

Original Article

Immunomodulatory activity of polysaccharides from *Brassica rapa* by activating Akt/NF- κ B signaling

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ABSTRACT

Objective: To investigate the immunomodulatory activity of polysaccharides from the roots of *Brassica rapa*.

Methods: The crude polysaccharide from roots of *B. rapa* (BRP) was extracted and purified to further investigate the active fraction of BRT for inducing macrophage phagocytosis.

Results: Effects on RAW264.7 cells demonstrated that BRP behaved better phagocytic capacity and had potent immunomodulatory activity, including increasing production of nitric oxide (NO), tumor necrosis factor α (TNF α) and upregulating mRNA levels of inducible NO synthase (iNOS) and TNF α . Furthermore, modulation of macrophage by BRP was indicated to be mediated via the activation of Akt and nuclear factor-kappa B (NF- κ B).

Conclusion: The beneficial effects of BRP could be used as an immunotherapeutic adjuvant in treatment of inflammatory diseases.

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1. Introduction

Macrophages play an essential role in the regulation of acquired immunity (Beurel & Jope, 2009), which could initiate the innate immune response by secreting pro-inflammatory factors, cytokines and chemokines. Stimulation of the innate immune system with immunomodulators can increase host resistance to unforeseen pathogenic threats (Schepetkin et al., 2008), and a number of innate system immunomodulators have been identified, including cytokines and natural substances isolated from microorganisms and botanical sources (Nworu et al., 2015; OuYang et al., 2013; Zhang et al., 2012). At present, traditional Chinese medicine (TCM) has been well accepted as a complementary and alternative therapy to improve the clinical symptoms (Jiang et al., 2016; Tian et al., 2012). A variety of polysaccharides isolated from TCM have also attracted a great deal of attention because of their broad spec-

trum of therapeutic properties and relatively low toxicity (Tzianabos, 2000), exhibiting various pharmacological activities, such as anti-tumor, anti-inflammatory and anti-atherosclerotic activities (Takata, Yamamoto, Konno, & Okubo, 2005; Thakur, Connellan, Deseo, Morris, & Dixit, 2011; Wong, Lai, & Cheung, 2011).

Brassica rapa L., the family of Cruciferae, has been widely used as a Uyghur plant with homology to medicine and food, which has long been credited with health benefits especially by long-lived people. *B. rapa* has been traditionally used to cure cough and asthma in Xinjiang Uyghur Autonomous Region, and the root extract and capsules made of *B. rapa* have been clinically proven to have anti-tumor and immunity-enhancing activity (Sun et al., 2010; Xiao & Zhang, 2010; Zhang, Ma, & Zhang, 2013). Studies have shown that phytochemicals of *B. rapa*, including vitamins (Domínguez-Perles, Mena, García-Viguera, & Moreno, 2014), glucosinolates (Jaehoon et al., 2015), flavonoids (Hirokazu et al., 2011), and polysaccharides (Wang et al., 2016; Xie et al., 2010), are associated with biological functions such as antioxidative, immunomodulatory effects and inhibition of malignant transformation and mutations (Kestwal, Lin, Bagal-Kestwal, & Chiang,

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2011). Wang *et al* have purified three fractions of BRP-1–1, BRP-2–1 and BRP-2–2 with average molecular weight of 1510, 1110 and 838 kDa, while the crude BRP (*B. rapa* polysaccharides) exhibited relatively higher antioxidant activity *in vitro* than purified fractions (Wang *et al.*, 2016). One of the major mechanisms of plant-derived polysaccharides to play a role of immunomodulator has been reported to contribute to its ability to affect complement activation and macrophage function (Schepetkin, Faulkner, Nelson-Overton, Wiley, & Quinn, 2005).

In the present study, the polysaccharide from *B. rapa* was prepared, and the effects of both BRP and *B. rapa* tablet (BRT, the tablet from whole roots of *B. rapa*) on immunomodulatory activities were investigated, including phagocytic activities, inflammatory effect and its proper signaling pathway.

2. Materials and methods

2.1. Chemicals and reagents

Neutral Red Cell Proliferation and Total Nitric Oxide Assay Kit were purchased from Beyotime biotechnology (Shanghai, China). Quantitative Analysis Mouse ELISA Kit was ordered from Boster Biological Technology Co., Ltd (Wuhan, China). Immobilon™ Western Chemiluminescent HRP Substrate Kit (WBKL S0100) was purchased from Merck Millipore (CA, USA). Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb (4060), Akt (pan) (C67E7) Rabbit mAb (4691), Phospho-IκBα (Ser32) (14D4) Rabbit mAb (2859), IκBα (44D4) Rabbit mAb (4812), Phospho-NF-κB p65 (Ser536) Rabbit mAb (3033), NF-κB p65 (D14E12) XP® Rabbit mAb (8242), TNFα (D2D4) XP® Rabbit mAb (11948), GAPDH (D4C6R) Mouse mAb (97166) Anti-rabbit IgG (HRP-linked Antibody, 7074) and Anti-mouse IgG (HRP-linked Antibody, 7076) were purchased from Cell Signaling Technology (MA, USA).

2.2. Phagocytosis of peritoneal macrophages *in vivo*

BRT was made from the roots of *B. rapa* and was provided by Xinjiang Uygur Autonomous Region Keping County Shengquan Industry Co., Ltd. KM mice (weight 18–22 g) were provided by Experimental Animal Center of Xinjiang Medical University, allowed to acclimate to a new SPF surrounding [temperature: (22 ± 2) °C, humidity: 40%–60%, light/dark cycle: 12 h] for 1 week, with food and water supplied *ad libitum*. Then mice were randomly divided into four groups with ten animals in each group, including one control group and three treatment groups with different oral dosage of BRT (3, 6, 12 g/kg/d). For the control group, mice were given an equal volume of water. Phagocytosis by peritoneal macrophages was detected using the method described by Wang *et al* (Wang *et al.*, 2009). One hour after the last oral administration, 0.4 mL 5×10^6 chicken red blood cells (CRBCs) was intraperitoneally injected into each mouse. One hour later the mice were euthanized with CO₂. The experiments were conducted in full compliance with the local, national, ethical and regulatory principles with the approval of the Institutional Animal Care and Use Committee at Xinjiang Medical University.

Saline (2 mL) was injected into the abdominal cavity and 0.2 mL ascites was collected and prepared for smear microscopy. The smears were fixed with acetone-methanol (1:1, volume percent) solution, then stained with filtered Giemsa-phosphate (1:9) dye for 30 min. The number of macrophages swallowing CRBCs was counted under an optical microscope and the phagocytic rate (PR) and phagocytic index (PI) were calculated. PR (%) = the number of macrophages swallowing CRBCs/the number of total macrophages × 100%, PI = the number of swallowed CRBCs/the

number of macrophages swallowing CRBCs (at least 100 cells were selected to test).

2.3. Extraction of BRP

B. rapa was collected in April 2015 from Keping county, Xinjiang Uygur Autonomous Region (79°2'48"E, 40°30'30"N), and identified by associate professor Jiang He (Xinjiang Institute of Materia Medica). A voucher specimen (KP20150428-001) was deposited in Xinjiang Institute of Materia Medica (Xinjiang, China). The powder of roots of *B. rapa* was extracted with 25-time the volumes of distilled water (volume to mass ratio) at 100 °C twice (2 h each time). All extracts were combined, filtrated, precipitated with 80% ethanol and the mixture was kept overnight at 4 °C. The sediments were dried to constant weight under vacuum at 40 °C, and then resolved into distilled water to a rough concentration of 5 mg/mL for deproteinizing by Sevag method (Zha *et al.*, 2014). After removal of the Sevag reagent, the extract was decolorized with 5% activated carbon (mass to volume ratio) at 60 °C for 30 min. The extracts were precipitated with 80% ethanol and dried to constant weight again. Finally, the polysaccharide from *B. rapa* was obtained, named as BRP, and its content was determined by phenol sulfuric acid method (Masuko *et al.*, 2005).

2.4. Size exclusion chromatography analysis

BRP and BRT were dissolved in ultrapure water to a final concentration of 5 mg/mL. Size exclusion chromatography (SEC) analysis was performed at 35 °C on a Thermo Scientific Dionex UltiMate 3000, equipped with CAD detection Dionex Corona Veo RS. A TSK-Gel G3000SWXL column (7.8 mm × 300 mm, 5 μm, Tosoh Bioscience, Montgomeryville, PA, USA) was used at 25 °C. The mobile phase, comprised of 100 mmol/L ammonium formate-acetonitrile-ultrapure water (5:30:65), was delivered at a flow rate of 0.5 mL/min.

2.5. Cell culture and reagents

The murine macrophage-like cell line RAW264.7 was purchased from National Infrastructure of Cell Line Resource and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO₂ humidified atmosphere.

2.6. Measurement of phagocytic capacity of macrophages *in vitro*

RAW264.7 cells were incubated with BRT and BRP (312.5, 625, 1250, 2500 and 5000 μg/mL) for 24 h, and then 100 μL of aseptic neutral red solution (0.075%) was added into each well and incubated for another 1 h. After washed with PBS, the cells were lysed by adding 150 μL cell lysis buffer (anhydrous ethanol/acetic acid, 1:1). The absorbance was measured at 550 nm using a Bio-Tek EXL800 microplate reader (Bio-Tek, Winooski, VT). The absorbance represented the phagocytic ability of the macrophages.

2.7. Biochemical assay

RAW264.7 cells were plated at a density of (5×10^5) cells/mL in 12-well culture plates and incubated in medium with and without BRT and BRP (312.5, 625, 1250, 2500 and 5000 μg/mL) for 24 h. 200 μL supernatants were saved for the measurement of nitric oxide (NO) according to the colorimetric method described previously (Schepetkin *et al.*, 2005).

2.8. Enzyme-linked immunosorbent assay (ELISA)

RAW264.7 cells were seeded into six-well plates at (1×10^6) cells/mL. After 24 h irritants incubated the culture medium was collected and centrifugated, and the supernatant was stored at -20°C for the following test. The tumor necrosis factor α (TNF- α) level was detected using commercial enzyme-linked immunosorbent assay (ELISA) kit (Boster, Wuhan, China). All experimental procedures were performed according to the manufacture's instructions and normalized by protein content (mg/mL).

2.9. Quantitative real-time PCR assay (qRT-PCR)

Total RNA from RAW264.7 cells was extracted using Mag-Bind Total RNA Kit, and then was used as substrate to synthesize the complementary DNA with First-Strand cDNA Synthesis SuperMix Kit. The relative levels of mRNA to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, accession number: NC_000072.7) were analyzed using Faststart universal SYBR Green master Kit on Bio-Rad CFX96 qPCR system (CA, USA). The data were analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method (Subbiah et al., 2015). The primer sequences are as follows: inducible NO synthase (iNOS) forward 5'-TCAGC TACGCCTTCAACACC-3' and reverse 5'-TTCCCAAATGTGCTTGT CACC-3'; TNF- α forward 5'-ACACCGTCAGCCGATTTGCTA-3' and reverse 5'-CCAAAGTAGACCTGCCCGGAC-3'; GAPDH forward 5'-CA TCTCCAGGAGCGACCC-3' and reverse 5'-AGACACCAGTAGACTC CACGACA-3'.

2.10. Western blot

After being treated with various irritants for 24 h, the cells were lysed by RIPA buffer (containing 4% protease and phosphatase inhibitor cocktail) for 10 min and centrifuged at 12 000 rpm for 10 min at 4°C . The protein of each sample was detected using BCA protein assay kit. The samples were separated through a 12% SDS-PAGE gel, and then transferred to PVDF membranes. After being blocked with 5% skimmed milk or BSA solution for 2 h, the membranes were probed with diluted primary antibodies against phosphor-Akt, Akt, phosphor-I κ B α , I κ B α , NF- κ B p65, phosphor-p65, TNF α and GAPDH in 5% BSA/TBST overnight at 4°C . After being washed with TBST for 3 times, the membranes were incubated with horse-

radish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. The blots were smeared with chemiluminescence (Millipore) and analyzed by ChemiDoc Imaging system (Bio-Rad, CA, USA).

2.11. Statistics

Data were shown as mean \pm standard deviation of at least three independent experiments. One-way ANOVA and Student's *t*-test were used to evaluate statistical significance with the SPSS statistics 23.0 software. Values of $P < 0.05$ were considered as statistically significant.

3. Results

3.1. BRT induces macrophage phagocytic activity in vivo

For phagocytosis of CRBCs, both PI and PR increased significantly in BRT 6 g/kg and 12 g/kg treatment groups when compared with control group ($P < 0.05$) (Fig. 1). Treatment of 3 g/kg BRT had no significant effect on rate and index of phagocytosis of CRBCs. The results indicated that BRT could enhance the ability of phagocytes to scavenge their none-self materials.

3.2. Purification and characterization of BRP

Polysaccharides from the roots of *B. rapa* were obtained following the method described in materials and methods, and determined by phenol sulfuric acid method. Based on the established standard curve (Fig. 2A), 7.03228 g BRP was calculated to be obtained from 20.0012 g roots of *B. rapa*, with a yield of 35.1619%. The content of main fractions 1, 2 and 3 in BRP was higher than that in BRT as shown in spectrum of size exclusion chromatography (Fig. 2B).

3.3. Key active component of BRT inducing macrophage phagocytic activity

One of the most distinguished features of macrophage activation would be an increase in pinocytic activity. Pinocytic activity of polysaccharides-activated macrophages was examined by the

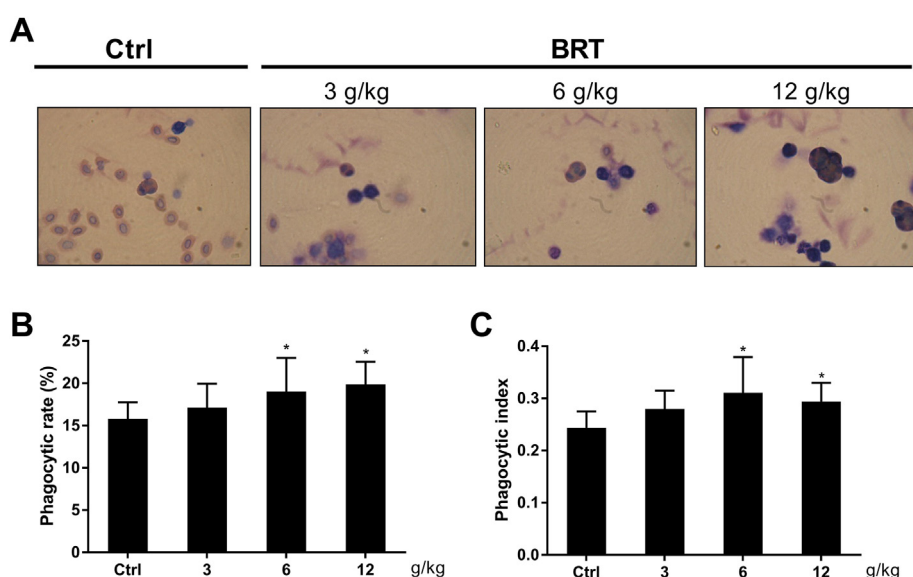


Fig. 1. Macrophage phagocytic activity *in vivo* induced by BRT (mean \pm SD, $n = 6$). Microscope images (A), PR (B), and PI (C) of CRBCs with BRT treatment (Ctrl stands for the group without BRT treatment; * $P < 0.05$ vs control).

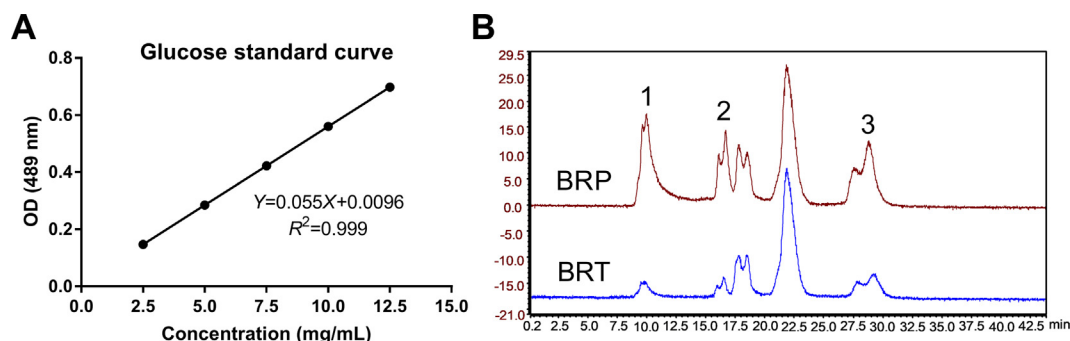


Fig. 2. Detection of BRP extracted from *B. rapa* by SEC. Glucose standard curve (A) and main fractions 1, 2 and 3 showed on BRP and BRT grams (B).

uptake of neutral red (0.1%). BRP (1250 $\mu\text{g/mL}$) showed the similar activity to phagocytose neutral red with 5000 $\mu\text{g/mL}$ BRT, and BRP behaved better in its phagocytic capacity than BRT at the same concentration (Fig. 3). The above results indicate that BRP significantly enhances the phagocytic ability of macrophages and could be the main active component of *B. rapa* promoting macrophage phagocytosis.

3.4. BRP stimulates secretion and transcription of NO and TNF- α in RAW 264.7

A minimum amount of NO production was determined in the culture supernatants of macrophages incubated with medium alone. Whereas, treatment with 1250 $\mu\text{g/mL}$ BRP resulted in a 4-

time increase in NO production ($P < 0.01$) and 2 times increase as treatment with BRT (Fig. 4A). iNOS mRNA level of RAW264.7 cells was enhanced by BRP significantly ($P < 0.05$) as compared with its control group, and presented a dose-dependent manner (Fig. 4B).

Untreated cells produced very little TNF- α . Incubation of these cells with BRP significantly enhanced TNF- α production ($P < 0.05$) and mRNA level ($P < 0.01$) in a concentration-dependent manner (Fig. 4C and D).

3.5. BRP activates Akt/NF- κB signaling pathway

Substance with immunomodulatory activity can regulate cytokine production. To further evaluate the signaling pathway involved in the immunomodulatory activity of BRP, the effect of BRP was assessed on macrophage TNF- α , phosphorylation of NF- κB , I $\kappa\text{B}\alpha$ and Akt activation in RAW264.7 macrophage cells using Western blot.

BRP ranging from 312.5 $\mu\text{g/mL}$ to 5000 $\mu\text{g/mL}$ was observed to increase the protein expression of TNF- α and stimulate NF- κB directed alkaline phosphatase expression ($P < 0.05$) (Fig. 5A and B). Akt and I $\kappa\text{B}\alpha$ phosphorylation were also increased by BRP ($P < 0.05$) (Fig. 5C and D). The results indicated that BRP was responsible for the observed effects and potently activation of the Akt/NF- κB pathway.

4. Discussion

There are multiple receptors on the phagocytes surface could recognize and internalize particle and clear none-self substance such as apoptotic cells, senescent erythrocytes, and inflammatory products (Brock, Serezani, Carstens, & Peters-Golden, 2008). Macrophages, as the most important professional phagocytes, play an important role in phagocytosis of pathogens and apoptotic cells, production of cytokines, and proteolytic processing and presentation of foreign antigens (Hume, 2006). NO, synthesized by macrophage-induced nitric oxide synthase, has been identified as a critical mediator of a variety of biological functions, including pathogen elimination and destruction of tumor cells by activated macrophages (Niedbala, Cai, & Liew, 2006). TNF α , also considered as a major immune and inflammatory mediator, owns cytostatic and cytotoxic effects in vivo and in vitro (Antwerp, Van Martin, Kafri, Green, & Verma, 1996). These reactive oxidants are involved in the regulation of apoptosis and immune homeostasis, which play critical roles in host defense and other physiological processes (Serhan & Savill, 2005).

Recently, a large amount of polysaccharides isolated from plants have been reported to process various biological activities, including immunomodulation, antitumor and anti-oxidation (Fei, Yuan, & Rashid, 2009). It has been reported that the union of

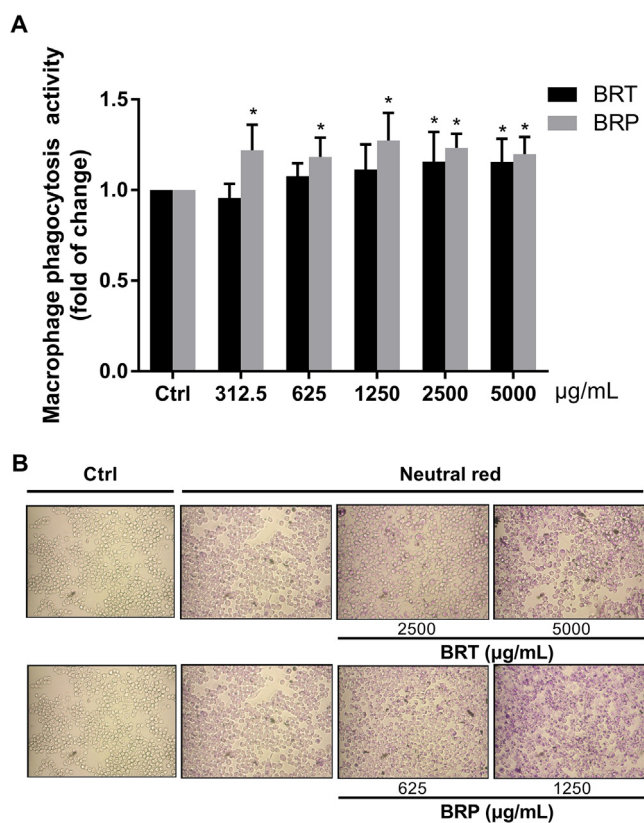


Fig. 3. Phagocytic ability of macrophages with the treatment of BRP and BRT (mean \pm SD, $n = 3$). (A) Absorbance compared with control and (B) microscope images of RAW264.7 cells after dyeing with neutral red ($*P < 0.05$ vs control).

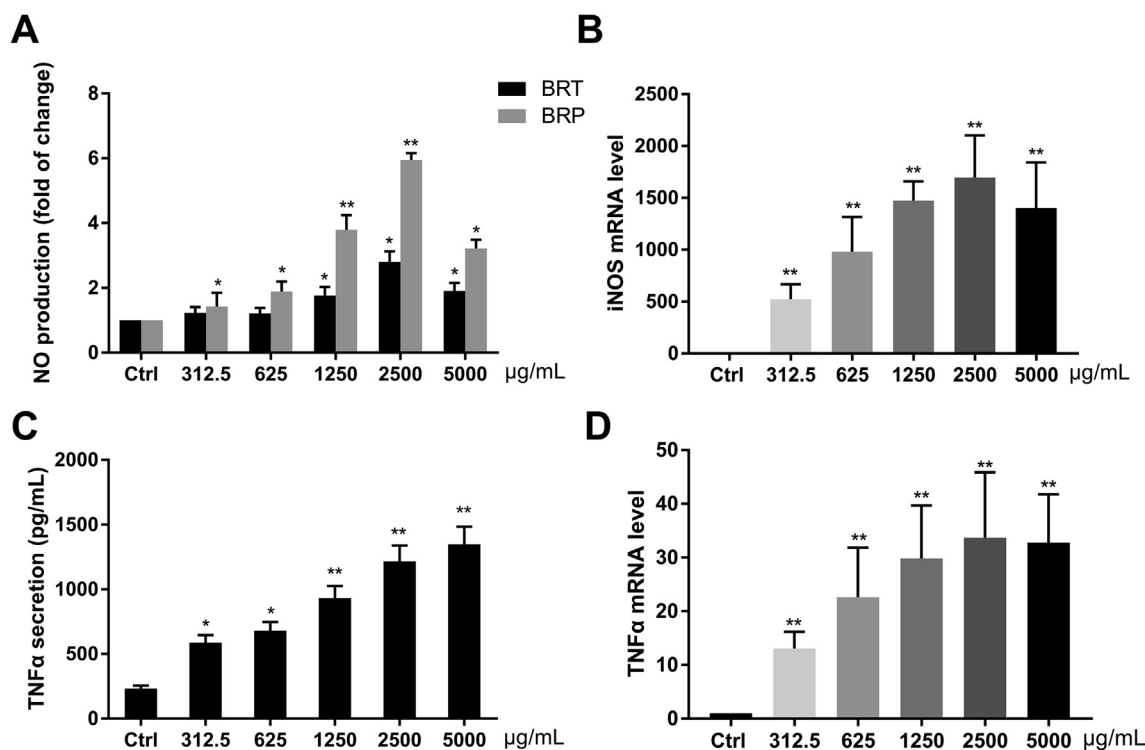


Fig. 4. Effect of BRP on macrophage NO and TNF α in RAW264.7 cells (mean \pm SD, $n = 3$). NO production of cells with BRP and BRT (A), iNOS mRNA levels of cells with BRP (B), TNF α production (C), and mRNA level (D) (* $P < 0.05$, ** $P < 0.01$ vs control).

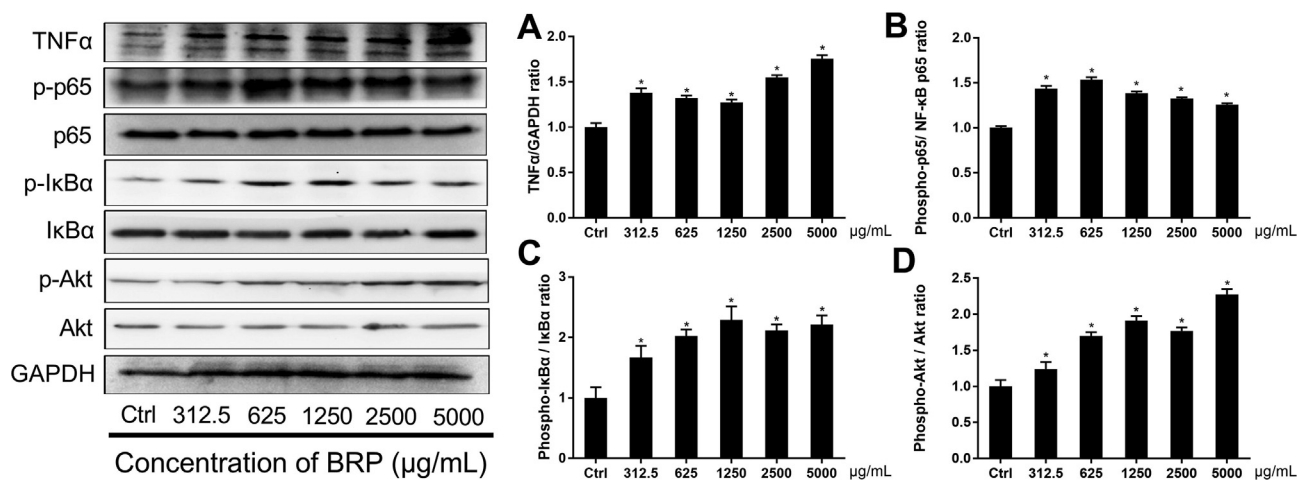


Fig. 5. Effect of BRP on cell signaling pathway in RAW264.7 macrophage cells (mean \pm SD, $n = 3$). The protein expression of TNF α (A), phosphorylation of NF- κ B (B), I κ B α (C) and Akt (D) (* $P < 0.05$, ** $P < 0.01$ vs control).

polysaccharide and its receptor on the surface of macrophages could increase the secretion of NO and TNF α -related macrophage immune responses (Schepetkin & Quinn, 2006).

B. rapa root has been traditionally used as a Uyghur folk medicine as well as health-care food to cure cough and asthma by Uyghur nationality. In previous studies, the antioxidant activity of crude BRP was determined to be better than that of the purified fractions of BRP (Wang et al., 2016; Xie et al., 2010). In addition, the chalcone glycosides isolated from *B. rapa* exhibited the effect to suppress LPS-induced iNOS expression and NO production via inhi-

bition of STAT1 (Hirokazu et al., 2014; Hirokazu et al., 2011), which left us a speculation that BRP could act an important role in activating immune system response. At first it was found that BRT (6 g/kg and 12 g/kg) had a significant effect on phagocytosis rate and index of CRBCs. Crude polysaccharides from the roots of *B. rapa* (BRP) were then precipitated with 80% ethanol and deproteinized by Sevag reagent, and immunomodulatory activity of BRP was investigated in this study. Compared with 1250 μ g/mL of BRT, 312.5 μ g/mL of BRP could already significantly enhance the phagocytic ability of macrophages, which indicated the benefit of BRT on immune

and inflammatory diseases might mostly result from BRP dealing to the improvement of deficient phagocytosis of macrophage. BRP significantly enhanced TNF α production and mRNA level in a concentration-dependent manner within 312.5–5000 $\mu\text{g}/\text{mL}$, while 2500 $\mu\text{g}/\text{mL}$ BRP exhibited the better effect on NO induction. iNOS is a key enzyme responsible for the production of NO and it plays an important role in the oxidative stress (Conforti & Menichini, 2011). As an immunomodulator, a dose between 2500 and 5000 $\mu\text{g}/\text{mL}$ of BRP was thought of the optimal concentration. In addition, the efficacy of BRP can be explicated by interference with Akt/NF- κB signaling pathways in RAW264.7 macrophages by activating the phosphorylation of Akt, I $\kappa\text{B}\alpha$ and NF- κB expression. Apparently, activation of NF- κB plays a key role in the transcriptional regulation of TNF α and iNOS genes. On the opposite, the generation of TNF α and NO due to cell activation can further activate the expression of NF- κB (Brasier, 2006).

5. Conclusion

By investigating the effects of BRT and BRP on RAW 264.7 macrophages, it was found that BRP behaved better phagocytic capacity, and had potent immune-stimulated activity via upregulating the iNOS and TNF- α pro-inflammatory cytokines, which was supported by the mRNA expression levels and secretion of cytokines. Furthermore, modulation of macrophage function by BRP was indicated to be mediated via activation of Akt/NF- κB signaling pathway. The present study showed beneficial effects of BRP which could be used as an immunotherapeutic adjuvant on inflammatory diseases, and provided a foundation for further studies of immunomodulatory mechanism of BRP.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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