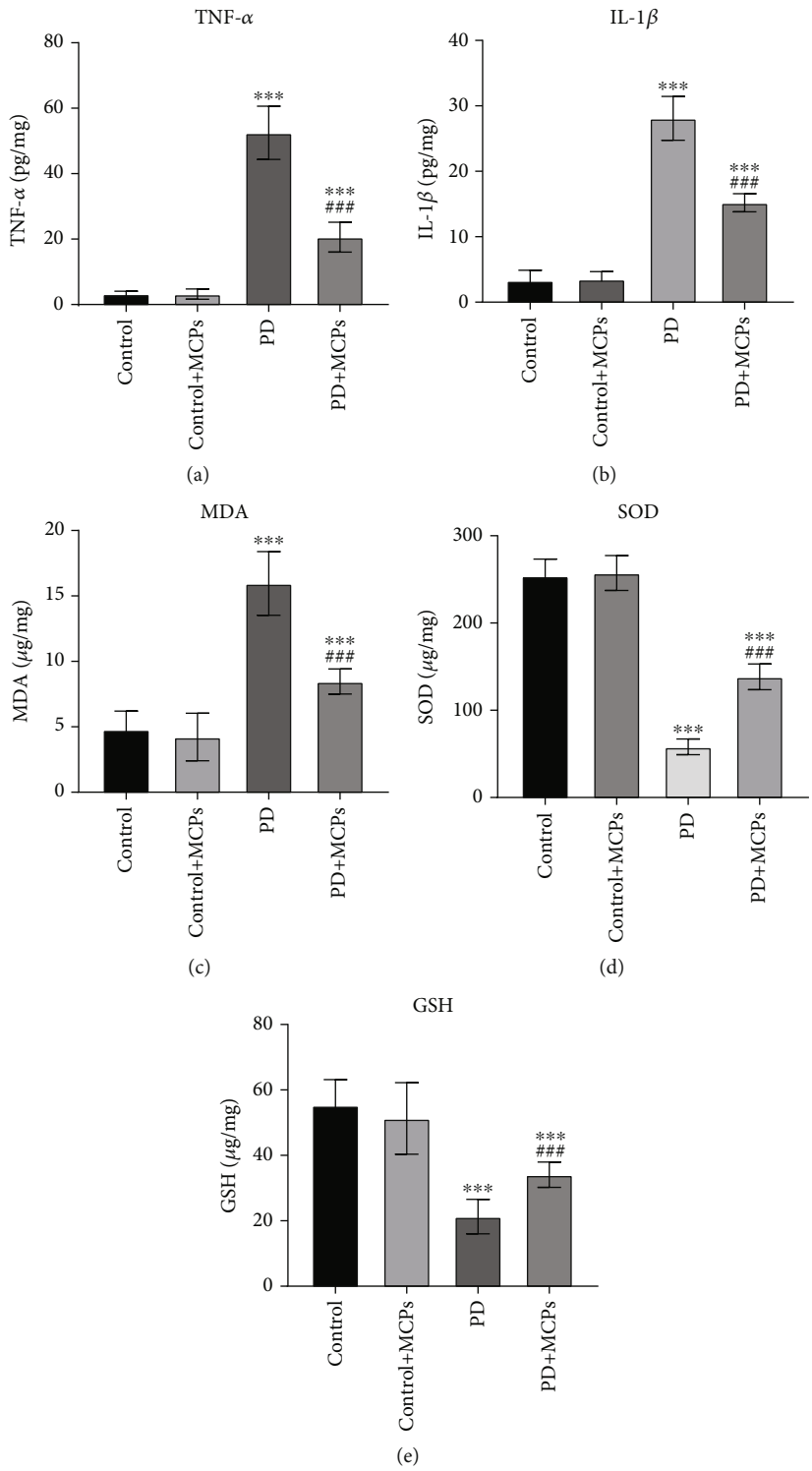


Figure 2: MCPs reduce the dyskinesia of MPTP-induced PD and regulate DA and 5-HT metabolism. (a) Pole climbing test score. (b) Rotarod test retention ability. (c–g) DA, DOPAC, HVA, 5-HT, and 5-HIAA levels in striatum tissue homogenate. Compared with the Control group (Control+MCPs group), $^{***}P < 0.001$, and compared with the PD group, $^{###}P < 0.001$.

temperature for 15 min in the dark, and placed in a flow cytometer to determine the cell apoptosis rate.

PI-Hoechst staining was used to observe cell apoptosis. The apoptotic cells were stained with PI (4 mM, Sigma-Aldrich, USA) and Hoechst 33432 (0.5 mg/mL, Sigma-Aldrich, USA) at 37°C for 10 min. PI-positive cells were counted under a fluorescence microscope at excitation and emission wavelengths of 535 nm and 615 nm, respectively.



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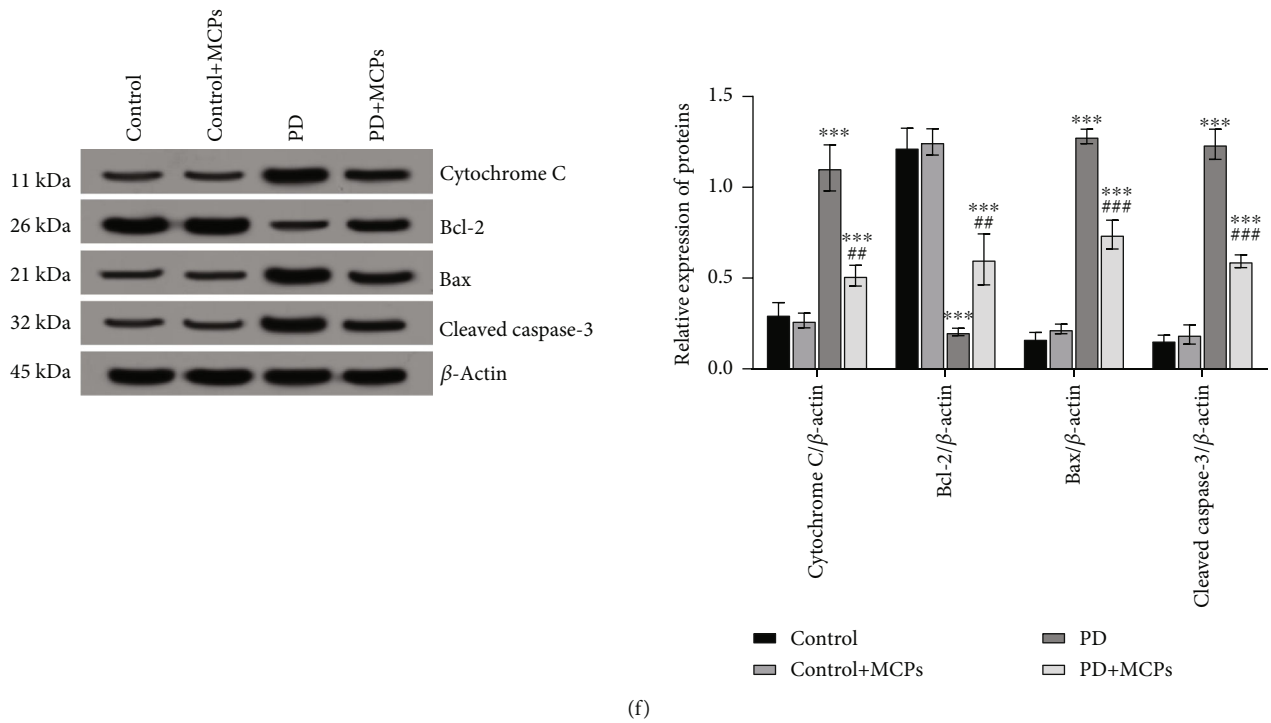
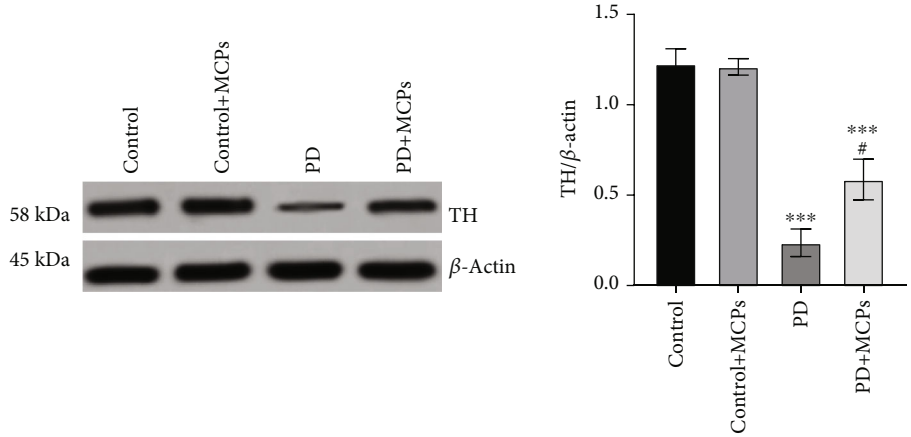


(f)



(g)

Figure 3: MCPs reduce the inflammation, oxidative stress, and apoptosis of PD induced by MPTP. (a, b) Expression of proinflammatory cytokines TNF-α and IL-1β in striatum tissue homogenate. (c–e) Expression of oxidative stress-related factors MDA, SOD, and GSH in striatum tissue homogenate. (f) The expression levels of oxidative stress-related protein Cytochrome C and apoptosis-related proteins Bcl-2, Bax, and cleaved Caspase-3 in striatal tissue homogenate. (g) Tyrosine hydroxylase (TH) protein level in striatum tissue homogenate. Compared with Control group (Control+MCPs group), $^{***}P < 0.001$, compared with PD group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

2.11. The Determination of Protein Levels by Western Blotting Method. RIPA strong lysate (Shanghai Biyuntian Biotechnology Co., Ltd., China) was used to extract total tissue/cell protein. First, lysed on ice for 30 minutes, centrifuged at 4°C, 12,000 g for 20 minutes, and extracted the supernatant. Then, protein quantification was performed by the BCA method (Thermo Fisher Scientific, USA). An equal amount of protein (50 mg) was electrophoresed on a 10% SDS polyacrylamide gel and, then, transferred to a PVDF membrane for blotting (Millipore, USA). Blocked in 5% skimmed milk at room temperature for 1 hour, with primary antibodies Bcl-2 (ab182858, Abcam, UK), Bax (ab32503, Abcam, UK), cleaved Caspase-3 (ab32042, Abcam, UK), Cytochrome C (ab133504, Abcam, UK), TLR4 (ab13556, Abcam, UK), MyD88 (ab133739, Abcam, UK), p-NF-κB p65 (ab183559, Abcam, UK), Tyrosine hydroxylase (ab137869, Abcam, UK), and THβ-actin (ab6276, Abcam, UK) incubated the PVDF membrane overnight at 4°C. The next day, horseradish peroxidase-conjugated secondary antibody (Abcam, UK) was added and incubated for 1 h at room temperature, and then, ECL luminescent developer (Shanghai Biyuntian Biotechnology Co., Ltd., China) was used to quantify protein levels in the imaging system. According to the manufacturer's recommendations, an enhanced chemiluminescence method (Thermo Fisher Scientific) was used to develop the western blot.

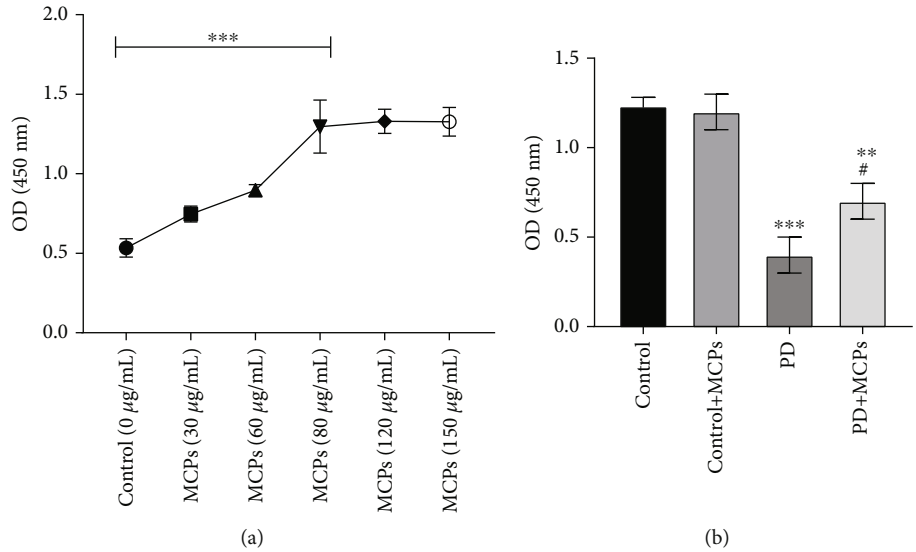


Figure 4: MCPs improve the viability of glioblastoma cells. (a) The effects of different concentrations of MCPs (0 μg/mL, 30 μg/mL, 60 μg/mL, 80 μg/mL, 120 μg/mL, 150 μg/mL) on cell viability. (b) In the PD cell model, the effect of MCPs on cell viability. Compared with the Control group (Control+MCPs group), $^{***}P < 0.001$, and compared with the PD group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

2.12. Statistical Analysis. The data were expressed as mean ± standard deviation. Statistical analysis was performed using the statistical software SPSS 19.0. Student's t-test was used for comparison between different groups, and one-way analysis of variance was used for comparison between groups under the same conditions. $P < 0.05$ was considered as statistically significant.

3. Results

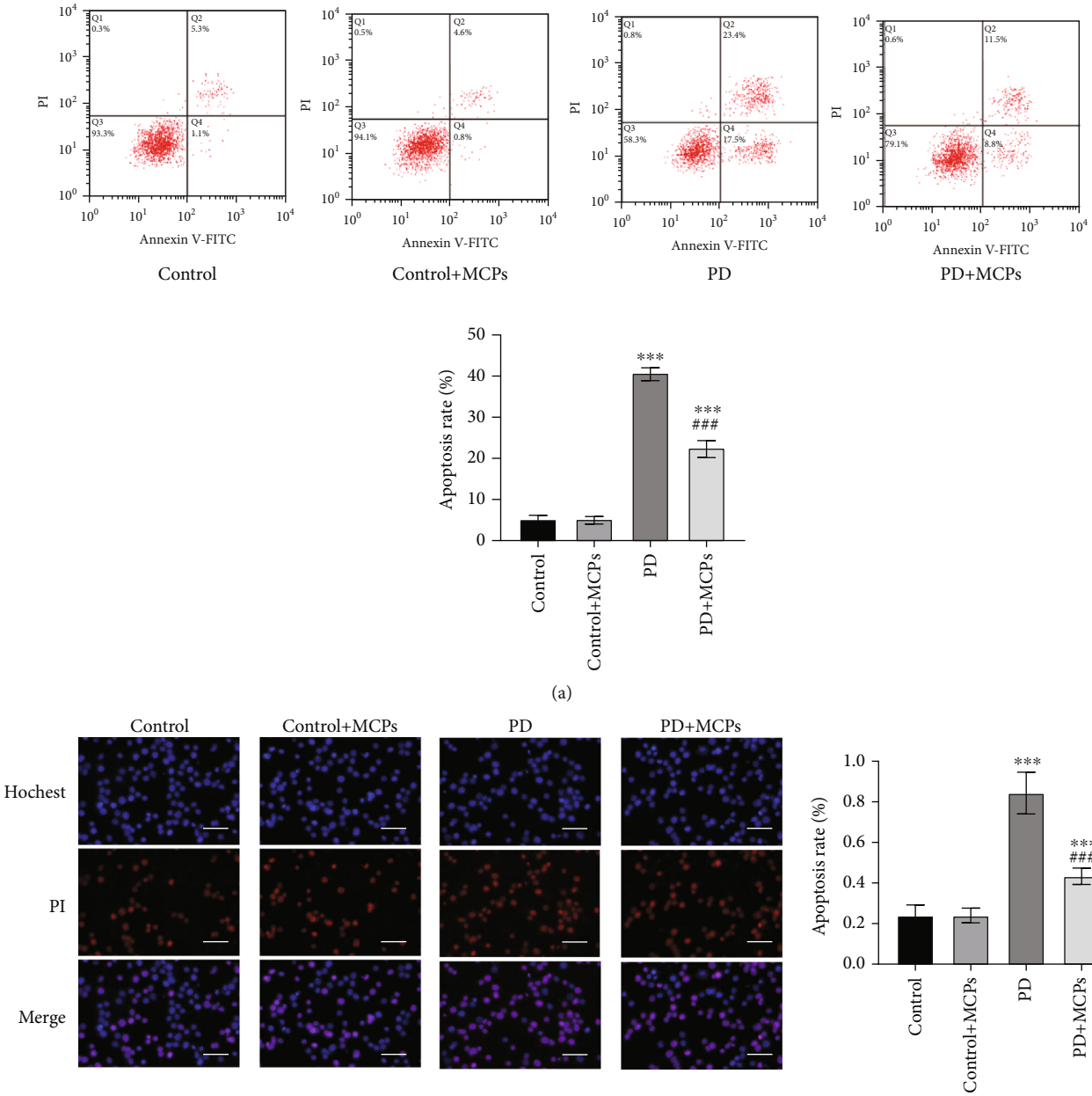
3.1. Analysis of MCPs. After filtering the bitter gourd precipitate, it was freeze-dried to obtain a yellow powder to obtain a polysaccharide with a yield of 3.2%. The total sugar content is 71.3 ± 1.1%, and the protein content is 9.1 ± 0.3%. The content of uronic acid in MCPs was 20.2 ± 0.3%. The molecular weight of polysaccharides was determined in the range of 85-100 kDa. In order to further study the composition of MCPs, we used different monosaccharide standards and recorded their retention time to identify the monosaccharide components of MCPs by HPLC. Analysis of monosaccharide components showed that according to HPLC retention time and peak area, MCPs contained arabinose, xylose, galactose, and rhamnose in a ratio of 1.01 : 1.13 : 4.17 : 1.67 (Figure 1).

3.2. Changes in Appearance and Weight of Mice. Throughout the experiment, the mice were visually observed and weighed daily. This study found that after administration of MPTP, the mice showed typical disease behaviors, including reduced exercise, hunchback, anorexia, and weight loss, but after treatment with MCPs, the symptoms of the mice gradually alleviated.

3.3. MCPs Reduce the Dyskinesia of MPTP-Induced PD and Regulate DA and 5-HT Metabolism. In this study, the pole climbing experiment and the Rotarod experiment were used to evaluate the exercise ability of mice. As shown in Figures 2(a) and 2(b), in the former, compared with the Control group, the pole climbing experiment time of the PD group mice was increased ($P < 0.05$), while the Rotarod experiment stay time was shortened ($P < 0.05$). In contrast, treatment with MCPs can significantly reverse the above changes (all $P < 0.05$). The above results indicate that the administration of MCP treatment can reduce the dyskinesia caused by MPTP.

Moreover, to evaluate the potential protective effect of MCP on brain function, this study used HPLC fluorescence detection to determine the concentration of striatal neurotransmitters DA, 5-HT, and their metabolites DOPAC, HVA, and 5-HIAA. As shown in Figures 2(c)–2(g), the levels of DA, DOPAC, and HVA in the brain tissue of the PD group significantly decreased ($P < 0.05$), while the levels of 5-HT and its metabolite 5-HIAA increased significantly (all $P < 0.05$), and the administration of MCPs can significantly reverse the changes in the levels of the above substances (all $P < 0.05$). The above results suggest that MCPs can participate in the metabolism of neurotransmitters in the brain by inhibiting the MPTP-induced decrease in striatal DA, 5-HT, and their metabolites.

3.4. MCPs Reduce the Inflammation, Oxidative Stress, and Apoptosis of PD Induced by MPTP. To investigate the inflammatory state of PD mice and the effect of MCP on the inflammatory response, the expression levels of proinflammatory cytokines TNF-α and IL-1β in the striatum were detected. As shown in Figures 3(a) and 3(b), the expression levels of TNF-α and IL-1β in the brain tissue of mice induced by MPTP were higher than those in the Control group (both $P < 0.05$). It shows that MPTP can cause inflammation and the release of proinflammatory cytokines, and the treatment of MCPs can significantly reduce the expression levels of these factors (all $P < 0.05$), suggesting that MCP has anti-inflammatory effects.



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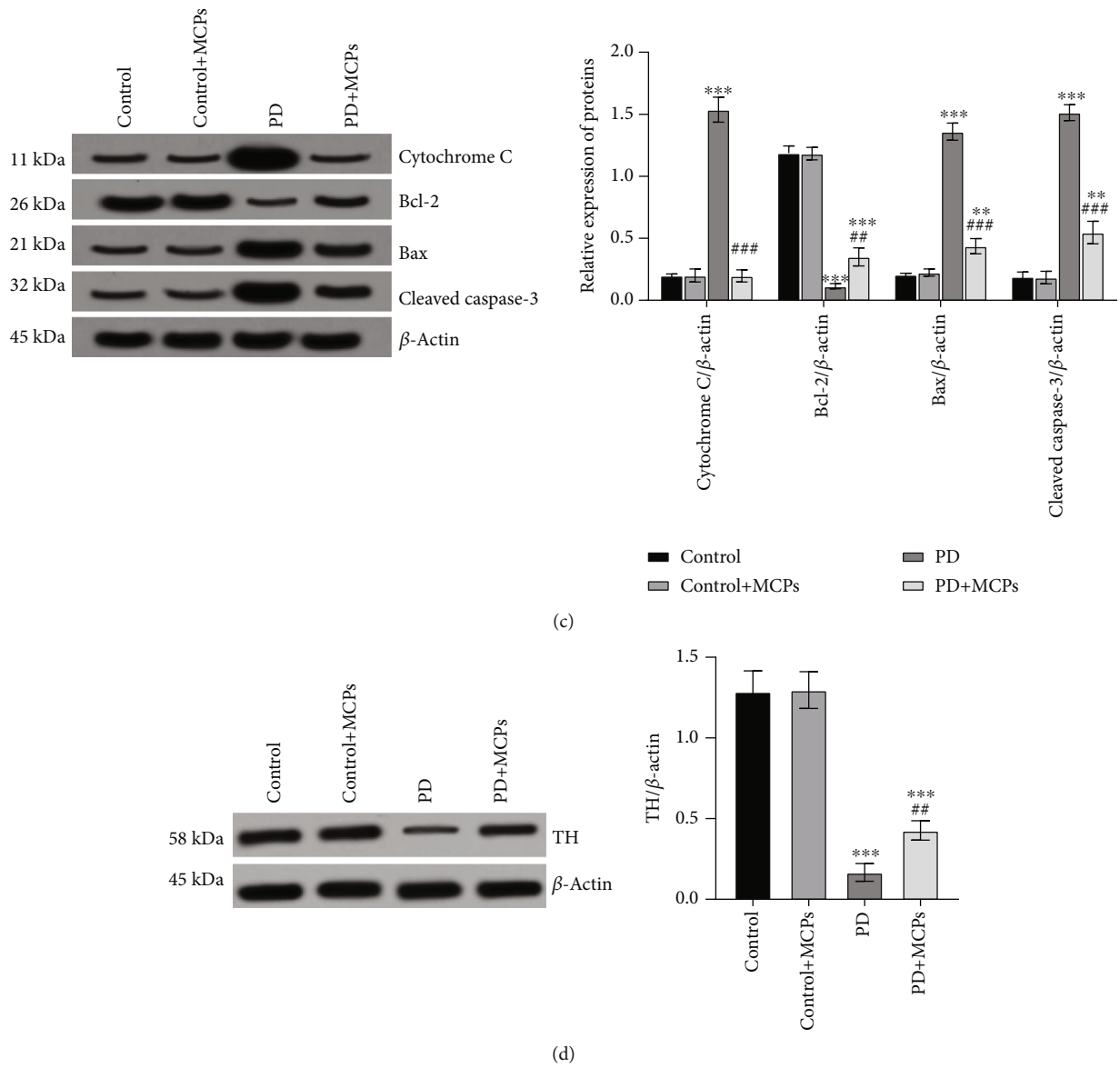


Figure 5: MCPs inhibit MPP^+-induced apoptosis and reduce oxidative stress. (a) Flow cytometry to detect cell apoptosis rate after Annexin V-FITC/PI staining. (b) PI staining fluorescence microscope to observe cell apoptosis. (c) The expression level of an oxidative stress-related protein Cytochrome C, and the expression levels of apoptosis-related proteins Bcl-2, Bax, and cleaved Caspase-3 in cells. (d) TH protein level in the cells. Compared with Control group (Control+MCPs group), $^{***}P < 0.001$, compared with PD group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

This study also detected the levels of GSH, SOD, and MDA in the striatum. As shown in Figures 3(c)–3(e), MPTP-induced MDA content in mice was significantly higher than that in the Control group ($P < 0.05$), while GSH and SOD were significantly lower ($P < 0.05$). MCPs treatment can significantly reverse the changes in the above factors (all $P < 0.05$), suggesting that MCPs have antioxidant effects.

Furthermore, this study also detected the expression of oxidation and apoptosis marker proteins in the striatum. As shown in Figure 3(f), the expression levels of oxidative factor Cytochrome C, proapoptotic proteins Bax, and cleaved Caspase-3 in the brain tissue of PD mice were significantly increased (all $P < 0.05$), while Bcl-2 protein was significantly reduced ($P < 0.05$), and MCPs treatment can significantly reverse the expression changes of the abovementioned proteins (all $P < 0.05$), suggesting that MCPs have antioxidant and anti-apoptotic effects. In addition, as shown in Figure 3(g), in order to study the effect of MCPs on dopaminergic neurons, we also detected the expression of TH in the striatum and found that the level of TH in the brain tissue of the PD model decreased ($P < 0.05$), while MCPs treatment can eliminate this effect ($P < 0.05$).

3.5. MCPs Improve the Activity of Glioblastoma Cells. This study first observed the effect of MCPs on the activity of glioblastoma cells. As shown in Figure 4(a), cells were given different concentrations of MCPs (0 μg/mL, 30 μg/mL, 60 μg/mL, 80 μg/mL, 120 μg/mL, 150 μg/mL) to culture for 24 h, and CCK-8 results showed that when the concentration

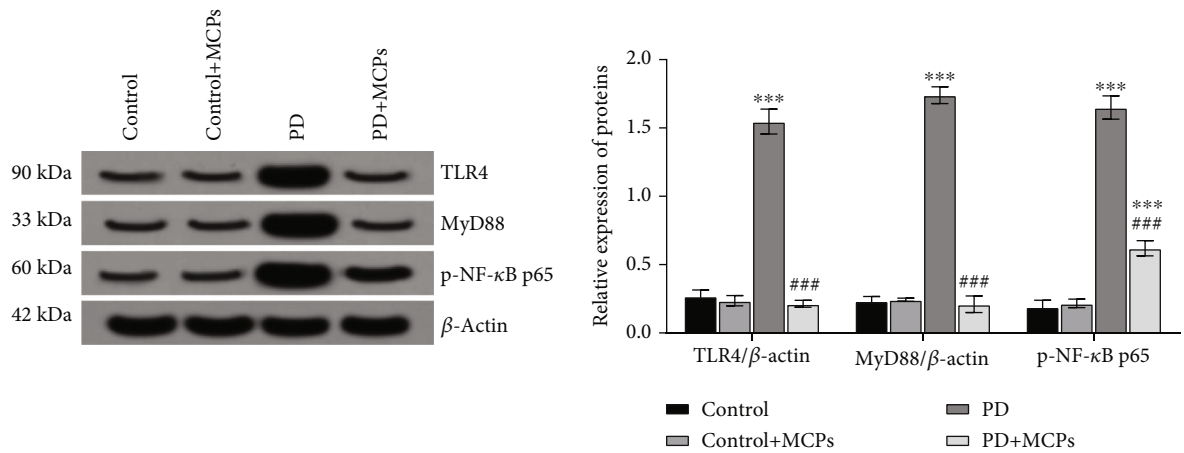


Figure 6: MCPs regulate the activation state of TLR4/MyD88/NF-κB signaling pathway. The expression level of TLR4, MyD88, p-NF-κB p65 protein in the striatum tissue homogenate. Compared with the Control group (Control+MCPs group), $^{***}P < 0.001$, and compared with the PD group, $^{###}P < 0.001$.

of MCPs was in the range of 0-80 μg/mL, it can promote the increase of SK-N-SH cell activity in a dose-dependent manner ($P < 0.05$). Therefore, 80 μg/mL was selected as the treatment concentration of MCPs in subsequent experiments.

Subsequently, to observe the effect of MCPs on the activity of MPP^+-induced injured cells, the changes of cell activity in the Control group, PD group, Control+MCPs group, and PD+MCPs group were observed by the CCK-8 method. As shown in Figure 4(b), compared with the Control group, SK-N-SH cell viability was significantly reduced after MPP^+ treatment ($P < 0.05$), and then, the cell viability of MCPs treatment increased ($P < 0.05$), suggesting that MCPs can reduce the damage induced by MPP^+ and improve cell viability.

3.6. MCPs Inhibit MPP^+-Induced Apoptosis and Reduce Oxidative Stress. As shown in Figures 5(a) and 5(b), compared with the Control group, MPP^+ can induce an apoptotic response in glioblastoma cells, with a higher apoptotic rate ($P < 0.05$), and a significant increase in apoptotic cells ($P < 0.05$). After cell injury, treatment with MCPs can significantly inhibit the occurrence of apoptosis, reduce the rate of apoptosis ($P < 0.05$), and reduce the number of apoptotic cells ($P < 0.05$), suggesting that MCPs have anti-apoptotic effects.

This study also detected changes in the expression of apoptosis and oxidative stress-related proteins. As shown in Figure 5(c), after treatment with MPP^+, SK-N-SH cells expressed a significant decrease in the level of antiapoptotic protein Bcl-2 ($P < 0.05$), while the levels of Bax and cleaved Caspase-3 increased significantly ($P < 0.05$). In addition, the expression of Cytochrome C, an oxidative stress index, increased in MPP^+-induced injury ($P < 0.05$), and MCPs treatment could reverse its change. It is suggested that in addition to antiapoptotic effects, MCPs also have antioxidative stress functions. As shown in Figure 5(d), the expression of TH in the PD cell model was reduced ($P < 0.05$), and treatment with MCPs could reverse this effect ($P < 0.05$).

3.7. MCPs Regulate the Activation State of TLR4/MyD88/NF-κB Signaling Pathway. As shown in Figure 6, compared with Control, MPP+ treatment can significantly promote the expression of TLR4, MyD88, and p-p65 proteins (all $P < 0.05$), while MCPs can inhibit the expression of these proteins (all $P < 0.05$).

3.8. The Verification of the Role of MCPs by Using TLR4 Inhibitors. In this study, TAK-242, a small molecule inhibitor of TLR4, was used to verify the protective effect of MCPs on MPP^+-induced damaged cells. As shown in Figure 7, this study found that the application of TLR4 inhibitors can significantly reverse the protective effect of MCPs (all $P < 0.05$).

4. Discussion

This study confirmed that MCPs have protective effects on MPTP- and MPP^+-induced PD models in mice and cells. In terms of motor function, we found that MCPs can reduce the damage of MPTP to mice's coordination and exercise ability and can inhibit the production of inflammatory factors and oxidative stress products in the brain, thereby increasing the level of dopamine. In terms of cell function, MCPs can inhibit MPP^+-induced apoptosis and oxidative stress, and we found that MCP exerts a protective effect by inhibiting the activation of the TLR4/MyD88/NF-κB pathway.

PD is a common age-related neurodegenerative disease, which seriously affects the quality of life [31]. Due to insufficient knowledge of PD pathology, current treatment focuses on symptom relief, rather than PD prevention and basic treatment [32]. Previous studies have found that plant ingredients such as ursolic acid [33], maidenhair fern [34], and Poria cocos [35] have protective effects in delaying the progression of PD and reducing symptoms of PD. MCPs account for about 6% of bitter melon powder, which are heteropolysaccharides, composed of galactose (Gal), glucose (Glu), arabinose (Ara), rhamnose (Rha), and mannose (Man) [36]. Tan and Gan [37] have reported that an acidic

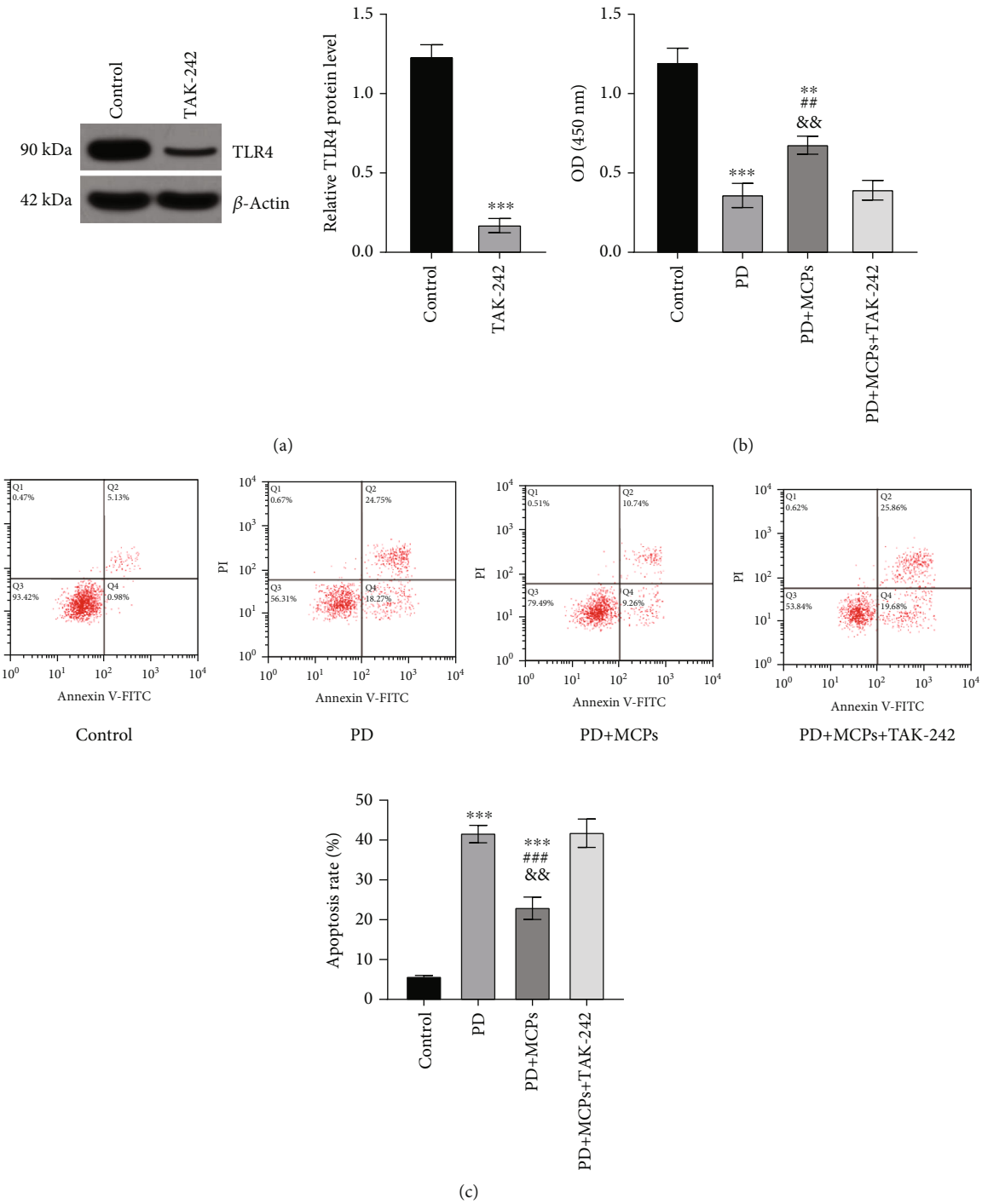
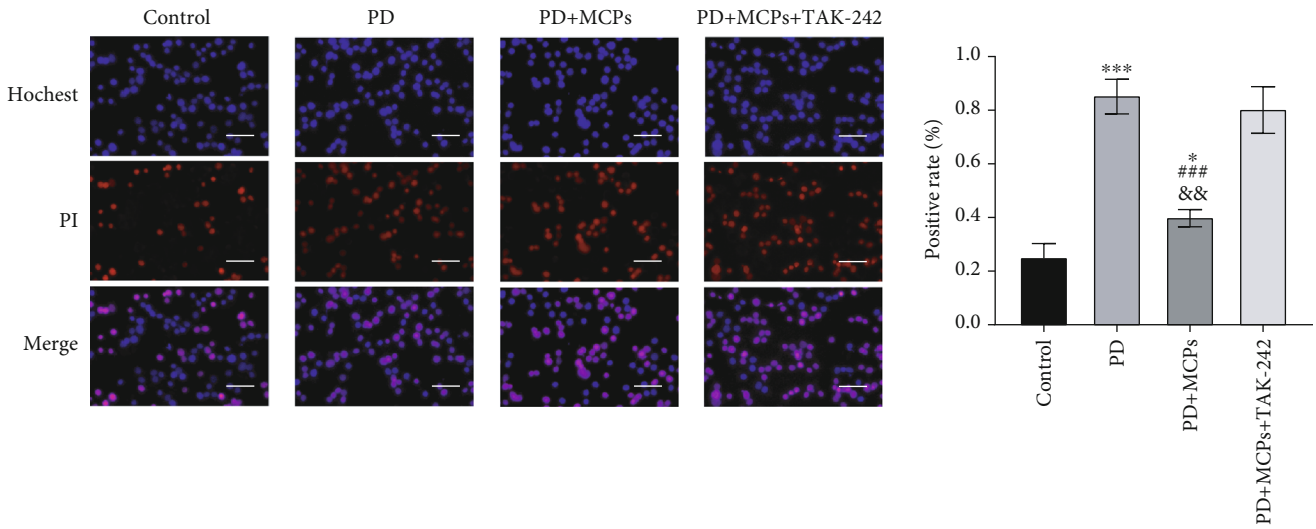


Figure 7: Continued.



(d)

Figure 7: Application of TLR4 inhibitor to verify the effect of MCPs. (a) Validation of TAK-242 inhibitor. (b) Detect the effect of MCPs on cell viability after applying TAK-22. (c, d) Detect the effect of MCPs on cell apoptosis after applying TAK-22. Compared with the Control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the PD group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, similar to PD+TAK-242 Ratio, $^{\&\&}P < 0.01$.

branched heteropolysaccharide isolated from bitter melon is mainly composed of Man, galacturonic acid (GalA), Rha, Glu, Gal, xylose (Xyl), and Ara, which has antioxidant and inhibitory properties. α-Amylase and inhibition of the angiotensin-converting enzyme. Recently, a water-soluble polysaccharide (MBP) was isolated from the fruit of the bitter gourd. Its main components are Ara, Xyl, Gal, and Rha, which have a significant hypoglycemic effect [38]. Raish proved that MCPs can improve oxidative stress, hyperlipidemia, inflammation, and apoptosis during myocardial infarction by inhibiting the NF-κB signaling pathway [39]. In addition, MCPs also have the ability to increase total volatile fatty acid production, regulate rumen fermentation pathways, and affect the number of cellulose-decomposing bacteria [40]. However, there is no relevant report on the effect of MCPs on PD.

The gradual decrease of striatal DA in PD patients is the cause of motor and nonmotor symptoms [41]. The MPTP-induced PD mouse model is similar to the symptoms of PD patients with abnormal muscle tone, posture, and physical decline. The pole-climbing experiment is considered to be a way to measure MPTP-induced movement changes in PD mice. We have found through research that MCPs treatment is effective for the climbing time of PD mice, reversing the negative effects of MPTP treatment. The Rotarod test also has a similar finding that MCPs treatment can significantly reverse the decrease in balance and coordination caused by MPTP. Furthermore, in this study, it was detected by high-performance liquid chromatography that the DA content in the striatum of MPTP-treated mice was decreased, while the levels of 5-HT and its metabolites were increased, and MCPs treatment can alleviate the above changes. The above results indicate that MCPs treatment can effectively improve the motor coordination ability of PD mice and promote the recovery of nerve function.

It is known that PD is related to oxidative stress, inflammation, and apoptosis [42]. Many studies have shown that mitochondrial dysfunction, oxidative stress, caspase release, and electron transport chain are the main features of PD neuron death [43–45], which is consistent with our research results. MPTP can induce the decrease of GSH and SOD levels in the mouse striatum and the increase of MDA levels. The treatment of MCPs can reverse the changes of these factors, suggesting that MCPs have antioxidant effects. It is also known that Cytochrome C is a signal molecule necessary for the death of apoptotic cells. It is released from the mitochondria into the cytoplasm and can act as an apoptotic protease activator to initiate an apoptotic response. Moreover, Cytochrome C plays an important role in oxidative stress and inflammation [46]. Therefore, this study detected the expression level of Cytochrome C in mouse striatum tissues and in glioblastoma cells and found that MCPs treatment can significantly reverse the increase in PD-induced Cytochrome C expression, further verifying the antioxidant capacity of MCPs. It also suggests that MCPs have antiapoptotic and anti-inflammatory effects. In addition, this study also detected the expression level of TH in mouse striatum and glioblastoma. It is known that TH is a monooxygenase that can affect the changes in the synthesis rate and release of catecholamines during nerve stimulation. Adaptive response to maintain an appropriate supply of neurotransmitters in nerve endings [47]. This study found that MCPs treatment can significantly reverse the decrease in TH in the PD model and exert neuroprotection.

Mohammad et al. once found in alcoholic gastritis that MCPs can improve mucosal oxidative stress, inflammation, and apoptosis by inhibiting the activation of the NF-κB signaling pathway [48]. In this study, it was found for the first time in animal and cell models of PD that MCPs have antioxidant, anti-inflammatory, and antiapoptotic effects. In terms

of anti-inflammatory response, this study detected the expression levels of inflammatory factors TNF-α and IL-1β in brain striatum tissue. In terms of apoptosis, this study observed cell apoptosis by flow cytometry and fluorescence staining and further verified by detecting the expression level of antiapoptotic protein Bcl-2, the proapoptotic proteins Bax, and cleaved Caspase-3. Experiments have found that MCPs treatment can significantly reverse the increased levels of TNF-α and IL-1β induced by MPTP or MPP^+, the decrease of Bcl-2 expression, and the increase of Bax and cleaved Caspase-3 expression. This is consistent with the results of Mohammad et al. and provides a more favorable experimental basis for the protective biological effects of MCPs. In addition to animal and cell function studies, this study also deeply explored the potential mechanisms of MCPs' protective effects. The TLR4/MyD88/NF-κB pathway is a key regulator involved in the inflammatory process [42, 49]. In this study, by observing the changes in this signaling pathway, it was found that MCPs can regulate the activation state of the TLR4/MyD88/NF-κB signaling pathway, and we also applied TLR4 inhibitors to verify the protective effects of MCPs. At present, there are a variety of selective inhibitors with TLR4 inhibitory function, among which TAK-242 is a bioavailable TLR4 inhibitor with extensive anti-inflammatory effects [50]. In this study, the combined use of MCPs and TAK-242 in the PD state found that the use of TAK-242 could reverse the protective effect of MCPs, thus verifying the effect of MCPs on the TLR4/MyD88/NF-κB signaling pathway.

In this study, MCPs were used to treat Parkinson's for the first time, using mouse animal models and human neuroblastoma cell models, starting from in vivo and in vitro experiments to study the protective effects of MCPs. It is worth noting that the dose concentrations in animal and cell models are similar, suggesting that the dose stability of MCPs, that is, the drug concentration plays a role in animals and cells when the drug concentration reaches 80-100 μg/mL. This result also provides information for population studies. The theoretical basis and reference. Although this study has confirmed the protection and resistance of MCPs in terms of motor function, oxidative stress, inflammation, and apoptosis in animal and cell models and found that MCPs interfere with TLR4/The activation state of MyD88/NF-κB pathway. However, this study also has shortcomings. In view of the diversity of mechanism studies, the protective effect of MCPs is not limited to the above studies, and mice and animal models cannot completely replace population studies, so follow-up studies are still needed to clarify the role and application prospects of MCPs.

In summary, this study found that in the PD model, MCPs can regulate the activation state of the TLR4/MyD88/NF-κB pathway, exert protective effects such as anti-inflammatory, antioxidative stress, and antiapoptosis, improve brain function, and provide a new method for the treatment of PD.

Data Availability

The data are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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